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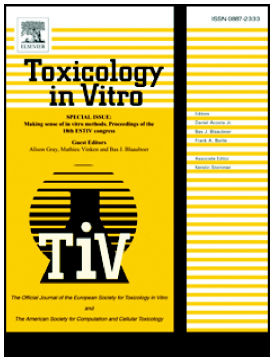

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Experimental evidence of oxidative stress in patients with L-2-hydroxyglutaric aciduria and that L-carnitine attenuates *in vitro* DNA damage caused by D-2-hydroxyglutaric and L-2-hydroxyglutaric acids

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Abbreviations: L-2-HGA, L-2-hydroxyglutaric aciduria; L-2-HG, L-2-hydroxyglutaric acid; D-2-HGA: D-2-hydroxyglutaric aciduria; D-2-HG, D-2-hydroxyglutaric acid; 2-HG, 2-hydroxyglutaric acid; LC, L-carnitine; L2HGDH, L-2-hydroxyglutarate dehydrogenase gene; D2HGDH, D-2-hydroxyglutarate dehydrogenase gene; IDH2, isocitrate dehydrogenase II gene; MRI, magnetic resonance imaging; IEM, inborn errors of metabolism; GC-MS/MS, gas chromatography coupled to mass spectrometry; LC-MS/MS, liquid chromatography- tandem mass spectrometry; DI, damage index; SSBs, single-strand breaks; DSBs, double-strand breaks; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

Abstract

D-2-hydroxyglutaric (D-2-HGA) and L-2-hydroxyglutaric (L-2-HGA) acidurias are rare neurometabolic disorders biochemically characterized by increased levels of D-2-hydroxyglutaric acid (D-2-HG) and L-2-hydroxyglutaric acid (L-2-HG) respectively, in biological fluids and tissues. These diseases are caused by mutations in the specific enzymes involved in the metabolic pathways of these organic acids. In the present work, we first investigated whether D-2-HG and L-2-HGA could provoke DNA oxidative damage in blood leukocytes and whether L-carnitine (LC) could prevent the *in vitro* DNA damage induced by these organic acids. It was verified that 50 μM of D-2-HG and 30 μM of L-2-HG significantly induced DNA damage that was prevented by 30 and 150 μM of LC. We also evaluated oxidative stress parameters in urine of L-2-HGA patients and observed a significant increase of oxidized guanine species and di-tyrosine, biomarkers of oxidative DNA and protein damage, respectively. In contrast, no significant changes of urinary isoprostanes and reactive nitrogen species levels were observed in these patients. Taken together, our data indicate the involvement of oxidative damage, especially on DNA, in patients affected by these diseases and the protective effect of LC.

Keywords: L-2-hydroxyglutaric aciduria; D-2-hydroxyglutaric aciduria; L-carnitine; DNA damage; Oxidative stress.

1. Introduction

D-2-hydroxyglutaric (D-2-HGA) and L-2-hydroxyglutaric (L-2-HGA) acidurias are two distinct inherited neurometabolic disorders biochemically characterized by increased levels of D-2-hydroxyglutaric acid (D-2-HG) and L-2-hydroxyglutaric acid (L-2-HG), respectively, in biological fluids and tissues (Kranendijk et al., 2012).

D-2-HGA was identified first by Chalmers et al (1980) and since then about 80 patients have been described worldwide (Wajner and Goodman, 2011). Affected patients present one of the two variants: D-2-HGA type I, which is caused by mutations in the D-2-hydroxyglutarate dehydrogenase gene (*D2HGDH*), whereas D-2-HGA type II is due to gain-of-function mutations in the isocitrate dehydrogenase II gene (*IDH2*) (Struys et al., 2005a, 2005b; Kranendijk et al., 2010a, 2011).These disorders may have a severe and deadly form or a milder form. Patients affected by the severe form usually present a neonatal or early infantile epileptic encephalopathy, with marked hypotonia, severe development delay, seizures and cardiomyopathy, whereas patients with the mild form commonly have psychomotor retardation, macrocephaly and hypotonia. Brain magnetic resonance imaging (MRI) features include delayed cerebral maturation, ventricular dilatation and sub ependymal cysts (Van der Knaap et al., 1999 a, b).

L-2-HGA was first identified by Duran et al (1980) with approximately 300 cases reported so far in literature (Balaji et al., 2014). This disorder is caused by mutations in the L-2-hydroxyglutarate dehydrogenase gene (*L2HGDH*) and unlike most other organic acidurias, L-2-HGA develops insidiously, without episodes of metabolic decompensation and is potentially associated with central nervous system tumors (Moroni et al., 2004; Topçu et al., 2005; Haliloglu et al., 2008; Patay et al., 2012). The

main neurological manifestations include progressive mental deficiency, variable motor impairment and cerebellar ataxia. Besides, most patients present macrocephaly, pyramidal and extrapyramidal signs, dystonia and a typical MRI profile with striking subcortical leucoencephalopathy and basal ganglia abnormalities (Wajner and Goodman, 2011; Isikay, 2014).

The mechanisms of the neurological symptoms presented by D-2-HGA and L-2-HGA patients are still poorly understood. However, considering that increased concentrations of D-2-HG and L-2-HG were associated with the appearance of neurological symptoms, these compounds may be potentially neurotoxic in these disorders (Van der Knaap et al., 1999a; Yilmaz, 2009; Patay et al., 2012; Marcel et al., 2012; Jovic et al., 2014). An *in vitro* study demonstrated that L-2-HG inhibits creatine kinase activity in cerebellum (da Silva et al., 2003). Furthermore, in an animal model of L-2-HGA it was demonstrated that L-2-HG induces oxidative stress by decreasing the antioxidant defenses and raising reactive oxygen species in striatum and cerebellum of rats, as well as important brain histopathological alterations (da Rosa et al., 2015). In what concern to D-2-HGA, it was shown that D-2-HG similarly disrupts redox homeostasis and causes histopathological alterations in rat striatum (da Rosa et al., 2014).

Oxidative stress may play an important role in the pathophysiology of some inborn errors of intermediary metabolism, since accumulation of toxic metabolites may lead to excessive production of free radicals (Barschak et al., 2006; Ribas et al., 2010). Oxidative damage, caused by reactive oxygen and nitrogen species, is an important mediator of neurodegeneration since brain has relatively low levels of antioxidant defenses, high lipid content and catecholamines, which are highly susceptible to free radical attack (Gülçin, 2006; Halliwell and Gutteridge, 2007). The hydroxyl radical, the

most dangerous reactive oxygen species, can induce a diversity of lesions in DNA, which include single-strand breaks, double-strand breaks, alkali-labile sites, oxidized purine and pyrimidine bases, and DNA-protein cross-links (Halliwell, 2001; Wajner et al., 2004; Garcia et al., 2006; Gülçin, 2012). In this context, if cellular repair mechanisms fail, mutations, deletions, cancer and even cell death could occur (Marnett, 2000; Cooke et al., 2006). Several studies have investigated DNA damage in some inborn errors of metabolism (IEM) (Filippon et al., 2011; Negretto et al., 2014; Marchetti et al., 2015) but there is no study in L-2-HGA and D-2-HGA. Besides, the mechanisms by which brain tumors develop in patients affect by L-2-HGA are not yet elucidated.

In the present study, we tested the *in vitro* effects of D-2-HG and L-2-HG on DNA damage by using the comet assay, as well as whether L-carnitine (LC), a drug commonly used to treat organic acidurias, could reduce this damage *in vitro*. Furthermore, we measured oxidative and nitrative stress parameters in urine of L-2-HGA patients.

2. Materials and Methods

2.1 *In vitro* study

2.1.1 Leukocytes preparation from blood of normal individuals

Venous blood was collected under sterile conditions in heparinized vials from six healthy volunteers. Leukocytes were isolated and incubated with 50 µM of

D-2-HG or 30 µM of L-2-HG at 37 ºC for 6 h (Tice et al., 2000). These concentrations were based on the plasma D-2-HG or L-2-HG levels in patients with these diseases (Gibson et al., 1993). In some experiments, LC (30 and 150µM) was co-incubated with 50 µM of D-2-HG or 30 µM of L-2-HG and DNA damage was determined afterwards. The final concentrations of LC in the assays were based in previous findings obtained from patients affected by organic acidurias and treated with LC (100 mg/Kg/dia), showing that plasma levels of this compound vary from 30 µmol/L at diagnosis to almost 100 µmol/L under LC supplementation (Ribas et al., 2010).

2.2 *In vivo* study

2.2.1 Urine collection from controls and patients with L-2-HGA

Eleven patients (the average age at the time of urine collection was 17.7 $\pm$ 12.99 years old) with L-2-HGA diagnosed at the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (HCPA), Brazil, were used in the experiments. The diagnosis was performed by urinary organic acid analysis using gas chromatography coupled to mass spectrometry (GC-MS/MS) (Wajner et al., 2009) and confirmed by enantiomer separation using stable-isotope dilution liquid chromatography- mass spectrometry (Struys et al., 2004). The control group consisted of samples from ten age-matched healthy children (the average age at the time of urine collection was 20.0 $\pm$ 8.12 years old).

2.2.1.1 Ethical considerations

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, Brazil (15-0601). Informed consent was obtained from all parents of the patients before sample collection.

2.1.3 Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al (1988) following the general guidelines for use of the comet assay (Tice et al., 2000; Singh et al., 1988). Aliquots of 100 µL from whole blood were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Slides were placed in lyses buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, pH 10-10.5) to remove cell proteins, leaving DNA as "nucleiods". After being treated with lyses buffer and alkaline buffer solution (300 mM NaOH and 1 mM EDTA, pH>13), the slides were submitted to a horizontal electrophoresis for 20 min at 4 ºC (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol (Nadin et al., 2001). After drying at room temperature overnight, gels were analyzed using an optical microscope. A hundred cells were analyzed by microscopy and a damage class was attributed for each cell. The damage classes were classified as: 0 = no tail (no damage); 1 = small tail smaller than the diameter of the head; 2 = tail length between one and two times the diameter of the head; 3 = long tail greater than twice the diameter of the head; 4 = longer tail than class 3. Thereafter, each damage class was multiplied by the number of cells found in each damage class. The damage index (DI)

was determined by the sum of these multiplications. The slides were analyzed under blind conditions at least by two different individuals.

2.2.2 Enantiomeric classification of D-2-HG or L-2-HG acids

Enantiomeric analysis was made in urine samples of each patient included in the study. Enantiomeric separation of the D-2-HG or L-2-HG was performed by liquid chromatography- mass spectrometry with stable-isotope-labeled internal standards. The analytes were derivatized by use of diacetyl- L-tartaric anhydride to obtain diastereomers, which were separated on an achiral C18 HPLC column and detected by MS/MS in multiple-reaction-monitoring mode (LC-MS/MS). Results were reported in mmol/mol creatinine (Struys et al., 2004).

2.2.3 Urinary oxidized guanine species

Urinary 8-hydroxy-2'-deoxyguanosine (from DNA), 8- hydroxyguanosine (RNA) and 8-hydroxyguanine (DNA and RNA) levels were determined by the DNA/RNA Oxidative Damage ELISA Kit (Cayman Chemical, USA). This assay is based on the competition between oxidatively damaged guanine species and an 8-OH-dG-acetylcholinesterase conjugate for a limited about of DNA/RNA Oxidative Damage Monoclonal antibody. Because the amount of tracer is held constant while the concentration of oxidatively damage guanine varies, the amount of tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of oxidatively damaged guanine in the well. The antibody-oxidatively damaged guanine complex binds to the goat polyclonal anti-mouse IgG that has been previously attached

to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The concentrations of oxidized guanine species (ng/mL) were calculated using a polynomial equation from the relative absorbance of a standard curve. After creatinine correction, the results were expressed as ng/mg creatinine.

2.2.4 Urinary 15-F2t-isoprostane levels

15-F2t-isoprostane, a product of arachidonic acid metabolism and a biomarker of lipid peroxidation, was measured by the *Urinary Isoprostane ELISA kit* (Oxford Biomedical Research, Inc., Oxford, MI, USA) according the kit instructions. In this assay, the analyte present in the urine samples competes with the horseradish peroxidase-conjugated 15-F2t-isoprostane for the binding to a specific antibody fixed on the microplate. 15-F2t-isoprostane concentrations were determined spectrophotometrically at 630 nm by the intensity of color developed after substrate was added to the wells. Results were expressed as ng of isoprostanes per mg of urinary creatinine (ng/mg creatinine).

2.2.5 Urinary di-tyrosine (di-Tyr) levels

It was measured the intensity of di-Tyr fluorescence according to the method described by Kirschbaum (2002) in order to determine the levels of protein oxidation in urine. For this assay, 50 µL of t urine was added to 950 µL of 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 minutes, di-Tyr concentration was measured using a fluorimeter (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg of urinary creatinine (FU/mg creatinine).

2.2.6 Reactive nitrogen species

Total levels of nitrate + nitrite (NO_3^- + NO_2^-) in urine were determined using the *Nitrate/Nitrite Colorimetric Assay Kit (LDH Method)* from Cayman Chemical® (Cayman Chemical Company, Ann Arbor, MI, USA). This method is based on the reduction of NO_3^- to NO_2^- using nitrate reductase. In this method, NO_2^- reacts with sulfanilamide producing a cationic intermediate, which reacts with N-(1-naphtyl) ethylenediamine. This reaction results in an azo product whose absorption can be measured at 540 nm. Results were reported as µmol/mg creatinine.

2.2.7 Urinary creatinine

Creatinine was determined using the Creatinine Kinetic kit of Bioclin® (Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil). Creatinine reacts with picric acid under alkaline conditions producing an orange colored derivative, whose absorbance was determined in a spectrophotometer at 510 nm. Results were expressed as mg creatinine/dL.

2.3 Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney test for comparisons between two groups and nonparametric Kruskall-Wallis followed by the Mann-Whitney U-test for comparisons of more than two groups. Differences were considered significant when $p<0.05$. The values were presented as medians (min; max). All analyses were performed using the GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA — version 5.0) software in a PC-compatible computer.

3. Results

We first investigated the *in vitro* effect of 50µM of D-2-HG and 30 µM of L-2-HG on DNA damage in leukocytes from whole blood of healty volunteers (figures 1A and 1B, respectively). We also investigated the *in vitro* effect of LC (30 µM and 150 µM) on DNA damage induced by these acids, as showed in figures 1C and 1D, respectively. Both D-2-HG and L-2-HG tested concentrations provoked DNA damage (arbitrary units) significantly higher than in the control group ($p<0.01$). LC at concentrations of 30 µM and 150 µM significantly reduced the DNA damage induced by 30 µM of L-2-HG and 50 µM of D-2-HG ($p<0.05$). Furthermore, there was a significant difference between the attenuation provoked by the two LC concentrations studied ($p<0.05$), since the reduction of DNA damage induced by 150 µM of LC was higher than that of 30 µM of LC.

Table 1 displays L-2-HG and D-2-HG urinary levels in the patients, confirming the L-2-HGA diagnosis in all patients studied. In order to investigate if there was oxidative DNA damage in these patients, we measured urinary oxidized guanine species. It was observed a significant increase in these species ($p<0.05$) when compared to the control group (figure 2A).

It was also analyzed lipid (isoprostanes) and protein (di-tyr) oxidative damage, as well as the nitrate plus nitrite content in the urine of patients with L-2-HGA. As described in figure 2B, it was observed a significant increase of di-Tyr excretion in urine of these patients when compared to controls ($p<0.05$), evidencing some degree of protein oxidation, but there were no significant differences between the two groups

regarding to isoprostanes and reactive nitrogen species levels (nitrate plus nitrite), as demonstrated in figure 2C and 2D, respectively.

4. Discussion

In this work we investigated whether D-2-HG and L-2-HG could induce *in vitro* DNA damage determined by the comet assay in peripheral blood leukocytes, as well as the role of LC upon DNA damage provoked by these metabolites. We also measured important parameters of oxidative stress in urine from L-2-HGA patients.

In previous studies, it was observed that L-2HG and D-2HG induce free radical production, as well as protein and lipid oxidation (Latini et al., 2003; Jellouli et al., 2014; da Rosa et al., 2014, 2015), but no report exists on DNA damage provoked by these organic acids. Thus, we found that 50 μM of D-2-HG and 30 μM of L-2-HG significantly increased leukocyte DNA migration (comet assay), indicating DNA damage. It is of note that the comet alkaline assay is a relatively simple, rapid, low-cost and sensitive technique for strand break detection in individual cells (Liao et al., 2009), but it cannot discriminate among the precise mechanisms of DNA damage.

We also verified a significant increase of guanine oxidized species (8-hydroxy-2'-deoxyguanosine, 8-hydroxyguanosine and 8-hydroxyguanine) in urine samples from L-2-HGA patients, indicating oxidative DNA damage. It is of note that guanine is a DNA base very prone to oxidation. Furthermore, in recent years it has become clear that both DNA and RNA can be damaged by oxidation in various diseases and that the repair processes that are initiated to correct the oxidative damage release various oxidized species into the urine (Shi et al., 2012). Oxidative DNA damage occurs as a result of the attack of reactive oxygen species to both nuclear and mitochondrial DNA

generating the oxidized form of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Halliwell and Gutteridge, 2007). Serum and urinary 8-OHdG reflect the equilibrium between its production and repair of DNA and the nucleotide pool (Cooke et al., 2006).

Oxidative damage to nucleic acids has been found in a variety of pathologies including cancer, neurodegenerative diseases and IEM (Roszkowski et al., 2011). In this scenario, it has been described high serum concentrations of 8-OHdG in phenylketonuric patients, an inborn error of phenylalanine metabolism, associated with oxidative damage, as well as a positive significant correlation between phenylalanine that accumulates in this disease and 8-OHdG levels (Deon et al., 2015). In the present study we demonstrated an increase of oxidized guanine species in the L-2-HGA patients corroborating our *in vitro* findings (by comet assay) and reinforcing the involvement of reactive oxygen species in this disease and that these species may potentially cause oxidative modifications in DNA bases. In case these findings are confirmed in cerebrospinal fluid of patients with D-2-HGA and L-2-HGA, it would contribute to clarify the pathophysiology of these diseases.

It is of note that oxidative modifications resulting in permanent DNA changes represent the first step involved in mutagenesis, carcinogenesis and aging (Halliwell and Gutteridge, 2007), and patients with L-2-HGA have a predisposition to brain tumors (Patay et al., 2012, 2015). While a study with a large number of patients (295 cases with the L-isomer variant of 2-hydroxyglutaric) revealed that 14 patients developed cerebral neoplasms (almost 5%), another study including 18 patients with this disorder showed 7 cases with cerebral neoplasms, showing that the prevalence of cerebral tumors can reach up to 40% (Moroni et al., 2004; Patay et al., 2012). In this particular, a recent study showed that 80% of gliomas of WHO grade II/III and secondary glioblastomas harbor isocitrate dehydrogenase I (IDH1) mutation and induce a gain of function that promotes

the conversion of isocitrate to 2-hydroxyglutaric acid (2-HG). Our present findings indicating that L-2-HG provokes DNA changes may possibly explain an oncogenic mechanism of sustained L-2-HG exposure to the brain in L-2-HGA (Patay et al., 2015).

It is of note that oxidative stress usually causes oxidative damage to cellular membrane phospholipids, proteins and DNA, eventually culminating in cell death (Halliwell and Gutteridge, 2007), so that therapy is necessary to prevent these deleterious effects. Furthermore, it is known that brain is highly susceptible to reactive species attack due to high oxygen consumption and low levels of antioxidant defenses and oxidative stress was postulated to contribute to the pathophysiology of many inherited neurometabolic disorders with neurodegeneration (Halliwell, 2006).

LC is a water soluble molecule of mammalian metabolism that plays an important role in normal mitochondrial oxidation of fatty acids (Walter et al., 1996). Several studies have demonstrated its protective effect against oxidative injury, especially due to its capacity to scavenge hydrogen peroxide and superoxide radical and also chelate transition metal ions (Gülçin, 2006). Moreover, LC has been reported to exhibit protective properties reducing DNA damage and accelerating the disappearance of SSBs induced by oxygen radicals and alkylating agents, due its scavenge capacity, trigger the repair enzyme poly(ADP-ribose)polymerase and other repair mechanism, besides enhancing cell energy production (Boerrigter et al., 1993; Garcia et al., 2006). LC was also shown to reduce lipid peroxidation and protein oxidation in phenylketonuria (Sitta et al., 2011) and maple syrup urine disease (Meska et al., 2014, 2015 a,b), as well as to decrease DNA damage induced by propionic and methylmalonic acid (Ribas et al., 2010). Besides, LC reduced lipid peroxidation in patients with 3-hydroxy-3-methyl-glutaric aciduria (dos Santos et al., 2015). Lastly, LC

supplementation has been considered safe, which makes it a promising candidate for prevention and treatment of oxidative alterations in many diseases (Ribas et al., 2014).

Therefore, in order to verify whether LC could be able to reduce DNA damage induced by D-2-HG and L-2-HG, we used the *in vitro* comet assay supplementing D-2-HG or L-2-HG with 30 µM and 150 µM of LC. Our results showed that LC was able to significantly reduce the levels of DNA migration caused by both organic acids.

In this study we also evaluated some oxidative and nitrative stress parameters in urine of L-2-HGA patients. We verified a significant increase of urinary di-tyr levels in L-2-HGA patients that is considered a marker of protein oxidative damage. Di-Tyr is formed by oxidation of tyrosine residues leading to the generation of a highly stable inter-phenolic bond that can lead to malfunction of enzymes, receptors and transport proteins, finally resulting in impairment of cellular metabolism (Kirschbaum, 2002; Halliwell and Gutteridge, 2007). In contrast, no significant alterations of isoprostanes, nitrate and nitrite levels were found in urine of L-2-HGA patients, ruling out a major role for the involvement of lipid peroxidation and reactive nitrogen species in these patients.

In summary, our results demonstrated for the first time that D-2-HG and L-2-HG acids induce *in vitro* DNA damage and that LC has an *in vitro* protective effect upon this damage. Another novel and important finding of the present study indicates that oxidative damage to DNA and proteins occurs in L-2-HGA patients. It is tempting to speculate that LC could be used in L-2-HGA patients as a potential adjuvant therapy to prevent or attenuate oxidative damage, as it is commonly used for many organic acidemias. However, further *in vivo* studies are necessary to clarify this issue since we have used urine samples only.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1. *In vitro* effect of **(A)** D-2-hydroxyglutaric acid (D-2-HG, 50 µM) and **(B)** L-2-hydroxyglutaric acid (L-2-HG, 30 µM) on DNA damage (comet assay) in leukocytes from whole blood. $^{**}p < 0.01$ compared to the control group (nonparametric Mann-Whitney); **(C)** L-carnitine (LC, 30 and 150 µM) on DNA damage induced by 50 µM of D-2-HG in leukocytes from whole blood. (a) $p<0.01$ compared to the control group; (b) $p<0.05$ compared to the 50 µM D-2-HG group; (c) $p<0.05$, compared to the 50 µM D-2-HG/30 µM LC group; **(D)** L-carnitine (LC, 30 and 150 µM) on DNA damage induced by 30 µM of L-2-HG in leukocytes from whole blood. (a) $p<0.01$ compared to the control group; (b) $p<0.05$ compared to the 30 µM L-2-HG group; (c) $p<0.05$, compared to the 30 µM L-2-HG/30 µM LC group (Kruskal-Wallis test followed by Mann-Whitney U-test). Data represent median (min; max) of six independent experiments (individuals).

Fig. 1A

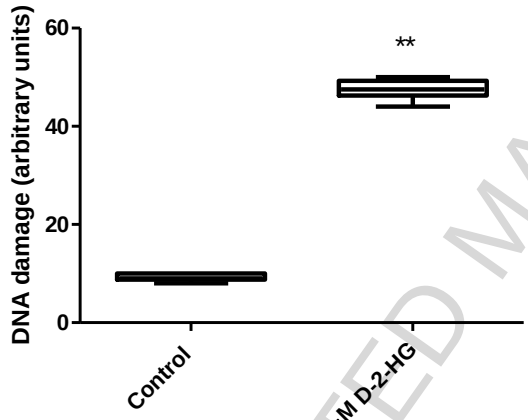
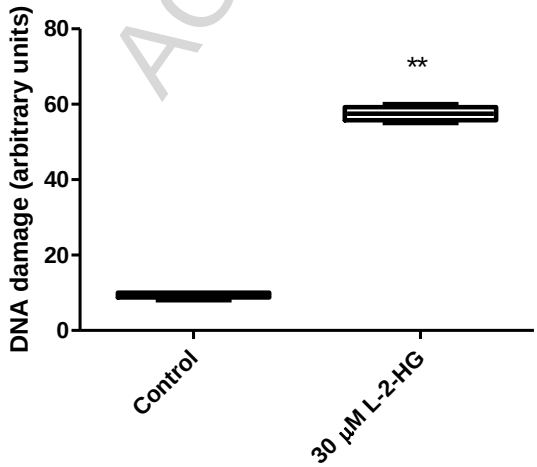


Fig. 1B



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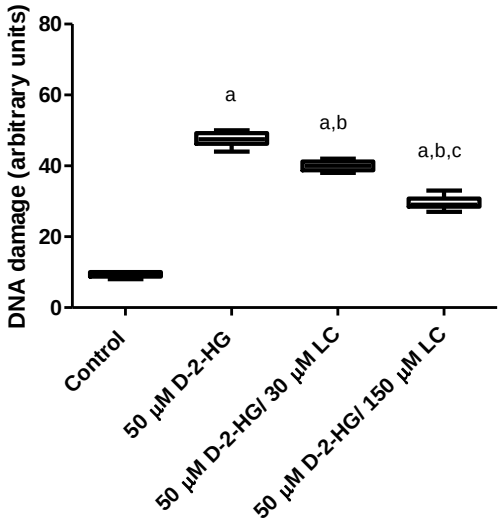


Fig. 1D

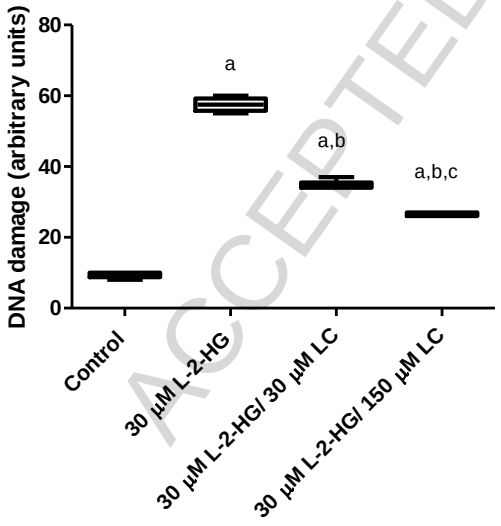


Figure 2. (A) DNA/RNA oxidative damage (urinary oxidized guanine species) and **(B)** protein oxidative damage (urinary di-tyrosine) in patients with L-2-HGA (n= 11) and controls (n=10) *p<0.05 compared to the control group (nonparametric Mann- Whitney test); **(C)** Lipid oxidative damage (urinary 15-F2t-isoprostane) and **(D)** Reactive nitrogen species (urinary nitrate + nitrite content) in patients with L-2-HGA (n= 11) and controls (n=10). Data represent median (min; max), p> 0.05 compared to the control group (nonparametric Mann- Whitney test).

Fig. 2A

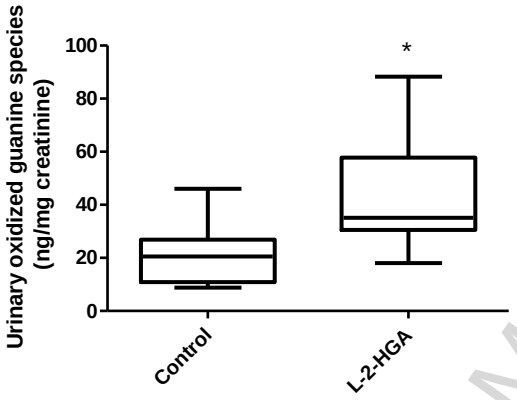
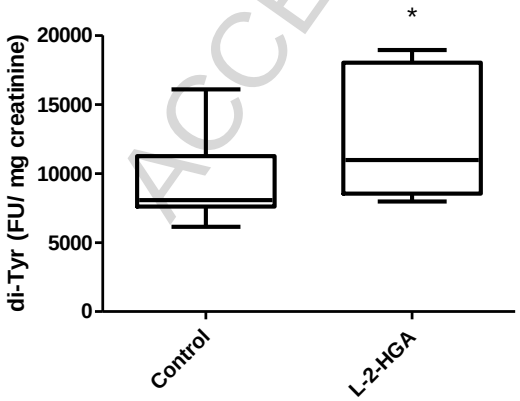


Fig. 2B



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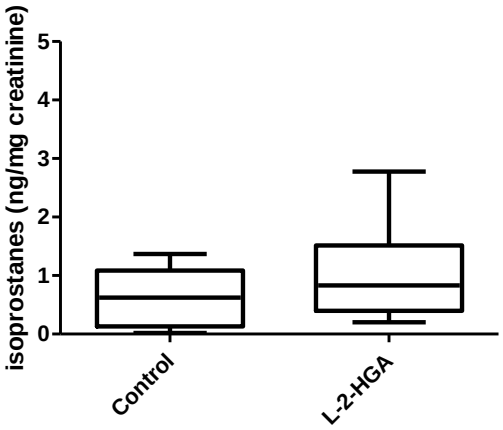

Fig. 2D

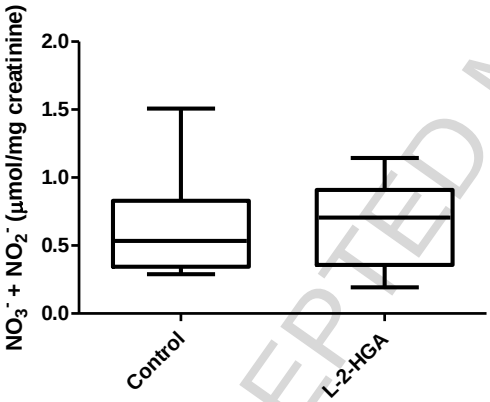

Table 1

Urinary D-2-hydroxyglutaric acid (D-2-HG) and L-2-hydroxyglutaric acid (L-2-HG) levels analyzed by LC-MS/MS in patients

Patient Sample	Creatinine mmol/L	L-2-HG µmol/L	L-2-HG mmol/mol creat	D-2-HG µmol/L	D-2-HG mmol/mol creat
1	14.4	6780	471	nd	-
2	4.3	2021	470	nd	-
3	12.3	2958	240	nd	-
4	11.2	5254	469	nd	-
5	7.4	2102	284	nd	-
6	15.5	4534	293	nd	-
7	22.2	6822	307	nd	-
8	14.2	3508	247	nd	-
9	18.4	3771	205	nd	-
10	22.2	2691	121	nd	-
11	7.0	2708	387	nd	-

nd: not detected

Highlights

- L-2-hydroxyglutaric and D-2-hydroxyglutaric acids induce *in vitro* DNA damage.
- L-carnitine attenuates *in vitro* DNA damage induced by L-2-hydroxyglutaric and D-2-hydroxyglutaric acids.
- Oxidative damage to proteins and DNA occurs in patients with L-2-hydroxyglutaric aciduria.