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# Disruption of brain redox homeostasis in glutaryl-CoA dehydrogenase deficient mice treated with high dietary lysine supplementation

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ABSTRACT

Deficiency of glutaryl-CoA dehydrogenase (GCDH) activity or glutaric aciduria type I (GA I) is an inherited neurometabolic disorder biochemically characterized by predominant accumulation of glutaric acid and 3-hydroxyglutaric acid in the brain and other tissues. Affected patients usually present acute striatum necrosis during encephalopathic crises triggered by metabolic stress situations, as well as chronic leukodystrophy and delayed myelination. Considering that the mechanisms underlying the brain injury in this disease are not yet fully established, in the present study we investigated important parameters of oxidative stress in the brain (cerebral cortex, striatum and hippocampus), liver and heart of 30-day-old GCDH deficient knockout (*Gcdh*$^{-/-}$) and wild type (WT) mice submitted to a normal lysine (Lys) (0.9% Lys), or high Lys diets (2.8% or 4.7% Lys) for 60 h. It was observed that the dietary supplementation of 2.8% and 4.7% Lys elicited noticeable oxidative stress, as verified by an increase of malondialdehyde concentrations (lipid oxidative damage) and 2-7-dihydrodichlorofluorescein (DCFH) oxidation (free radical production), as well as a decrease of reduced glutathione levels and alteration of various antioxidant enzyme activities (antioxidant defenses) in the cerebral cortex and the striatum, but not in the hippocampus, the liver and the heart of *Gcdh*$^{-/-}$ mice, as compared to WT mice receiving the same diets. Furthermore, alterations of oxidative stress parameters in the cerebral cortex and striatum were more accentuated in symptomatic, as compared to asymptomatic *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys overload. Histopathological studies performed in the cerebral cortex and striatum of these animals exposed to high dietary Lys revealed increased expression of oxidative stress markers despite the absence of significant structural damage. The results indicate that a disruption of redox homeostasis in the cerebral cortex and striatum of young *Gcdh*$^{-/-}$ mice exposed to increased Lys diet may possibly represent an important pathomechanism of brain injury in GA I patients under metabolic stress.



## 1. Introduction

Glutaric aciduria type I (GA I, McKusick 23167; OMIM #231670) is a neurometabolic disorder caused by a severe deficiency of the activity of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) due to heterogeneous mutations in the GCDH gene. It is biochemically characterized by tissue accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA) and glutarylcarnitine and high urinary excretion of GA and 3OHGA. Some GA I patients only excrete 3OHGA in the urine. Affected patients present macrocephaly associated with frontotemporal atrophy at birth. Between 6 months and 3 years most untreated children suffer acute encephalopathic crises commonly precipitated by infectious illness or vaccination. These episodes coincide with massive striatal necrosis, clinically manifested

*Abbreviations:* CAT, catalase; DCF-DA, 2-7-dihydrodichlorofluorescein diacetate; DCFH, 2-7-dihydrodichlorofluorescein; DTNB, 5-5-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GA I, glutaric aciduria type I; GA, glutaric acid; *Gcdh*$^{-/-}$, deficient knockout mice; GCDH, glutaryl-CoA dehydrogenase; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose-6-phosphate dehydrogenase; 3OHGA, 3-hydroxyglutaric acid; KO, knockout; Lys, lysine; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NADP$^{+}$, nicotinamide adenine dinucleotide phosphate; NeuN, neuronal nuclei; PAF, paraformaldehyde; PBS, phosphate buffered saline; PBST, PBS plus 0.1–0.3% Triton X-100; SPSS, Statistical Package for the Social Sciences; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid-reactive substances; WT, wild type.

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as dystonia and dyskinesia and other neurological symptoms [1–3]. Patients can also develop chronically progressive neurological dysfunction without undergoing acute episodes [2,4,5]. Cranial MRI findings usually show a pattern of progressive spongiform white matter changes (leukoencephalopathy) with cortical hypoplasia, subdural hemorrhages and degeneration of the basal ganglia with loss of medium spiny neurons, as well as astrogliosis [4,6–9].

Despite a great deal of experimental work on GA I neuropathology in the last few years, the comprehension of the pathomechanisms of brain injury in this disorder still remains partial. However, it has been suggested that the accumulating organic acids GA and 3OHGA behave as neurotoxins through three major synergistic mechanisms, excitotoxicity, disruption of mitochondrial energy homeostasis and oxidative stress in vulnerable regions of the brain [10–35]. However, the pathophysiological significance of these data is uncertain since most data were obtained in tissues with normal GCDH activity, whereas some results are contradictory [22,36] possibly due to differences between the experimental approaches utilized.

A knockout (KO) mice model of GA I ($Gcdh^{-/-}$) developed by Koeller and collaborators [37] proved to mimic the biochemical phenotype (accumulation of GA and 3OHGA in tissues and body fluids) and some of the neurological abnormalities (leukoencephalopathy) observed in GA I patients, but did not induce the characteristic striatum necrosis even under metabolic or infectious stressors. Zinnanti and colleagues [38,39] improved this model by exposing the KO animals to high protein or lysine (Lys) (4.7%) dietary intake that gave rise to higher brain GA concentrations and striatum degeneration. It was also shown that the neuropathological findings observed in the high dietary Lys-treated mice were comparable to those found in human patients, including neuronal loss and vacuolization, blood–brain barrier breakdown, myelin disruption and gliosis mostly in the striatum and deep cortex. These investigators also demonstrated a disturbance of mitochondrial energy metabolism, revealed by lower concentrations of ATP and phosphocreatine and alterations of other bioenergetics markers in the KO mice supplemented by high Lys [39]. However, a setback of this model is that most 4-week-old $Gcdh^{-/-}$ mice receiving high Lys supplementation presented hypoactivity, followed by paralysis, seizures and death after 5–7 days, paralleled with striatal and cortical pathological abnormalities after 48 h of this diet. These clinical and pathological findings may possibly interfere with the investigation of the underlying mechanisms of brain damage in this model since the obtained results may be the consequence rather than the cause of neural cell injury and death.

We recently found that the striatum and to a lesser degree the cerebral cortex of 30-day-old $Gcdh^{-/-}$ mice are vulnerable to oxidative damage 24 h after an acute Lys intraperitoneal administration (8 μmol/g) [40]. It is of note that some of these animals were symptomatic, presenting hypoactivity. In the present study we aimed to evaluate a large spectrum of important parameters of redox homeostasis in brain structures (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) from 30-day-old $Gcdh^{-/-}$ and WT mice submitted to a normal (0.9%) or high (2.8 or 4.7%) Lys diets for 60 h. We used a moderately increased amount of Lys dietary supplementation (2.8% Lys), besides the proposed high diet with 4.7% Lys supplementation, because the later diet usually gives rise to severe neurological alterations and provokes death in the $Gcdh^{-/-}$ mice [38,39]. Histopathological studies were also carried out in the brain of these animals submitted to a high (4.7%) Lys diet.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffers used for each technique.

### 2.2. Animals

$Gcdh^{-/-}$ and WT mice of C129SvEv background were generated from heterozygotes and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were kept on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air-conditioned constant temperature ($22 \pm 1$ °C) colony room, with free access to water and 20% protein (w/w) commercial chow. At 27 days of age, WT and $Gcdh^{-/-}$ animals were submitted to a normal (0.9% Lys) or high dietary Lys intake (2.8 or 4.7% Lys) for 60 h. WT and $Gcdh^{-/-}$ mice were killed 60 h after the beginning of the diet and then the parameters of oxidative stress were immediately measured in the various tissues (see detailed protocol below).

### 2.3. Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of the United States of America, NIH, publication n 85-23, revised in 2011, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

### 2.4. Tissue preparation

The mice were anesthetized with the mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardially perfused during 5 min with saline solution. After perfusion, the brain, liver and heart were rapidly removed and placed on a Petri dish on ice. The olfactory bulb, pons, medulla, and cerebellum were discarded from the total brain, and the cortex, striatum and hippocampus dissected and weighed. The tissues were homogenized in 9 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C to discard nuclei and cell debris [41]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters. We emphasize that supernatants are widely used as a valid model system to evaluate important pro-oxidant and antioxidant parameters of oxidative stress [41–46] because they contain the whole cell machinery including preserved organelles such as mitochondria (the major source of free radical generation) and enzymes that are necessary for free radical production and scavenging [43,46–48]. Tissue slices (400 μm) were also prepared from the cerebral cortex and the peripheral structures for DCFH oxidation measurement.

### 2.5. Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS levels were measured according to the method described by Yagi [49] with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/ mg protein. Results were expressed as percentage of controls.

### 2.6. 2-7-Dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [50] by using 2-7-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and incubated with tissue slices (30 mg) during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2-7-dichlorofluorescein (DCF) in the presence of reactive species. The DCF fluorescence intensity parallels to the amount of reactive species formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 mM) and the levels of reactive species were calculated as pmol DCF formed/mg protein. Results were expressed as percentage of controls.

### 2.7. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [51]. Tissue supernatants with approximately 0.3 mg of protein were diluted (1:20, v/v) in 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/ mg protein. Results were expressed as percentage of controls.

### 2.8. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [52] using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 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, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

### 2.9. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [53] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on $O_2^{\bullet-}$, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer containing 1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and tissue supernatants (approximately 1 μg of protein). A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results were calculated as U/mg protein and expressed as percentage of controls.

### 2.10. Catalase (CAT) activity

CAT activity was assayed according to Aebi [54] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of $H_2O_2$ consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

### 2.11. Glucose-6-phosphate dehydrogenase (G6PD) activity

G6PD activity was measured by the method of Leong and Clark [55] in a reaction mixture containing 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ and tissue supernatants (approximately 3 μg of protein). The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PD unit corresponds to 1 mmol of substrate transformed per minute and the specific activity was calculated as U/mg protein and expressed as percentage of controls.

### 2.12. Histopathological studies

WT and *Gcdh*$^{-/-}$ mice were anesthetized with 90:10 mg/kg ketamine/xilazine mixture and intracardially perfused with 4% paraformaldehyde (PAF) in 0.01 M PBS, pH 7.4. After fixation, brains were quickly removed, maintained overnight at 4 °C in 10% PAF and changed to phosphate buffered saline (PBS) until sectioning. A 1000S Leica vibratome was used to obtain 30–50 μm thick consecutive coronal series. Sections were stored either free-floating at 4 °C or mounted on gelatin-coated slides for histological analysis. Representative sections were stained with 1% boraxic methylene blue to evidence gross neuroanatomical features. In some experiments, the cerebral cortex and striatum were dissected. Myelin content was estimated by histochemistry against Sudan III and Sudan IV (Sudan Black). Immunohistochemistry was performed in striatum to study the different cell populations and oxidative stress prototypic markers. For each animal and staining procedure, 5 to 8 equivalent sections were immunostained. Striatal neuronal population was recognized by labeling against the pan-neuronal marker neuronal nuclei (NeuN), whereas the astrocytic population was studied by using antibodies against S100β and glial fibrillary acidic protein (GFAP). Microglia was recognized by Iba1 immunoreactivity. Immunostaining against Ki67 was performed to look for proliferating cells. A monoclonal antibody reported as recognizing GCDH positive cells were also employed. To perform immunohistochemical assays, free-floating sections were washed with PBS, submitted to antigen retrieval by boiling in 10 mM pH 6.0 sodium citrate during 10 min, then washed twice for 10 min. After that, slices were permeabilized with PBS plus 0.1–0.3% Triton X-100 (PBST) and treated with blocking buffer (PBS + 0.3% Triton X-100 + 5% bovine serum albumin) for 30 min. Finally slices were incubated with pairs of antibodies i.e. anti-NeuN (Millipore, 1:250) together with anti-GFAP (1:400), or anti-Ki67 (1:200, Abcam), or anti-S100β (1:500) with anti-nitrotyrosine (Millipore, 1:300), or anti-Ki67 (Abcam, 1:300) with anti-iNOS (Santa Cruz, 1:300). All dilutions were made in PBST. After a 4 °C overnight incubation, sections were rinsed in PBS, and incubated at room temperature for 90 min with 1:800 dilutions of corresponding secondary antibodies conjugated to fluorescent probes (Molecular Probes). Sections were then washed, mounted in glycerol and imaged in a FV300 Olympus confocal microscope provided with 405, 488, 546 and 633 nm lasers. Primary or secondary antibodies were omitted in negative controls [29].

### 2.13. Protein determination

Protein concentrations were measured by the method of Lowry et al. [56] using bovine serum albumin as standard.

### 2.14. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples to compare the parameters between and WT and *Gcdh*$^{-/-}$ mice. Only significant *t* values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried

out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

## 3. Results

We first found that WT mice treated with 0.9%, 2.8% or 4.7% dietary Lys did not present apparent motor or behavioral alterations. The same occurred with the *Gcdh*$^{-/-}$ mice receiving normal or 2.8% Lys intake. In contrast, 20–25% of *Gcdh*$^{-/-}$ mice fed a 4.7% Lys became hypoactive approximately 48–60 h after the diet start. Unless otherwise stated, the results presented below refer to asymptomatic mice. We also observed in a separate experiment that the hypoactive mice died after approximately 5–7 days of diet.

Regarding the oxidative stress parameters, we observed that these biochemical measurements did not differ in all examined structures (cerebral cortex, striatum, hippocampus, liver and heart) from asymptomatic WT and *Gcdh*$^{-/-}$ mice receiving normal chow (0.9% Lys). However, significant alterations of most of these parameters were observed in the brain of asymptomatic *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys for 60 h, as discussed below with more details. Furthermore, there were no differences in the magnitude of the alterations of the oxidative stress parameters in *Gcdh*$^{-/-}$ mice fed with either 2.8% or 4.7% Lys, implying that a "moderately" increased Lys intake (2.8%) was sufficient to impair redox homeostasis in the absence of symptoms.

### 3.1. Lys dietary overload induces lipid peroxidation in the cerebral cortex and striatum of *Gcdh*$^{-/-}$ mice

Lys overload (2.8% and 4.7%) resulted in a significant increase of malondialdehyde (TBA-RS) levels in the cerebral cortex [2.8%: $t_{(8)}=3.313$; $P<0.01$; 4.7%: $t_{(8)}=5.049$; $P<0.001$] (Fig. 1A) and in striatum [4.7%: $t_{(8)}=3.501$; $P<0.01$] (Fig. 1B) of *Gcdh*$^{-/-}$ mice as compared to WT mice. In contrast, this measurement was not altered in the hippocampus, liver and heart of *Gcdh*$^{-/-}$ animals receiving 2.8% or 4.7% Lys diet (results not shown).

### 3.2. Lys dietary overload increases reactive species formation (DCFH oxidation) in the cerebral cortex of *Gcdh*$^{-/-}$ mice

We also observed that DCFH oxidation was significantly increased in the cerebral cortex of *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys dietary supplementation, as compared to WT [2.8%: $t_{(8)}=4.646$; $P<0.01$; 4.7%: $t_{(8)}=-2.412$; $P<0.05$] (Fig. 2). However, this parameter was not changed in liver and heart of *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys dietary supplementation (results not shown).

### 3.3. Lys dietary overload decreases reduced glutathione (GSH) levels in the cerebral cortex and striatum of *Gcdh*$^{-/-}$ mice

We verified that GSH levels were markedly diminished in the cerebral cortex [2.8%: $t_{(8)}=2.626$; $P<0.05$; 4.7%: $t_{(8)}=3.390$; $P<0.01$] and striatum [2.8%: $t_{(8)}=3.555$; $P<0.05$; 4.7%: $t_{(8)}=2.764$; $P<0.01$] of *Gcdh*$^{-/-}$ that received 2.8% or 4.7% Lys dietary intake as compared to WT mice (Figs. 3A and B). In contrast, GSH values were not changed in the hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice treated with 2.8% or 4.7% Lys diet (results not shown).

### 3.4. GSH levels and DCFH oxidation are inversely correlated in the cerebral cortex of *Gcdh*$^{-/-}$ mice on a high Lys diet

Furthermore, the reduction of GSH concentrations (decrease of the most important brain antioxidant defense) was inversely correlated with DCFH oxidation (increased production of reactive species) ($r=-0.6662$, $P<0.05$), suggesting that free radicals were probably involved in the decrease of the non-enzymatic antioxidant defenses (Fig. 4).

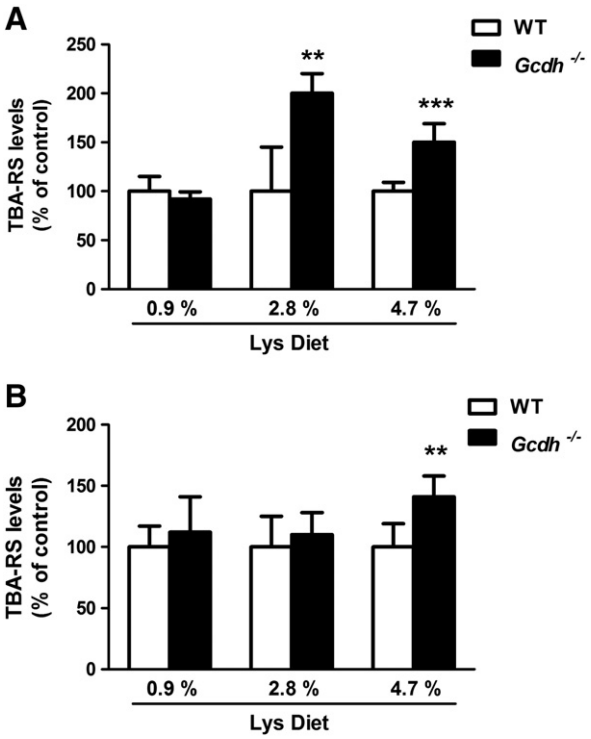


**Fig. 1.** Effect of lysine (Lys) dietary overload on thiobarbituric acid-reactive substances (TBA-RS) levels in the cerebral cortex (A) and the striatum (B) of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals) expressed as percentage of control (Control: A 0.9%: 1.49 ± 0.28; 2.8%: 1.12 ± 0.11; 4.7%: 1.30 ± 0.10; B 0.9%: 1.25 ± 0.21; 2.8%: 0.89 ± 0.22; 4.7%: 1.12 ± 0.21; [nmol TBA-RS/mg protein]). $^{**}P<0.01$, $^{***}P<0.001$, compared to WT mice (Student's *t*-test for unpaired samples).

### 3.5. Lys dietary overload increases the activities of superoxide dismutase and catalase in the cerebral cortex of *Gcdh*$^{-/-}$ mice

High Lys intake (2.8% and 4.7%) induced a significant increase in the activities of SOD and CAT in the cerebral cortex of *Gcdh*$^{-/-}$ mice [SOD [2.8% Lys: $t_{(8)}=2.835$; $P<0.05$; 4.7% Lys: $t_{(8)}=2.719$; $P<0.05$]; CAT [2.8% Lys: $t_{(8)}=4.253$; $P<0.01$; 4.7% Lys: [$t_{(12)}=3.424$; $P<0.01$]] (Figs. 5B and C). In contrast, no alterations of these antioxidant enzyme activities were found in striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice receiving either 2.8% or 4.7% Lys dietary supplementation (results not shown). Furthermore, GPx and G6PD activities were not changed in the cerebral cortex (Figs. 5A and D),

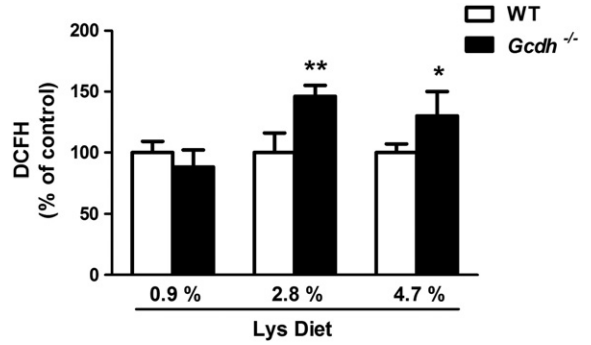


**Fig. 2.** Effect of lysine (Lys) dietary overload on 2-7-dihydrodichlorofluorescein (DCFH) oxidation in the cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: 0.9%: 8.64 ± 0.89; 2.8%: 6.59 ± 0.87; 4.7%: 7.12 ± 0.52; [pmol DCF/mg protein]). $^{*}P<0.05$, $^{**}P<0.01$, compared to WT mice (Student's *t*-test for unpaired samples).

as well as in the striatum, hippocampus and in the peripheral tissues (heart and liver) of *Gcdh*$^{-/-}$ mice receiving 0.9%, 2.8% or 4.7% Lys, as compared to WT mice (results not shown).

### 3.6. Disruption of redox homeostasis is significantly greater in the cerebral cortex and striatum of symptomatic *Gcdh*$^{-/-}$ mice submitted to 4.7% Lys dietary overload

In a separate set of experiments we submitted *Gcdh*$^{-/-}$ mice submitted to a 4.7% Lys diet and compared the changes of redox homeostasis between symptomatic and asymptomatic animals. Fig. 6 shows that alterations of oxidative stress parameters were more evident in the cerebral cortex and striatum of symptomatic as compared to asymptomatic *Gcdh*$^{-/-}$ mice submitted to 4.7% Lys dietary overload for 60 h. We can observe in the figure a significantly higher TBA-RS ($t_{(9)} = 7.174$, $P<0.001$) and SOD activity ($t_{(9)} = 6.505$, $P<0.001$) and a nonsignificant tendency of GSH reduction in symptomatic *Gcdh*$^{-/-}$ mice in the cerebral cortex. The striatum of symptomatic *Gcdh*$^{-/-}$ mice presented a significant reduction of GSH levels ($t_{(9)} = 4.658$, $P<0.001$), an increase of SOD activity ($t_{(9)} = 3.391$, $P<0.01$) and a nonsignificant increase of TBA-RS levels as compared to asymptomatic *Gcdh*$^{-/-}$ mice.

### 3.7. Lys dietary overload does not affect gross brain anatomy and white matter integrity in the cerebral cortex and striatum but provokes oxidative damage in *Gcdh*$^{-/-}$ mice submitted to 4.7% Lys dietary overload

We found no significant changes neither in the gross brain anatomy of both WT and *Gcdh*$^{-/-}$ mice submitted to high Lys nor in the myelination in the cerebral cortex and striatum of high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, and no ventricular enlargement, indicating that this short treatment (60 h) did not significantly affect the gross white matter integrity of these brain structures (data not shown). Furthermore, the number of striatal neurons (estimated by immunostaining against the panneuronal marker NeuN per area unit) remained unchanged in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice as compared to WT. However, NeuN neuronal staining in high Lys treated animals was weaker and occupied cell cytoplasm. A significant injury response was also discarded since Ki67 immunostaining was similar in WT and *Gcdh*$^{-/-}$ mice (Fig. 7A). Furthermore, no changes in S100b+ and GFAP+ cell number were observed in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, discarding prominent astrogliosis, despite that S100+ cells appeared swollen with increased immunoreactivity (Fig. 7B). Interestingly, the striatum of high Lys (4.7%) exposed *Gcdh*$^{-/-}$ mice showed an increased number of neurons stained with nitrotyrosine (Fig. 8A), as well as stained with iNOS, indicating an induction of oxidative damage (Fig. 8B). Furthermore, *Gcdh*$^{-/-}$ positive neurons were observed only in WT mice, as expected (Fig. 8B, left panel).

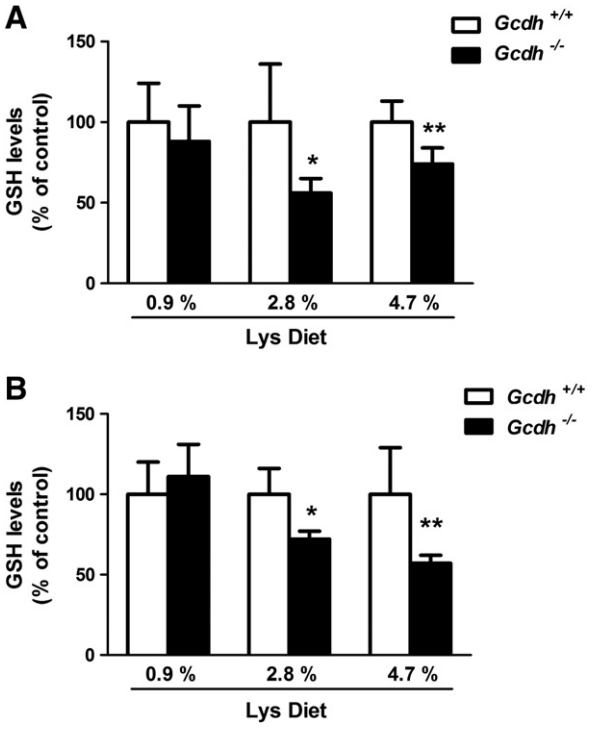


**Fig. 3.** Effect of lysine (Lys) dietary overload on GSH levels in the cerebral cortex (A) and the striatum (B) of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: A 0.9%: 8.35 ± 2.56; 2.8%: 9.74 ± 3.57; 4.7%: 5.86 ± 0.79; B 0.9%: 10.2 ± 0.95; 2.8%: 16.2 ± 2.71; 4.7%: 12.8 ± 2.93; [nmol GSH/mg protein]). *$P<0.05$, **$P<0.01$, compared to WT mice (Student's *t*-test for unpaired samples).

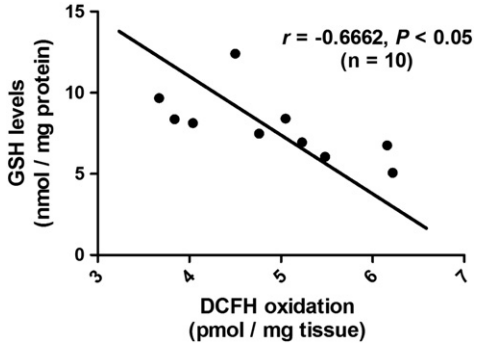


**Fig. 4.** Correlation between GSH levels and DCFH oxidation in the cerebral cortex of *Gcdh*$^{-/-}$ *mice on a high Lys diet*. Mice were fed with a 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet and GSH concentrations (nmol/mg protein) and DCFH oxidation (pmol/mg tissue) correlated.

## 4. Discussion

A large number of works investigated the molecular mechanisms implicated in the neuropathology of GA I, but the exact underlying processes are still poorly established probably because most studies were carried out in the brain tissue with normal GCDH activity.

Considering that astrogliosis (reactive astrocytes), that is usually associated with increased production of reactive oxygen species, was found in the striatum and cerebral cortex of the *Gcdh*$^{-/-}$ mice submitted to 4.7% dietary Lys [39] and also in the brain of GA I patients [4,8,9], it seems justified to investigate redox homeostasis in Lys-treated *Gcdh*$^{-/-}$ mice. In this regard, we have recently verified that oxidative stress is induced in vivo in the striatum and cerebral cortex of 30-day-old *Gcdh*$^{-/-}$ mice submitted to an acute intraperitoneal injection of Lys [40]. However, some of these results were obtained in tissues from symptomatic mice with hypoactivity and partial paralysis. In the present work we comprehensively evaluated oxidative stress parameters in brain structures (cerebral cortex, striatum and hippocampus) and peripheral tissues (liver and heart) from young (30-old-day) asymptomatic WT and *Gcdh*$^{-/-}$ mice chronically exposed to a normal (0.9% Lys) or special diets enriched with Lys (2.8% and 4.7%) for 60 h.

The utilization of a moderately increased (2.8%) Lys diet aimed to test whether this treatment could induce disruption of redox homeostasis without causing severe symptoms or death in the Lys supplemented animals, which occurs in *Gcdh*$^{-/-}$ mice receiving a 4.7% Lys enriched dietary supplementation [38]. It is of note that 2.8% Lys-treated animals did not present symptoms, whereas the 4.7% Lys dietary supplementation provoked hypoactivity in approximately one fourth of the *Gcdh*$^{-/-}$ mice at the time of sacrifice (60 h of diet). However, our objective was first to compare parameters of redox homeostasis between WT and *Gcdh*$^{-/-}$ asymptomatic mice, and therefore we only used animals with no symptoms in these experiments.

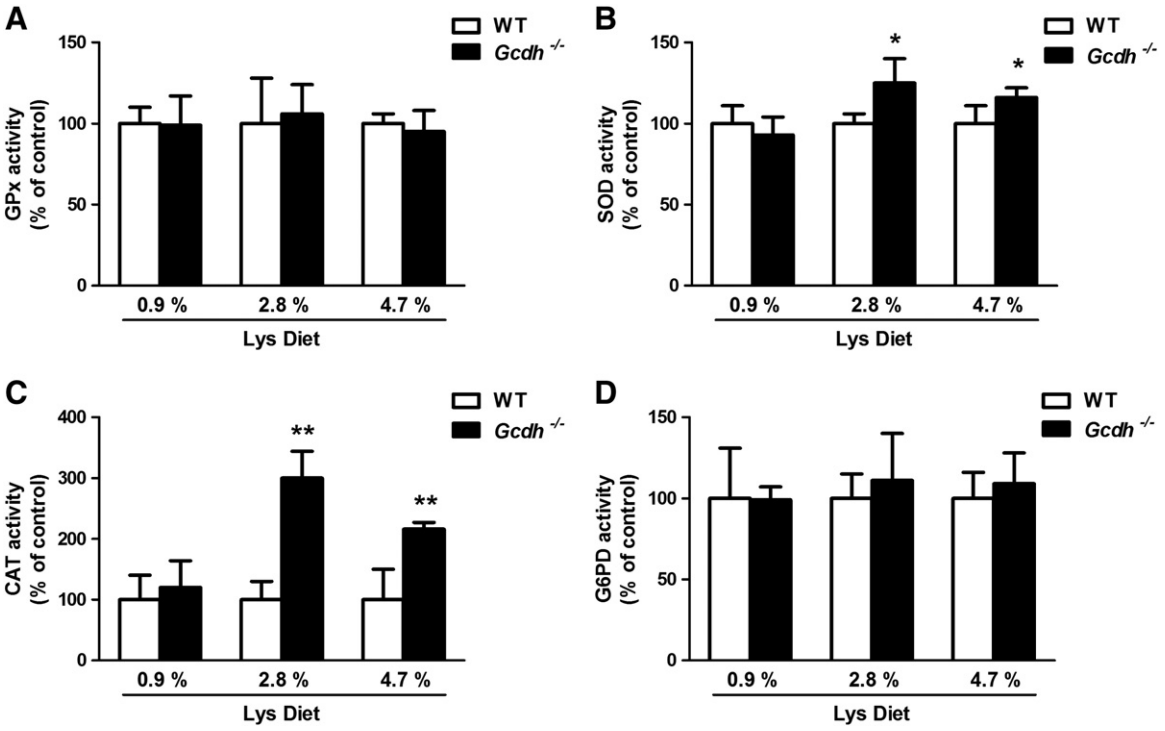


**Fig. 5.** Effects of lysine (Lys) dietary overload on the activity of the antioxidant enzymes glutathione peroxidase (GPx; A), superoxide dismutase (SOD; B), catalase (CAT; C) and glucose-6-phosphate dehydrogenase (G6PD; D) in the cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: GPx: 0.9%: 14.3 ± 1.46; 2.8%: 12.0 ± 3.36; 4.7%: 13.7 ± 0.90; SOD: 0.9%: 7.90 ± 0.86; 2.8%: 6.83 ± 0.43; 4.7%: 6.98 ± 0.78; CAT: 0.9%: 0.46 ± 0.03; 2.8%: 0.30 ± 0.12; 4.7%: 0.73 ± 0.18; G6PD: 0.9%: 5.34 ± 1.00; 2.8%: 4.76 ± 0.72; 4.7%: 4.93 ± 0.82; [U/mg protein]). *$P$<0.05, **$P$<0.01, compared to WT mice (Student's $t$-test for unpaired samples).

Overall we found that the evaluated parameters of redox homeostasis did not differ between WT and *Gcdh*$^{-/-}$ mice receiving a normal chow (0.9% Lys) in all brain structures and peripheral tissues, which is in accordance with our previous results [40] and reinforces the view that under basal conditions cellular redox status is preserved in the *Gcdh*$^{-/-}$ animals under a normal diet. However, chronic dietary Lys overload provoked a selective disruption of redox homeostasis in the brain. We observed that asymptomatic *Gcdh*$^{-/-}$ mice exposed to either a 2.8% or a 4.7% Lys enriched diet presented significant and similar alterations of oxidative stress parameters in the cerebral cortex and striatum, but not in hippocampus, liver and heart, as compared to WT mice. The data described in details below indicate that 2.8% Lys diet was sufficient to compromise redox homeostasis in *Gcdh*$^{-/-}$ mice and that the brain is more vulnerable to Lys overload, as compared to peripheral structures.

Significantly increased TBA-RS (malondialdehyde) levels were demonstrated in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice after 60 h of high (2.8% or 4.7%) Lys diet. These findings reflect

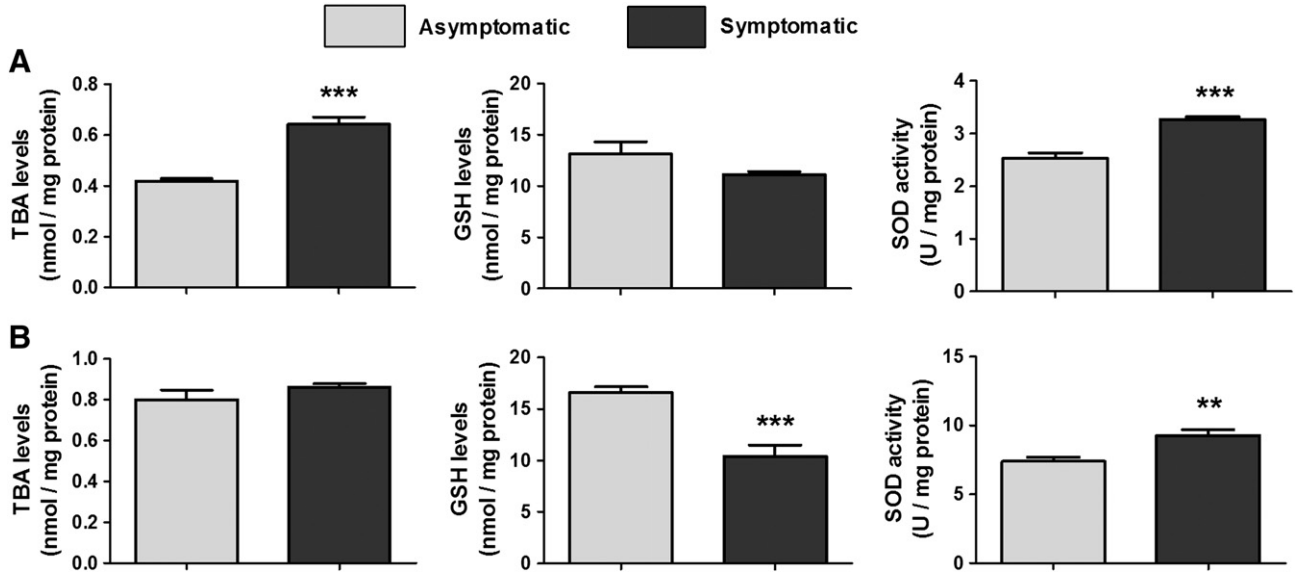


**Fig. 6.** Oxidative stress parameters (TBA-RS levels, GSH concentrations and SOD activity) in the cerebral cortex (A) and the striatum (B) of asymptomatic and symptomatic *Gcdh*$^{-/-}$ mice fed a 4.7% Lys diet. Values are mean ± standard deviation for 5–6 independent experiments (animals). Data were presented as nmol TBA/mg protein, nmol GSH/mg protein and U/mg protein for TBA-RS levels, GSH concentrations and SOD activity, respectively. **$P$<0.01, ***$P$<0.001, compared to asymptomatic mice (Student's $t$-test for unpaired samples).

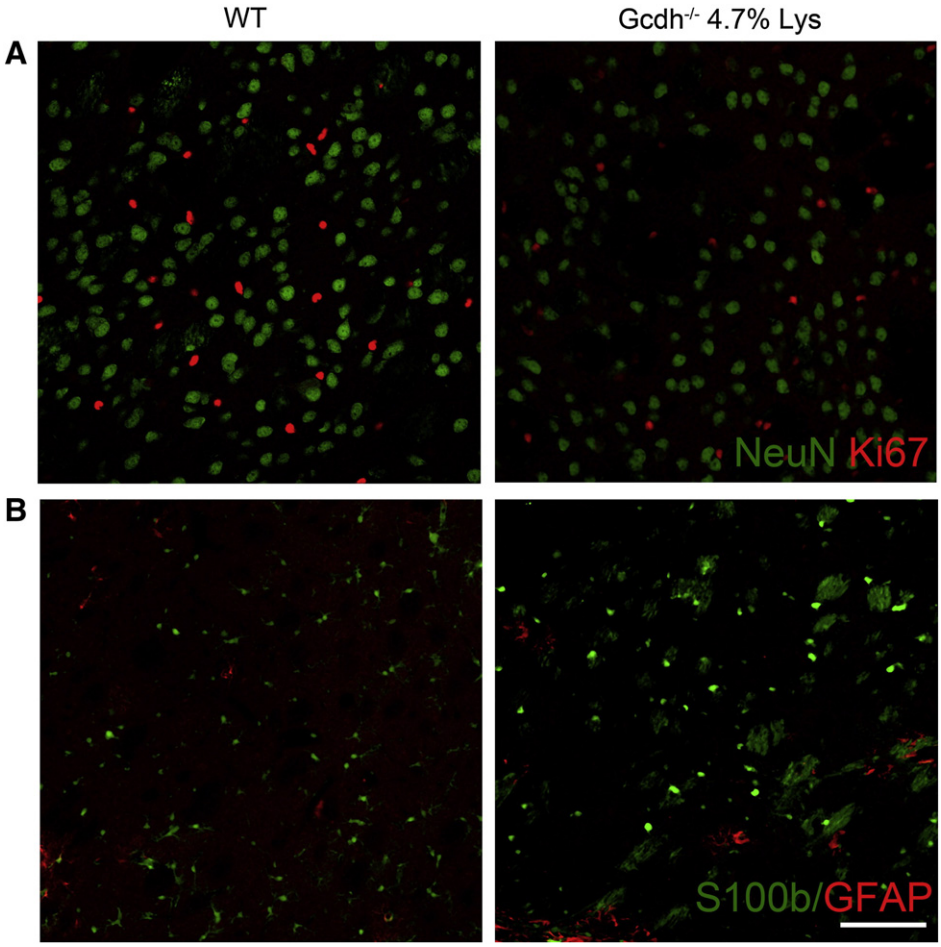


**Fig. 7.** Histopathological findings in the striatum from the *Gcdh*$^{-/-}$ and WT mice on a high Lys (4.7%) diet. (A) *Gcdh*$^{-/-}$ striatal neuronal NeuN positive population did not suffer significant changes neither in the number nor in the gross immunoreactivity, as compared to WT mice (left panel). Furthermore, Ki67 immunostaining was similar in *Gcdh*$^{-/-}$ and WT mice, discarding a significant injury response. (B) No changes in S100b+ and GFAP+ cell number were observed in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, discarding prominent astrogliosis, although a mild increase in GFAP immunoreactivity and an apparent swelling of S100b positive cells were observed in comparison to WT mice (left panel). Calibration bar indicates 50 μm.

induction of lipid peroxidation since malondialdehyde is an end product of membrane fatty acid oxidation [57]. We also observed in *Gcdh*$^{-/-}$ mice fed a high Lys diet a marked increase of DCFH oxidation, which is considered a reliable measurement of reactive species formation [58]. It is therefore presumed that the lipid oxidative damage observed following Lys supplementation could be secondary to the induction of reactive species generation.

Regarding GSH, its levels were significantly reduced in the cerebral cortex and striatum of the *Gcdh*$^{-/-}$ animals that received 2.8% or 4.7% Lys diets. Considering that endogenous GSH, the major naturally-occurring brain antioxidant, is used to evaluate the non-enzymatic antioxidant capacity of a tissue to prevent the damage associated with free radical processes [57], it can be presumed that the antioxidant defenses in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice were compromised by Lys enriched diets.

A significant inverse correlation between GSH concentrations and DCFH oxidation (DCF levels) was also demonstrated in the present study, suggesting that the decrease of the brain antioxidant defenses was probably secondary to increased induction of reactive species formation. Therefore, it is feasible that the marked decline of this important cerebral antioxidant defense (GSH) reflects the rapid consumption of this antioxidant by increased reactive species. Otherwise, we cannot exclude the possibility that Lys-induced lipid oxidative damage may have occurred due to GSH depletion.

Moreover, SOD and CAT activities were increased in the cerebral cortex of *Gcdh*$^{-/-}$ animals treated with either 2.8 or 4.7% Lys, with no alterations in the other structures evaluated. In this context, it is widely known that reactive species may upregulate the expression of numerous genes via signaling mechanisms [57,59,60]. Therefore, we presume that overexpression of SOD and CAT at the gene level might have taken place in order to compensate the increased formation of reactive species (superoxide and hydrogen peroxide, respectively), as previously observed in other pathological conditions with oxidative stress [16,61,62].

Our present data indicate that the cerebral cortex and the striatum are more vulnerable to oxidative stress than hippocampus and the peripheral tissues (heart and liver) of *Gcdh*$^{-/-}$ mice submitted to high Lys diet, which mimic the human condition evidencing that these brain regions are more affected and present structural abnormalities in GA I patients.

Interestingly, in a separate set of experiments we compared the oxidative stress parameters in the cerebral cortex and striatum from symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys diet. Symptomatic *Gcdh*$^{-/-}$ mice presented a more accentuated disruption of redox homeostasis, as compared to asymptomatic animals, indicating a probable association between neurologic symptoms and disturbance of redox status.

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue, our present results strongly indicate that oxidative stress, a deleterious cell condition leading to tissue damage, occurred in *Gcdh*$^{-/-}$ mice submitted to high Lys supplementation [57]. At this point, it

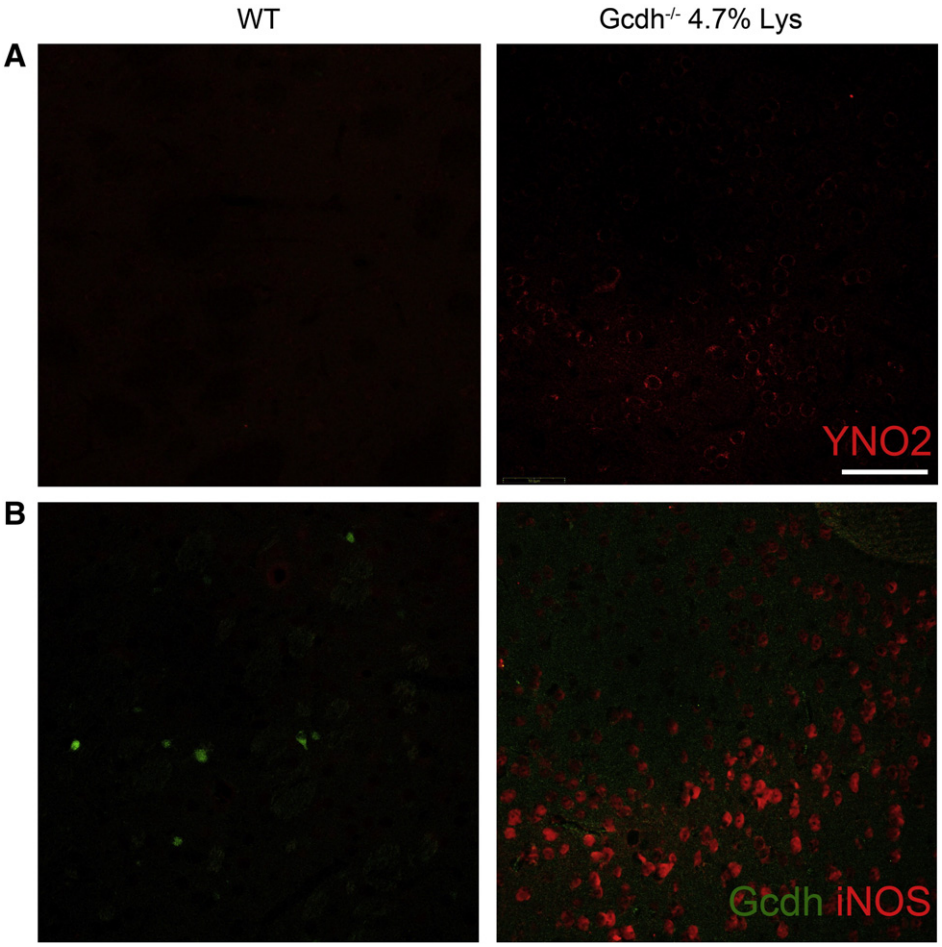


**Fig. 8.** Oxidative damage markers in the striatum from the *Gcdh*$^{-/-}$ and WT mice on a high Lys diet. (A) Striatal sections from the *Gcdh*$^{-/-}$ mice showed an increased number of nitrotyrosine (YNO2) positive cells and an impressive increase in the number of inducible nitric oxide synthetase (iNOS) (B), as compared to WT mice (left panels). Furthermore, *Gcdh*$^{-/-}$ positive neurons were observed only in WT mice (B). Calibration bar indicates 30 μm.

should be emphasized that the brain has low antioxidant defenses, a high metabolic rate associated with superoxide generation and increased amounts of polyunsaturated fatty acids compared with other tissues, that make this tissue more susceptible to free radical attack [57,63]. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, as well as in epileptic seizures and demyelination [63–71].

On the other hand, it is known that Lys can easily cross the blood–brain barrier in young mice being thereafter converted to GA in the central nervous system [72]. In this scenario, it has been demonstrated that the brain levels of GA increase considerably in *Gcdh*$^{-/-}$ mice supplemented by a high Lys diet [38,39] and this was probably the case in our present study. Taken into consideration these observations and also results from previous studies performed in vitro and in vivo in rat brain showing that GA is able to elicit oxidative stress in this tissue [11,15,26–28], it is conceivable that GA was possibly involved in the disruption of cell redox homeostasis as here observed.

On the other hand, it is unlikely that brain Lys accumulation could be responsible for the results obtained in the present study since it was previously shown that serum and brain Lys levels did not differ between WT, symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice fed a 4.7% Lys supplemented diet [38]. Therefore, distinctly from GA that significantly accumulates in the brain from the knockout mice with high Lys diet, it is presumed that Lys was not responsible to the compromised redox homeostasis observed in the brain of these animals.

Our results on the histopathological findings indicate that the brain of *Gcdh*$^{-/-}$ mice treated with high Lys (4.7%) did not show major structural alterations upon 60 h of treatment. This is interesting and suggests that the significant alterations of oxidative stress parameters were not due to cell death in the central nervous system. On the other hand, we cannot ascertain whether the data described by Zinnanti and collaborators [39] showing an impairment of energy homeostasis paralleled with striatum abnormalities in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice were consequence or cause of brain injury since the biochemical alterations with reduced ATP and phosphocreatine concentrations were obtained during or after neuronal loss and astrogliosis. Furthermore, the increased number of neurons stained with nitrotyrosine (YNO2) and iNOS in striatum of *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys diet reinforces our findings indicating oxidative damage in the brain of these animals. Moreover, the fact that neuronal NeuN staining was weaker and more diffuse and the observations that S100 + cells appeared swollen with increased immunoreactivity in high Lys treated *Gcdh*$^{-/-}$ animals than in WT mice suggest that neural cells may suffer metabolic perturbations previously to lose viability and that oxidative damage may be associated with these findings representing a relevant pathomechanism of ulterior brain injury in the *Gcdh*$^{-/-}$ mice fed a high Lys diet [73]. This is probably the case since symptomatic *Gcdh*$^{-/-}$ mice fed 4.7% Lys presented a more accentuated disruption of redox homeostasis in the cerebral cortex and striatum than asymptomatic *Gcdh*$^{-/-}$ mice, suggesting that oxidative stress was probably associated with the symptoms.

Furthermore, considering that one-fourth of *Gcdh*$^{-/-}$ mice fed a 4.7% Lys supplemented diet present hypoactivity after 48–72 h and die 5 to 7 days later [[], our own observations], whereas the animals with the same genetic background submitted to 2.8% Lys dietary intake are practically asymptomatic for a long time (even 14 days after diet start), it is conceivable that a moderately increased Lys overload (2.8%) may represent a better model of GAI, allowing investigation of underlying mechanisms responsible for brain damage in this disorder.

The present results are in line with the current therapeutic procedure of restricting Lys dietary intake to GA I patients, especially in the first few years of life, where the brain is highly vulnerable to degeneration [3,23]. Furthermore, we have proven that a 3-fold higher Lys diet (2.8%) applied to the genetic mice model established by Koeller and collaborators [37] does not induce death. It seems feasible that this new GA I model using 2.8% Lys-supplemented diet may represent a valuable tool to study long term effects of the putative toxic accumulating metabolites in the central nervous system given the increased number of children who are growing to adolescence and adulthood due to neonatal screening and/or aggressive treatment. The use of this model may be also essential for the development of novel specific therapies.

In conclusion, the present results provide experimental evidence that the cerebral cortex and striatum of 30-day-old *Gcdh*$^{-/-}$ mice submitted to high Lys intake are particularly susceptible to oxidative stress. Furthermore, disruption of redox homeostasis was more evident in symptomatic *Gcdh*$^{-/-}$ mice exposed to a high Lys diet indicating an association between symptoms and alterations of brain redox status in this model of GA I. However, we cannot rule out that other mechanisms such as excitotoxicity, mitochondrial dysfunction and breakdown of the blood–brain barrier [31,33–35,74–77] also contribute in a synergistically way to cause brain damage in GA I.

## Conflicts of interest

There are no conflicts of interest between the authors.

## Acknowledgments

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