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ORIGINAL PAPER

Biochemical regulatory processes in the control of oxidants and antioxidants production in the brain of rats with iron and copper chronic overloads

Christian Saporito-Magriñá[1] · Fabiana Lairion[1] · Rosario Musacco-Sebio[1] · Julian Fuda[2] · Horacio Torti[2] · Marisa Gabriela Repetto[1,3]

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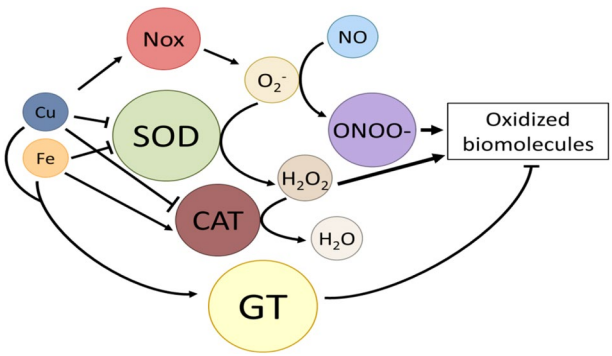
Abstract

Iron [Fe(II)] and copper [Cu(II)] overloads in rat brain are associated with oxidative stress and damage. The purpose of this research is to study whether brain antioxidant enzymes are involved in the control of intracellular redox homeostasis in the brain of rats male Sprague–Dawley rats (80–90 g) that received drinking water supplemented with either 1.0 g/L of ferrous chloride ($n = 24$) or 0.5 g/L cupric sulfate ($n = 24$) for 42 days. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione transferase (GT) activities in brain were determined by spectrophotometric methods and NO production by the content of nitrite concentration in the organ. Chronic treatment with Fe(II) and Cu(II) led to a significant decrease of nitrite content and SOD activity in brain. Activity of NADPH oxidase increased with Cu(II) treatment. Concerning Fe(II), catalase and GT activities increased in brain after 28 and 4 days of treatment, respectively. In the case of Cu(II), catalase activity decreased whereas GT activity increased after 2 and 14 days, respectively. The regulation of redox homeostasis in brain involves changes of the activity of these enzymes to control the steady state of oxidant species related to redox signaling pathways upon Cu and Fe overload. NO may serve to detoxify cells from superoxide anion and hydrogen peroxide with the concomitant formation of peroxynitrite. However, the latest is a powerful oxidant which leads to oxidative modifications of biomolecules. These results suggest a common pathway to oxidative stress and damage in brain for Cu(II) and Fe(II).

Graphical abstract



Keywords Iron · Copper · Brain · Redox dyshomeostasis · Oxidative stress · Antioxidants

Abbreviations

ATP7A	ATPase copper exporter protein
BBB	Blood–brain barrier
CDNB	1 Chlorine-2,4 dinitro benzene

✉ Marisa Gabriela Repetto
mrepetto@ffyb.uba.ar

Extended author information available on the last page of the article

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C_{50}	Metal content necessary to produce the half of the maximal effect
Ctr1	Copper transporter receptor 1
Cu	Copper
Cu(II)	Divalent copper ion
Fe	Iron
Fe(II)	Divalent iron ion
GPx	Glutathione peroxidase
GS-DNB	Glutathione-dinitrobenzene
GSH	Glutathione
GT	Glutathione transferase
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NO^-	Nitroxyl anion
NO^+	Nitrosonium cation
NO	Nitric oxide
N_2O_3	Dinitrogen trioxide
O_2	Oxygen
$ONNO^-$	Peroxynitrite
O_2^-	Superoxide anion
$^1O_2$	Singlet oxygen
RNS	Reactive nitrogen species
ROOH	Organic hydroperoxides
$ROO^{\cdot}$	Hydroperoxyl radical
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
$t_{1/2}$	Time necessary to produce the half of the maximal effect
Tf	Transferrin
TfR	Transferrin receptor

Introduction

The excessive generation of reactive oxygen and nitrogen species (ROS and RNS respectively), oxidative stress and the consequent oxidative damage along with autoimmune and inflammatory processes contribute to the pathogenesis and development of neurodegenerative disorders as Parkinson, Alzheimer, amyotrophic lateral sclerosis, Friedreich`s ataxia, and Huntington diseases [1–3].

Reactive oxygen and nitrogen species are continuously generated in living organisms due to aerobic respiration and extracellular stimuli [4]. These species are necessary for important biological and physiological functions, but when their levels surpass the physiological threshold and can no longer be controlled by antioxidant reservoirs, oxidative stress ensues. This condition sustained in time leads to irreversible oxidative modifications of biomolecules and eventually their loss of function [5].

The concept of oxidative stress as an imbalance between pro-oxidants and antioxidants, in favor of the former ones [6, 7] acknowledges the physiological production de oxidants and the existence of operative antioxidants. Oxidative stress implies that there is a controlled situation of highly dynamic quasi-equilibrium between oxidants and antioxidants in physiological conditions. The steady-state concentration of reactive species (hydrogen peroxide, H_2O_2: 1×10^{-8} M, superoxide anion, O_2^-: 2.5×10^{-11} M, hydroperoxyl radical, $ROO^{\bullet}$: 2×10^{-9} M, singlet oxygen, $^1O_2$: 1×10^{-16} M and hydroxyl radical, $OH^{\bullet}$: 6×10^{-18} M) [5] depends on the balance between the rate of production of these species and their rate of removal. The function of antioxidant enzymes is to decrease the production rate and increase the rate of all removal processes [5]. H_2O_2 and organic hydroperoxides (ROOH) are among all ROS quantitatively the predominant species by factor of $10–10^6$ [5]. However, in pathological situations the steady state concentration of specific ROS may rise above others not accompanied by the rest and the relative concentrations may vary [5].

The redox hypothesis amplifies the classical concept of oxidative stress, stating that oxidative stress is due to oxidative processes which alter the redox balance of the thiol groups, low molecular weight molecules as glutathione (GSH) or proteins involved in signaling pathways and regulation of physiological functions. ROS such as O_2^-, H_2O_2, $HO^{\bullet}$ and the secondary or final products from lipid or protein oxidation ($^1O_2$, $ROO^{\bullet}$, $RO^{\bullet}$, ROOH) are responsible for the oxidation of thiol groups [8].

The concept of nitrosative stress is used to describe the cellular damage produced by nitric oxide (NO) and other RNS as peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), nitroxyl anion (NO^-) and nitrosonium cation (NO^+) and is implicated in the pathogenesis of neurodegenerative diseases [9]. NO is involved in the control of physiologic functions and pathologic processes, the steady state concentration of NO is 5×10^{-8} M. In physiologic situations, this molecule acts as a modulator of neurotransmission processes, immune response and regulator of vessel tone. However, if produced in excess, NO becomes toxic for cells, tissues and organs, undergoing redox reactions and promoting cellular damage and nitrosative stress. The excessive production of NO, generated as a consequence of a pro-inflammatory response and loss of endogenous antioxidants content such as GSH in glial cells may be an important source of increased $ONOO^-$ levels in Parkinson disease [3] as well as the direct interaction of NO with thiol groups of specific proteins can trigger neurodegeneration and could explain the association between nitrosative stress and Alzheimer disease [10, 11].

The concept of stress is associated to adaptation, as a general association of stress with stress response [12]. Oxidative stress is a reversible process whereas oxidative damage is considered irreversible. These processes are associated to the

participation of transition metals such as iron (Fe) and copper (Cu) in oxidative reactions. The redox active ions of these metals, Fe(II) and Cu(II), are able to undergo intracellular redox cycling reactions and are thereby potentially toxic for cells [13–18]. Fe(II) and Cu(II) are involved in the production of strong oxidants in the brain, as H_2O_2 and ROOH, and free radicals such as $HO^{\bullet}$, $ROO^{\bullet}$ and the alkoxyl radical ($RO^{\bullet}$) [18], generated through Fenton's reaction [19].

A previous research showed that acute Fe(II) and Cu(II) overloads induces oxidative stress and damage in in liver [13] and brain [14] with changes in the activity of antioxidant enzymes [15, 16]. In liver of rats exposed to chronic Fe(II) and Cu(II) overloads, the decrease of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity is a common response to reduce oxidative processes, controlling the O_2^- and H_2O_2 intracellular concentration [20]. However, the biochemical pathways involved in the regulation of redox homeostasis and control of oxidative processes in the liver are different for Fe(II) and Cu(II) chronic overloads. Oxidative damage for chronic Fe(II) overload is controlled by reductive molecules as GSH and NADPH in liver as a response to lipid and protein oxidation induced by Fenton–Haber Weiss free radical reactions with ROOH and $ONOO^-$production. Cu(II) overload involves NO as a signaling molecule associated to the upregulation of antioxidant enzymes activities to decrease and prevent the liver damage [20].

The brain is extremely sensitive to oxidative damage because it metabolizes 20% of total oxygen used by the body at rest, has a high content of oxidizable molecules such as polyunsaturated fatty acids and a high content of several transition metals [21]. The accumulation of metals, ROS, RNS and their oxidation products in brain leads to oxidative and nitrosative stress and damage that culminates in cytotoxicity and eventual cell death [21].

The mechanisms involved in the intracellular protection against oxidative damage associated to the chronic overloads of Fe(II) and Cu(II) in brain may be useful in understanding the biochemical processes responsible of the toxic effects of redox active metals in neurodegenerative disorders and pathologies associated to metal accumulation as Wilson´s disease [22, 23].

The aim of this research is to study whether brain antioxidant enzymes are involved in the control of intracellular redox homeostasis in the brain of rats exposed to chronic iron and copper overload.

Experimental

Animal and housing

Adult male rats of 80–90 g were purchased from School of Pharmacy and Biochemistry, housed in cages of $44 \times 29 \times 15$ cm, and received standard pellet food (GANAVE, Argentina) and water ad libitum. After coming from the Animal House of this institution, rats were acclimatized to laboratory conditions for 7 days before the experiments in controlled laboratory conditions (24– °C, 45–50% humidity and alternating 12 h light–dark cycles). Animals were sacrificed at the corresponding time between day 0 and 42 of treatment [20], and animal care was given in compliance with the Guidelines for Ethical Treatment in Animal Experimentation of the American Physiological Society (Bethesda, MD, USA), according to the Argentine Regulations (ANMAT). The School of Pharmacy and Biochemistry has approved this protocol (*Comité Institucional de Uso y Cuidado de Animales de Laboratorio*, CICUAL-FFyB, Res. D 3685/16).

Experimental model of in vivo Fe and Cu chronic overloads

Rats received chronic overload of either ferrous chloride ($FeCl_2$, 1.0 g/ L, 0.1% w/v, $n = 24$) or cupric sulfate ($CuSO_4$, 0.5 g/ L, 0.05% w/v, $n = 24$) in drinking water and the standard diet ad libitum for 42 days [24]. Animals that received the same diet and drinking water without metals were considered as the control group ($n = 27$). The limit in metal concentration established by most national and supranational regulations for drinkable water is 0.2–0.3 mg/L of Fe (RD 140 in Spain, European Commission) and 1.3 mg/mL of Cu (US Environmental Protection Agency).

The dose of metals used in this experiment was assayed in a previous research by Musacco Sebio et al. [17] and Lairion et al. [20]. The chosen concentration for iron chronic overload was 1.0 g $FeCl_2$/L (dose: 7–19 mg Fe(II)/day or 60–90 mg Fe(II)/day/kg) and 0.5 g $CuSO_4$/L for copper treatment (dose: 3–9 mg Cu(II)/day or 30–40 mg Cu(II)/day/kg) because they are toxic to rats but are below LD_{50} (150 mg Fe(II)/day/kg and 60 mg Cu(II)/day/kg) [20].

A total of 75 animals were used divided into 9 groups of 3 control animals for controls and 8 groups of 3 animals for each treatment: (Fe(II) or Cu(II), each group was sacrificed at different times from the beginning of the experiment (after 2 days, 4 days, 7 days from the start of treatment and subsequently every 7 days until 42 days.) The control group included three animals at time zero. The number of biological replicates and independent animals used for measurement of each data point in the experiments was three.

This research protocol includes experiments that cause no or minimal stress or discomfort in male Sprague Dawley rats. The administration of chemical agents in the chronic model was carried out by adding the agent to the drinking water. The animals were checked daily regarding their health status. While no blood tests were carried out, and no changes in pulse rate or temperature were evidenced, a decreased

locomotor activity and higher anxiety-like behavior in open-field tests of Cu(II) overloaded rats were observed (data not shown).

Post-operative management consists of euthanasia of previously anesthetized animals by cervical dislocation [25]. The application of an intraperitoneal injection for the administration of anesthesia (urethane, dose 1 g/kg ip), for which no clinical effects other than the discomfort of the injection are expected. The type of immobilization used for surgical procedures, to obtain samples of the tissues under study, was of a chemical type, with rats anesthetized with intraperitoneal injection of urethane.

Iron and copper content in brain

The contents of Fe and Cu in the brain were determined by atomic absorption. After acid mineralization, samples of about 100 mg were incinerated for 4 h in a graphite muffle at 500 °C until the disappearance of brown colored vapors. Standard solutions provided by Merck (from 0 to 3 mg/ L) were used for calibration of the assay that was made in an atomic absorption spectrometer (Bück model 200A, East Norwalk, CT, USA). The results were expressed in µg of metal per g of wet organ [26].

Brain homogenate preparation

Brain homogenates were prepared with organs rapidly excised, weighed, cut and washed with ice cold saline solution as was previously indicated by Semprine et al. [14, 16, 18] from the original methods [27, 28].

Redox homeostasis in brain

Indirect measurement of nitric oxide production

The content of nitric oxide (NO) in brain homogenates was determined by the accumulation of nitrite, as a screening assay to determine whether NO synthesis was involved in Cu and Fe overload. Samples were mixed with 1% sulfanilamide and 0.1% naphthyl-ethylenediamine for 10 min at 25 ºC, and nitrite concentration was measured spectrophotometrically at 550 nm with the Griess reaction, with standard solutions of sodium nitrite at different concentration. The results were expressed as µmol/ mg protein [29].

Activity of NADPH oxidase system as a cellular source of superoxide anion

The activity of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) enzyme system in brain homogenates was determined by measuring the consumption of NADPH (100 µM) by decreasing the absorbance at 340 nm ($e_{340} = 6.22 \times 10^3\ M^{-1}\ s^{-1}$) every 10 min, for 30 min at 37 °C. As NADPH is oxidized by a wide variety of enzymes besides NADPH oxidase, to verify that the NADPH consumption measured is primarily from NADPH oxidase, apocynin (200 µM) was used as a control for this assay. Enzyme activity was expressed in µmoles of NADPH per min per g of organ [30].

Antioxidant enzyme activity in brain

The enzymatic activities of superoxide dismutase (SOD, Cu, Zn-SOD, SOD1), catalase, glutathione peroxidase (GPx) and glutathione transferase (GT) were determined by spectrophotometric methods to obtain new insights regarding participation of antioxidant enzymes in the toxic mechanism of Cu and Fe. Therefore, this research focused on studying their activity rather than their expression as it should better correlate with the actual protection against the damage induced by such metal overload. SOD was measured as the inhibition of the autocatalytic adrenochrome formation rate at 480 nm [31]. Results were expressed in units of SOD (amount of cytosolic SOD that produces 50% inhibition) per g of organ. For adrenochrome 1 unit = 0.15/g/mL of SOD = 4 nM of SOD [32]. Catalase activity was determined by measuring the decrease in absorption at 240 nm due to the dismutation of hydrogen peroxide (H_2O_2) [33]. The results were expressed as units equivalent to pmoles of H_2O_2 consumed per g of organ [34]. GPx activity was assayed following the oxidization of NADPH at 340 nm. The activity was expressed in nmoles of GPx per min per g of organ [35]. The determination of the GT activity was carried out using the reagent 1 chlorine -2.4 dinitro benzene (CDNB), which in the presence of glutathione forms GS-dinitro benzene (GS-DNB), absorbs at 340 nm. Enzyme activity was expressed in units (U) per g of organ. One U equals 1 µmol of GSH per minute [36].

Evaluation of brain metal content and time necessary to produce the half of the maximal antioxidant effect

The rat brain metal content (C_{50}) and time ($t_{1/2}$) necessary to produce half of the maximal effect on biomarkers of redox homeostasis (NO, NADPH oxidase and antioxidant enzymes activities) were evaluated from the plot of enzyme activities versus the kinetics of Fe(II) and Cu(II) chronic overloads or accumulation of Fe or Cu in the brain [16, 20].

Materials

Chemicals were purchased from Sigma-Aldrich Chemical Co, St Louis, Mo.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using Anova with Tukey's post-test with the program SPSS statistics (Statistical Package for the Social Sciences). The curve fitting were made using the Sigma Plot program for windows, version 11.0, Systat Software, Inc. Differences were considered statistically significant at $p < 0.05$.

Table 1 Metal content accumulated in the brain of rats with chronic Fe(II) and Cu(II) overloads, expressed in micrograms per kilogram of organ for each treatment

Time (days)	Fe	Cu
0	34 ± 2	6 ± 1
2	40 ± 4	10 ± 3
4	44 ± 6	48 ± 4**
7	66 ± 5*	54 ± 5**
14	75 ± 3*	58 ± 7**
21	90 ± 4*	66 ± 8**
28	125 ± 6*	75 ± 5**
35	170 ± 5*	96 ± 5**
42	175 ± 5*	100 ± 6**

*$p < 0.05$; **$p < 0.01$

Results

Iron and copper content in brain

There were no differences between the amount of $FeCl_2$ (1 g/L) and $CuSO_4$ (0.5 g/L) received by the animals because the amount of water drunk by the rats and the body weight, measured daily during the 42 days of treatment, increased both as treatment time progressed [20]. However, brain Fe(II) and Cu(II) content increased significantly (94%, $p < 0.05$) after 7 days of supplementation with Fe(II) and 4 days with Cu(II) (eightfold, $p < 0.01$) (control: 34 ± 2 µg Fe/g and 6 ± 1 µg Cu/g) (Table 1).

Redox regulation and biochemical response in rat brain

Nitrite is a final metabolite of NO metabolism and may be considered as an indirect marker of NO production or NO consumption. Chronic overload with Fe(II) caused a significant decrease of 90% of nitrite content in brain at day 7 while chronic overload with Cu(II) led to a 60% decrease at day 4 (control: 133 ± 12 µmol/ g brain, $p < 0.01$). The basal values of NO production were recovered by the end of the study (Fig. 1A). The decreased nitrite concentration in brain of rats with chronic overloads of Fe(II) and Cu(II) shown in Fig. 1 indicates an early increase in NO consumption until day 14 of treatment. The estimated $t_{1/2}$ for Fe(II) was of 5 days (Fig. 1A) with a C_{50} of 100 µg Fe/ g brain (Fig. 1B) and 3 days (Fig. 1A) with a C_{50} of 23 µg Cu/ g brain (Fig. 1B). It should be noted that while the determination of nitrite

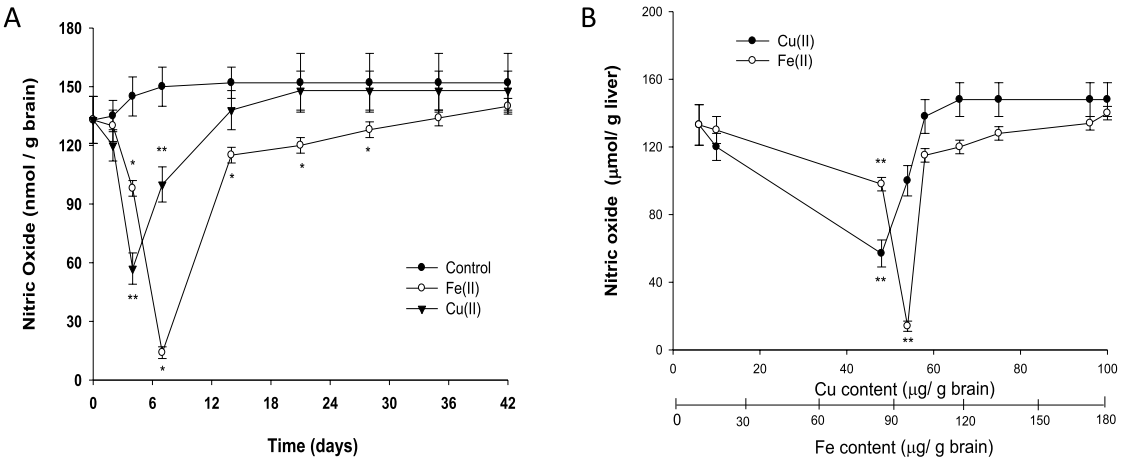

Fig. 1 Fe(II) and Cu(II) overloads decreased significantly NO in rat brain, after 7 and 4 days of the beginning of the treatment respectively. The content of nitrite due to chronic overload of Fe(II) and Cu(II) was determined and compared with respect to the control group during administration in drinking water for 42 days of Fe(II) (1.0 g/ L, 0.1%) or Cu(II) (0.5 g/ L, 0.05%). **A** With respect to time of treatment; B with respect to metal content in the brain (*, $p < 0.05$; **, $p < 0.01$)

content is useful as a simple assay to infer an enhanced flux of NO metabolism, it may be further metabolized to nitrate ions in the presence of blood. Therefore, these results are intended to be comparative between the treated and the control groups instead of absolute nitrite content in the organ.

Enzymatic response involved in the control of redox homeostasis in rat brain

Chronic Fe(II) overload did not produce significant changes in the activity of the enzyme NADPH oxidase in brain homogenates with respect to basal activity (control: 0.14 ± 0.02 µmol/min g brain). However, chronic overload with Cu(II) produced an 80% increase in the activity of the enzyme NADPH oxidase in brain homogenates with respect to the basal activity (control: 0.14 ± 0.02 µmol/min g brain), 2 days after starting the chronic treatment (Fig. 2A). The estimated $t_{1/2}$ for Cu(II) was of 1 day (Fig. 2A) with a C_{50} of 8 µg Cu/g brain (Fig. 2B).

Antioxidant enzyme response to Fe(II) and Cu(II) induced oxidative stress in brain

The activity of the cytosolic isoform of SOD was decreased 35% with chronic Fe(II) overload after 7 days of the beginning of treatment with respect to control group and 45% since day 2 of chronic treatment with Cu(II) (Fig. 3A), recovering the basal levels with time of treatment (control: 119 ± 10 U SOD/ g brain, $p < 0.01$). The estimated $t_{1/2}$ for Fe(II) overload was 3 days (Fig. 3A) with a C_{50} of 52 µg Fe/ g brain (Fig. 3B), and 1 day for Cu(II) treatment (Fig. 3A) with a C_{50} of 8 µg Cu/ g brain (Fig. 3B).

Catalase activity increased 10% ($p < 0.01$) at day 28 of chronic treatment with Fe(II) with $t_{1/2} = 14$ days (Fig. 4A), $C_{50} = 120$ µg Fe/ g brain (Fig. 4B); and decreased 54% ($p < 0{,}01$) at the second day of Cu(II) chronic administration with $t_{1/2} = 2$ days (Fig. 4A), and $C_{50} = 12$ µg Cu/ g brain (Fig. 4B) (control: 119 ± 15 pmol/g brain).

Regarding the activity of the enzyme glutathione peroxidase (GPx), no statistically significant changes were found with either of the two metals (control: 3.0 ± 0.2 µmol/min g brain).

Chronic overload with Fe(II) produced a significant increase of 13% in the activity of the enzyme glutathione transferase (GT) in brain homogenates from the fourth day after starting chronic treatment, reaching a maximum of 45% increase at from day 14. Chronic Cu(II) overload produced a 22% increase at 14 days of treatment (control: 2.3 ± 0.1 U/g brain) (Fig. 5A). The estimated $t_{1/2}$ for Fe overload was 8 days (Fig. 5A) with a C_{50} of 97 µg Fe/g brain (Fig. 5B) and for Cu 18 days (Fig. 5A) with a C50 of 61 µg Cu/g brain (Fig. 5B).

Brain metal content and time necessary to generate half of the maximal effect on biomarkers of redox control

The brain metal content (C_{50}) and the time ($t_{1/2}$) necessary to generate half of the maximal effect (50%) of NO content, NADPH oxidase, SOD, catalase and GT activities are summarized in Table 2.

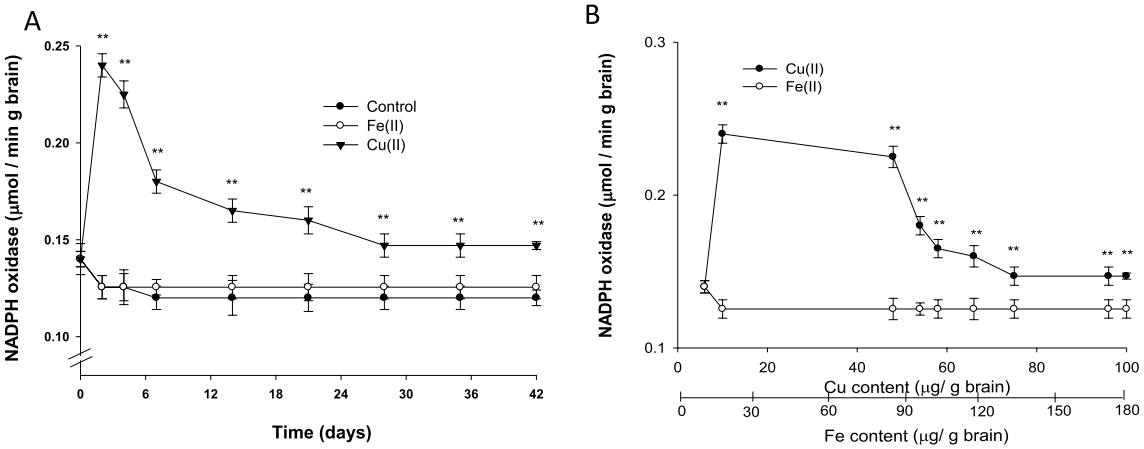

Fig. 2 Differential response of NADPH oxidase activity against chronic Fe(II) and Cu(II) overloads. Fe(II) overload did not showed changes in NADPH oxidase activity and Cu(II) overloads increased significantly this enzyme activity. Activity of the NADPH oxidase due to chronic overload of Fe(II) and Cu(II) was determined and compared with respect to the control group during administration in drinking water for 42 days of Fe(II) (1.0 g/ L, 0.1%) or Cu(II) (0.5 g/ L, 0.05%). **A** With respect to time of treatment; **B** with respect to metal content in the brain (**, $p < 0.01$)

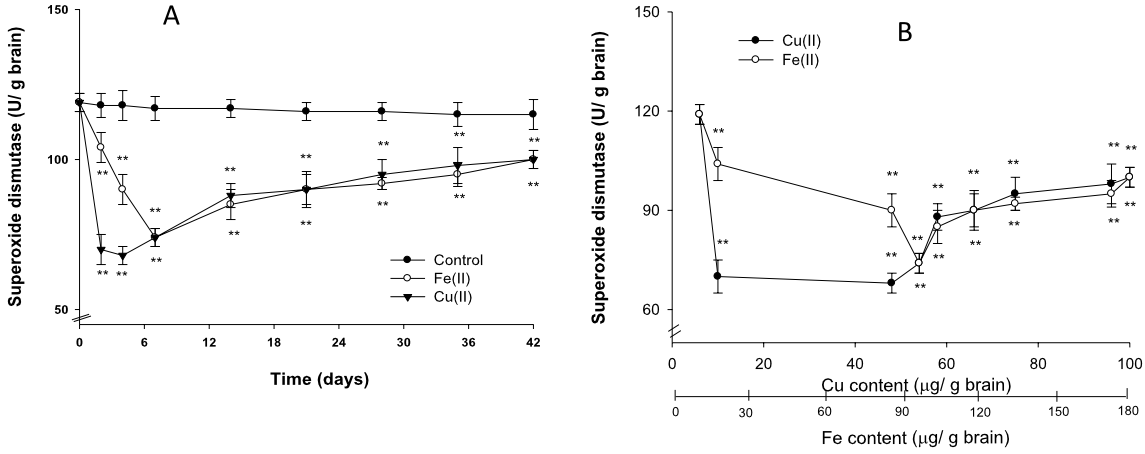

Fig. 3 Fe(II) and Cu(II) overloads decreased significantly SOD enzyme activity. Activity of the SOD due to chronic overload of Fe(II) and Cu(II) was determined and compared with respect to the control group during administration in drinking water for 42 days of Fe(II) (1.0 g/ L, 0.1%) or Cu(II) (0.5 g/ L, 0.05%). **A** With respect to time of treatment; B with respect to metal content in the brain (**, $p < 0.01$)

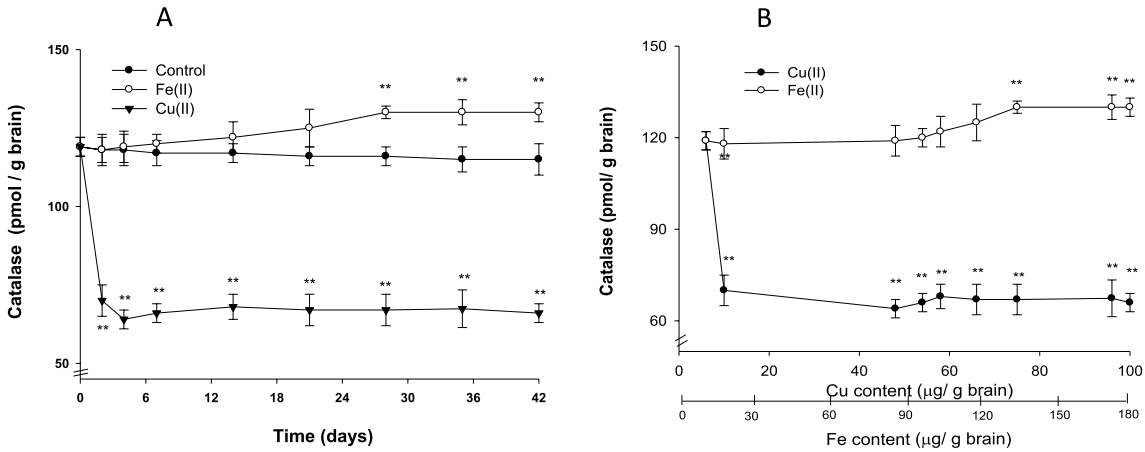

Fig. 4 Differential response of catalase activity against chronic Fe(II) and Cu(II) overloads. Fe(II) chronic overload increased catalase activity, however, Cu(II) treatment decreased the activity of the enzyme. Activity of the catalase due to overload of Fe(II) and Cu(II) was determined and compared with respect to the control group during administration in drinking water for 42 days of Fe(II) (1.0 g/ L, 0.1%) or Cu(II) (0.5 g/ L, 0.05%). **A** With respect to time of treatment; **B** with respect to metal content in the brain (**, $p < 0.01$)

Discussion

The brain of mammals actively concentrates Fe and Cu. In fact, this organ shows the highest content of these metals, after the liver. Due to the ability of Fe and Cu to participate in redox reactions, the metal content must be strictly controlled to avoid the formation of hazardous oxidant species. In humans, the physiological concentration of Fe in brain is about 210 μg/g dry brain or 40 μg/g wet brain (1.3% body weight) and that of Cu about 24 μg/g dry brain or 6 μg/g wet brain (8% body weight). The rat brain contains about 35-40 μg Fe/g wet brain and 2 μg Cu/g wet brain [37].

In the design of this research, we used a single dose of each metal (1.0 g Fe(II)/L or 0.5 g Cu(II)/ L) because such doses reproduce the oxidative damage observed in rats exposed to chronic Fe(II) and Cu(II) overloads in previous studies [17, 18, 20]. The results shown in the Table 1 were analyzed considering the change in the content of each metal in the brain of the rats that received the chronic overload with respect to its corresponding control for each day of treatment. Fe and Cu build up within the brain in a dose and time dependent manner as in the case of liver. However, there is a lag phase of around 4 days before Fe and Cu begin to accumulate within the organ. This is likely due to the presence of the blood–brain barrier (BBB), which prevents

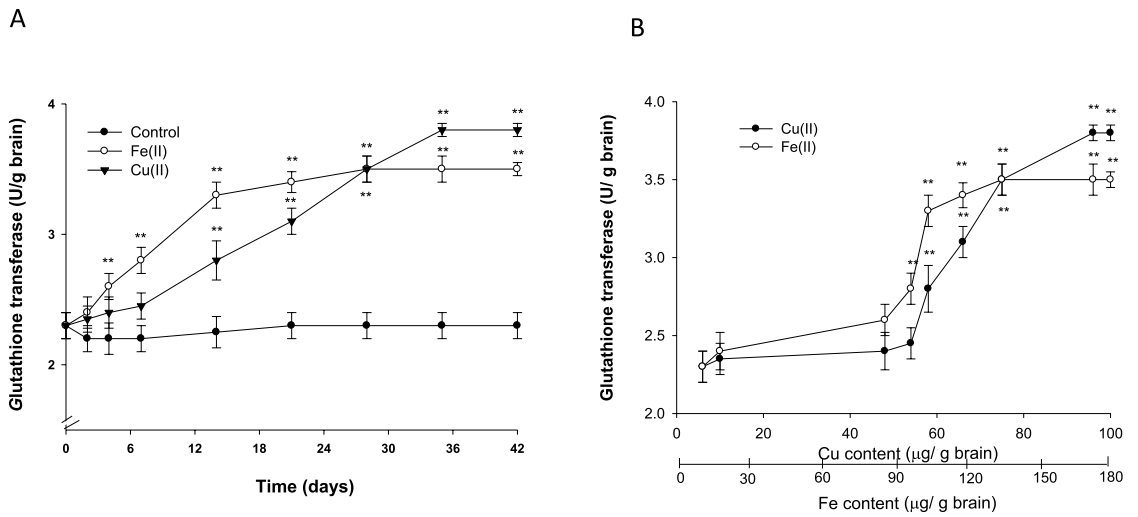

Fig. 5 Fe(II) and Cu(II) chronic overloads increased glutathione transferase activity. Activity of the glutathione transferase due to chronic overload of Fe(II) and Cu(II) was determined and compared with respect to the control group during administration in drinking water for 42 days of Fe(II) (1.0 g/ L, 0.1%) or Cu(II) (0.5 g/ L, 0.05%). **A** With respect to time of treatment; **B** with respect to metal content in the brain (**, $p < 0.01$)

Table 2 Brain metal content (C_{50}) and time ($t_{1/2}$) necessary to produce 50% of maximal effect on nitric oxide content, NADPH oxidase and antioxidant enzymes activities in brain Fe(II) and Cu(II) overloads

	C_{50} Fe (µg/g)	C_{50} Cu (µg/g)	$t_{½}$ Fe (days)	$t_{½}$ Cu (days)
Metal content	–	–	18	28
Molecules				
Nitric oxide	100	23	5	3
NADPH oxidase	–	8	–	1
Antioxidant enzymes				
Cu,Zn-SOD	52	8	3	1
Catalase	120	12	14	2
Glutathione transferase	97	61	8	18

the metals from freely diffusing into the interstitial space of the brain. Thus, allowing its entrance only through specific transporters.

The movement of Fe across the BBB is regulated and there is no passive flux from the blood to the brain for this metal. The entry of Fe into the brain parenchyma takes place through transferrin-mediated receptors (TfR) and once inside the brain, Fe is transported from the interstitial fluid to the neurons by Tf [38]. Regarding Cu, it is absorbed in the intestine as Cu(I) through the Ctr1 transporter, which is specific for this oxidation state. The reduction step from dietary Cu(II) to Cu(I) is carried out by intestinal reductases. Cu is internalized into the brain via the BBB by the transporter Ctr1 and the Cu-importing ATPase ATP7A [38] and keeping in its reduced form [Cu(I)] mainly by glutathione [39].

In rats, the chronic supplementation with either Fe(II) or Cu(II) increases metal content in brain after day 4 of treatment, with $t_{1/2}$ of 18 and 28 days, respectively (Table 2). The time profile of accumulation of Fe and Cu in the brain is much slower than the increase in the content of both metals in the liver (4 days) [20].

Fe and Cu do not freely diffuse into the brain and a well-orchestrated mechanism is required for each metal to reach the brain cells. Regarding the kinetics of metal distribution, the net flux of metal ions due to the increased loads would involve an enhanced steady state in all intermediate steps within the route to the final destination of the metal. The content of Cu or Fe ions in each overload involves increases in the interstitial space but such accumulation will be especially important in cells that will become a sink for Cu or Fe due to their capacity to store it. Therefore, once the storages can no longer prevent the accumulation of free Cu or Fe ions, metal-induced damage will ensue. Metal dyshomeostasis has been related to Parkinson's and Alzheimer's disease. We have recently determined the metal concentration specific brain areas which are also involved these pathologies such as hippocampus, striatum and cerebral cortex, in a chronic model of Cu overload. Our results indicated that in control rats there are no significant differences in metal content within the studied brain areas. Nevertheless, after 60 days of receiving daily Cu(II) in drinking water, the metal accumulation takes place especially in striatum and hippocampus (nearly a 100% increase in comparison to control rats in both areas, unpublished data).

The results obtained indicate that mild increases in Fe content in brain (20–30%) generate oxidative stress.

However, an eightfold increase in Cu content in the organ is required to generate 50% of the maximum oxidative damage after day 4 of the beginning of the treatment (Tables 1 and 2).

The intracellular mechanisms of Fe and Cu toxicity include the capacity to interfere with the function of cell membranes and enzymatic activity, its affinity for thiol groups, formation of stable complex with electron donor compounds and participation in redox reactions mediated by ROS and RNS [4].

Data showed in this research indicate that nitrite content in brain is decreased in both Fe(II) and Cu(II) overloads, possibly due to the intracellular inhibition of NO production or increased NO consumption. The NO, besides its function as a signaling molecule, has antioxidant function because it may protect cells from oxidative damage, trapping O_2^- and generating $ONOO^-$ as the reaction product, or regulating the activity of antioxidant enzymes to protect cells from oxidative damage caused by toxic agents. Nevertheless, $ONOO^-$ is a powerful oxidant itself, capable of oxidizing biomolecules [40].

Nitrite can be oxidized to nitrate in blood, but the analysis of this metabolite was performed in brain homogenate. Therefore, blood only became in contact with the interior of cells upon homogenization, after which, samples were kept in ice at all times. Additionally, the analysis was performed by comparing the values obtained from the treated groups with the values obtained from the control groups. Thereby, as increases in nitrite concentration in the treated groups were observable we could infer that the steady state of NO in the treated group was higher than the control group.

Non-phagocytic NADPH oxidase system generates small amounts of O_2^-, acting as an intracellular or extracellular signal involved in the control of cellular functions. The O_2^- and NO production mechanisms are separately regulated, but gamma interferon (IFN-γ) act as a trigger for both pathways. The simultaneous activation of both pathways may be hazardous for cells due to the formation of $ONOO^-$ and $HO^\bullet$ [41]. Fe(II) and Cu(II) chronic overloads decreased SOD activity, increasing levels of intracellular O_2^- in brain by hampering the conversion of O_2^- into H_2O_2 and oxygen (O_2).

Cytosolic SOD decreased with overload at 7 days. This is indeed very interesting because it is possible that the steady state concentration of specific oxidant species is what is actually driving the SOD response. To address this event, in a future work we will evaluate the transcriptome of brain homogenates to establish a correlation between the transcription levels and the expression levels, with the intention to obtain some insight into the transcription network that regulates these responses.

The physiological levels of H_2O_2 should not be affected by chronic Fe(II) and Cu(II) overloads because SOD activity were decreased at the beginning of the treatment. Nevertheless, O_2^- may be decreased as a consequence of the antioxidant function of NO, because O_2^- reacts with NO to produce $ONOO^-$. These processes take place earlier in rats treated with Cu(II) (Table 2).

We have previously observed in rat brain that protein oxidation and GSH consumption take place prior to the enhanced production of $HO^\bullet$ radical and the consequent phospholipid oxidation with chronic Fe(II) and Cu(II) overloads [18]. The $t_{1/2}$ for oxidative damage to proteins in brain previously reported [18] and the inactivation of SOD observed in this research are simultaneous for each metal, suggesting a common oxidative mechanism involving enzyme inactivation and loss of biological activity. GPx activity was not affected in the brain of rats with Fe(II) and Cu(II) overloads. However, increased GT activity was observed. GT becomes important in the detoxification of organic peroxides, resembling the activity of Se-dependent GPx.

Concerning Fe(II) overload in brain, no changes in NADPH oxidase activity was observed, while the SOD activity was decreased simultaneously with the decrease in the content of nitrite in the organ (Figs. 1, 2 and 3). These events are prior to the oxidative stress and damage to lipids observed previously [18]. In brain, NO consumption occurs simultaneously to the inhibition of SOD activity. The oxidation state of Fe may be modulated by O_2^- and the concentration of this species is regulated by SOD and NO [42]. These results indicate that the steady state concentration of O_2^- in brain is controlled by decreased activity of SOD and reaction with NO, because both processes have been shown to occur simultaneously in the time course. The main source of O_2^- in brain upon Fe(II) overload is due the hampered catalytic function of SOD (Fig. 6).

Regarding catalase activity, it was preserved until day 28, from when a greater enzymatic activity was observed, perhaps due to the fact that excess Fe preserves the integrity of the heme group in this enzyme. However, the early decrease in the enzymatic activity of SOD and its subsequent recovery, as well as the increase in the activity of GT and catalase, would indicate an adaptive response to oxidative stress mediated by ROS (mainly O_2^-, ROOH, H_2O_2 and $HO^\bullet$) and RNS (NO and $ONOO^-$). The identity of the crucial reactive species involved in the genesis of oxidative stress and the main mechanism associated with the antioxidant response observed in this model requires further research. However, these results outline participation of the above mentioned species (Figs. 5 and 6, Table 2).

Concerning Cu(II) overload, high levels of metal inhibits the activity of SOD and catalase, in accordance with early protein oxidation showed in a previous study of oxidative damage in brain [18]. NO is decreased in brain simultaneously with decreased of SOD and

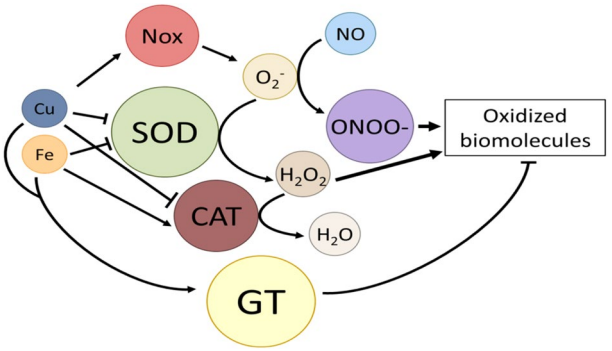

Fig. 6 Fe(II) chronic overload decreased SOD activity and increased catalase and GT activities, rising O2- levels in rat brain. Cu(II) chronic overload increased significantly NADPH oxidase activity but decreased SOD and catalase activities, rising O2- and H2O2 levels in rat brain. High levels of O2- react with NO to produce ONOO-, generating oxidative damage to lipids and proteins in rat brain. However, increased GT activity suggests antioxidant protection by decreasing levels of ROOH with Fe(II) and Cu(II). Blunt arrows (T) indicate inhibitory effects while sharp arrows (→) indicate activating effects

catalase activities, and with a concomitant upregulation of NADPH oxidase activity, sustaining high levels of O_2^-. These events are prior to oxidative stress and damage to lipid observed previously [18], while the activation of the enzyme GT occurs after a period of time as a consequence of oxidative damage and the accumulation of oxidized products ($ONOO^-$, $ROO^•$; $RO^•$ and ROOH) and metal in the organ. The activity of NADPH oxidase and SOD regulate the steady state concentration of O_2^-, increasing it by NADPH oxidase and decreasing by SOD activities. These results indicate that the steady state concentration of O_2^- in brain is controlled as a consequence of the increased activity of NADPH oxidase enzyme system and reaction with NO to form $ONOO^-$. NO can neutralize the cytotoxicity of O_2^- but generates $ONOO^-$, which is yet another powerful oxidant. The main source of O_2^- in brain of rats overloaded with Cu(II) is the activated NADPH oxidase system (Fig. 6). These effects contribute to the increase of O_2^-, H_2O_2 and $HO^•$ levels, which induce the oxidation of proteins (day 4) and phospholipids (day 10–12) previously reported [18].

In agreement with the data obtained, the slight differences in antioxidant enzyme profile observed in the responses to chronic overload of Fe(II) and Cu(II) highlight the existence of common molecules which may act as signaling compounds, and on the other hand, possible differences between the toxic mechanisms of these metals beyond their ability to induce nitrosative and oxidative stress and damage by a Fenton/Haber–Weiss mechanism that has been demonstrated in acute [13, 14] and chronic toxicity [17, 18], in liver and brain, involving cellular antioxidant response [15, 16, 20]. These results are framed within the classic concept of oxidative stress, which postulates that oxidizing species increase while endogenous antioxidants decrease [6–8].

High levels of $ONOO^-$ and ROOH in liver and brain may explain the increased levels of singlet oxygen ($^1O_2$) reported previously [13, 14, 17, 18]. The generation of $HO^•$ by a Fenton like homolytic scission of H_2O_2 and organic ROOH, immediately leads to intracellular oxidative stress and damage with increasing levels of $ROO^•$ and $^1O_2$ as products of phospholipid peroxidation and protein oxidation. These processes occur with simultaneous decrease in GSH and thiol group (-SH) content in the organ. Increased lipid peroxidation, protein oxidation, decreased GSH levels, and the de-excitation of the electronically excited species derived from lipid peroxidation, which is $^1O_2$ and excited carbonyl groups generation was evaluated in preliminary research [14, 18]. Regulation of antioxidant enzymes represents a compensatory response to oxidative stress, up-regulated by less reactive ROS (O_2^-, H_2O_2) or RNS (NO) and down-regulated by products de oxidation processes, or strongly oxidizing species or more reactive ROS ($HO^•$ and $ROO^•$) and RNS ($ONOO^-$).

The time profile of the effects of Fe(II) and Cu(II) chronic overloads on antioxidant enzyme activity and ROS and NOS production showed a decreased activity of SOD until day 14 of treatment, whereas after day 14, SOD and GT steadily increased their activities. The fact that their activities reach the basal values by the end of the treatment suggests an adaptive response which could be mediated by the enhanced production of NO and metal content in the organ (Figs. 1, 3 and 5). The NO and $ONOO^-$ are involved in the regulation of the activity of antioxidant enzymes and the redox homeostasis in rat brain after Fe(II) and Cu(II) chronic overloads, understanding this as the regulation of the adaptive response to oxidative stress. NO and $ONOO^-$ in the presence of Fe(II) or Cu(II) can oxidize lipids and proteins, and $ROO^•$ generated can act by upregulating GT activity. The nitrite concentration begins to rise after day 14 along with increments in the activity of SOD and GT. By the end of the treatments, nitrite reaches the basal levels.

The increase in SOD, catalase and GT activities after day 14 of chronic treatment with Fe(II) allows the cell to cope with the oxidant burden due to the enhanced steady state levels of intracellular of O_2^-, H_2O_2 and $ROO^•$, which are generated as a consequence of increased lipid and protein oxidation. These events are accompanied by increments in the rate of production of NO and the consequent nitrite content in brain.

The decreased activity of NADPH oxidase and increases in SOD and GT activities after day 14 of chronic treatment with Cu(II), along with the rising levels of nitrite content in brain, allows the cell to control the steady state concentrations of O_2^- and $ROO^•$. However, as catalase activity remains lower than control, the intracellular level of

H_2O_2 increases and its concentration cannot be regulated (Figs. 1, 2, 3, 4, 5 and 6).

In summary, the time course of oxidant (NO and metals) accumulation in brain and antioxidant response outline the biochemical processes occurring in the organ (Fig. 6). Recovering 50% of SOD activity is a late process that is produced with a greater content of Fe and Cu in the organ and at physiological concentration of NO (Figs. 1B and 3B), indicating that the increase of SOD activity is a regulatory process of redox homeostasis in rat brain with chronic Fe(II) and Cu(II) overload (Table 2). Catalase activity is regulated by different mechanisms for Fe(II) and Cu(II) treatments. Fe(II) increases catalase activity whereas Cu(II) decreases it, indicating an irreversible inhibition mediated by oxidative damage to protein.

The dip of NO content observed at day 6 is linked to changes in enzymatic activity and the subsequent effects on the generated or metabolized species by such enzymes. While it could be plausible that a portion of the synthesized NO is metabolized to nitrate, there is a very good correlation over time between the SOD decrease and the nitrite decrease. SOD is the main sink for O_2^{-}. Therefore, the decreased in SOD is indirectly linked to an enhanced steady state of O_2^{-}. Considering that the reaction between O_2^{-} and NO is a diffusion controlled reaction because the reaction rate is circa 7×10^9 L mol^{-1} s^{-1} [43], any increase in the steady state concentration of O_2^{-} would lead to a concomitant decrease in the steady state concentration of NO. It must also be mentioned that a decreased NADPH oxidase was observed. However, NADPH oxidase activity, while expressed in microglia and specific neurons in certain regions of the brain, in physiological conditions it is not the main driving force in the formation of O_2^{-} [44]. Therefore, as was observed, the decreased activity of SOD would be giving rise to an enhanced steady state concentration of O_2^{-}. Thus, it is very likely that the final concentration of end products of NO metabolism is increased, including nitrite and nitrate anions as well.

The subsequent enzyme activation of SOD, catalase and GT with Fe(II) or SOD and GT with Cu(II) chronic overloads would indicate that antioxidant defense system is activated to protect the liver from oxidative damage. Antioxidant response with chronic overload of Fe(II) and Cu(II) responds to ROO$^{\bullet}$, as a signaling compound, by regulating GT activity, the main molecule responsible for redox control in brain overloaded with Fe(II) seems to be NO while in the case of Cu(II), H_2O_2 could serve as a regulatory molecule. The upregulation of these antioxidant enzymes could be associated to the signaling function of ROO$^{\bullet}$, NO and H_2O_2. Notably, the increase in SOD activity was observed when the enzymatic activation of GT remained constant.

Conclusion

Oxidative stress in brain with Fe(II) and Cu(II) chronic overloads involves the participation of $ONOO^{-}$ as oxidant compound. The biochemical mechanisms of regulating redox homeostasis and control of oxidative stress and damage in the brain involves a common pathway but different antioxidant processes [45, 46]. Decrease in NO content and inhibition of SOD activity are common processes in brain chronic toxicity with Fe(II) and Cu(II) overloads. The increase of NADPH oxidase activity and decrease of activity of catalase with Cu(II) are initial responses that produce nitrosative and oxidative damage to proteins mediated by $ONOO^{-}$, H_2O_2 and O_2^{-}. Oxidative damage in chronic Fe(II) and Cu(II) overloads are controlled by GT, as a response to the oxidative processes. As a consequence, reversible activation of SOD, increase in NO levels, and inhibition of NADPH oxidase system (in the case of Cu(II)), decrease and prevent the oxidative damage in brain.

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Author contributions CS-M: methodology, validation, investigation, visualization, FL: validation, formal analysis, methodology, investigation, writing-original-draft, RM-S: methodology, investigation, JF: investigation, HT: investigation, resources, MGR: conceptualization, formal analysis, writing-original-draft, writing, review and editing, project administration, funding acquisition.

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Authors and Affiliations

Christian Saporito-Magriñá[1] · Fabiana Lairion[1] · Rosario Musacco-Sebio[1] · Julian Fuda[2] · Horacio Torti[2] · Marisa Gabriela Repetto[1,3]

[1] Facultad de Farmacia Y Bioquímica, Departamento de Ciencias Químicas, Cátedra de Química General E Inorgánica, Universidad de Buenos Aires, Junin 956, CP: 1113AAD Buenos Aires, Argentina

[2] Facultad de Farmacia Y Bioquímica, Departamento de Fisicomatemática, Cátedra de Física, Universidad de Buenos Aires, Buenos Aires, Argentina

[3] Instituto de Bioquímica Y Medicina Molecular Prof. Alberto Boveris(CONICET, IBIMOL), Consejo Nacional de Investigaciones Científicas Y Técnicas, Buenos Aires, Argentina