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Copper Increases Brain Oxidative Stress and Enhances the Ability of 6-Hydroxydopamine to Cause Dopaminergic Degeneration in a Rat Model of Parkinson's Disease

Antón Cruces-Sande[1] · Ana Isabel Rodríguez-Pérez[2,3] · Paloma Herbello-Hermelo[4] · Pilar Bermejo-Barrera[4] · Estefanía Méndez-Álvarez[1,3] · José Luis Labandeira-García[2,3] · Ramón Soto-Otero[1,3]

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Abstract

Redox properties enable copper to perform its essential role in many biological processes, but they can also convert it into a potentially hazardous element. Its dyshomeostasis may have serious neurological consequences, and its possible involvement in Parkinson's disease and other neurodegenerative disorders has been suggested. The in vitro and ex vivo ability of copper to increase oxidative stress has already been demonstrated, and the aim of the present study was to assess in vivo the capacity of copper to cause brain oxidative damage and its ability to increase the dopaminergic degeneration induced by 6-hydroxydopamine. We found that chronic copper administration (10 mg Cu^{2+}/kg/day, IP) causes its accumulation in different brain areas (cortex, striatum, nigra) and was accompanied by an increase in TBARS levels and a decrease in protein free-thiol content in the cortex. A decrease in catalase activity and an increase in glutathione peroxidase activity were also observed in the cortex. The intrastriatal administration of Cu^{2+} caused an increase in some indices of oxidative stress (TBARS and protein free-thiol content) in striatum and nigra, but was unable to induce dopaminergic degeneration. However, when copper was intrastriatally coadministered with 6-hydroxydopamine, it increased dopaminergic degeneration, a fact that was also accompanied by an increase in the assayed indices of oxidative stress, a decrease in catalase activity, and an augmentation in glutathione activity. Evidently, copper cannot cause neurodegeneration per se, but may potentiate the action of other factors involved in the pathogenesis of Parkinson's disease through oxidative stress.

Keywords Copper · 6-Hydroxydopamine · Oxidative stress · Glutathione peroxidase · Catalase · Dopaminergic degeneration

Introduction

Copper is a trace element with important roles in many physiological pathways, acting as an enzyme cofactor or a structural component in several proteins. Thus, it is involved in general oxidative metabolism (cytochrome c oxidase) and antioxidant defense (Zn,Cu-containing superoxide dismutases), which makes it indispensable for all eukaryotic cells. More specifically, in the brain, copper participates in the synthesis of neurotransmitters (dopamine-β-monoxygenase) and neuropeptides (peptidylglycine-α-amidating enzyme) [1, 2]. Copper's ability to cycle between its oxidized state (Cu^{2+}) and its reduced state (Cu^{+}) allows these copper-containing proteins to act as electron carriers or redox catalysts. However, this property also implies its potential involvement in reactions related to Fenton chemistry, where the interaction between a transition metal and hydrogen peroxide leads to the formation of very reactive, unstable radicals, able to attack biological macromolecules [3, 4]. Evidently, the presence of copper in the brain, together with the high metabolic rate of brain and its proportional low antioxidant defense, makes this organ very sensitive to oxidative stress [5–7]. For these reasons, under physiological conditions, copper concentration is

✉ Ramón Soto-Otero
ramon.soto@usc.es

1 Laboratory of Neurochemistry, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain

2 Laboratory of Neuroanatomy and Experimental Neurology, Department of Morphological Sciences, Faculty of Medicine, CIMUS, University of Santiago de Compostela, Santiago de Compostela, Spain

3 Networking Research Center on Neurodegenerative Diseases (CIBERNED), Institute of Health Carlos III, Madrid, Spain

4 Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Chemistry, University of Santiago de Compostela, Santiago de Compostela, Spain

closely regulated by proteins devoted to facilitate its uptake, efflux, and supply to copper-containing proteins [2, 8].

Brain copper is derived from peripheral copper transported across the blood-brain barrier and/or blood-cerebrospinal fluid barrier, mainly as a free ion [9]. Brain cells take up copper through the membrane copper transporter CTR1. Once inside the cell, copper is shuttled to its specific targets through a series of cytosolic chaperones (Atox1, CCS, Cox17). Surplus copper is chelated by metallothioneins and glutathione in order to control its reactivity [10, 11]. When cellular copper concentration reaches certain threshold, P-type ATPases (ATP7A and ATP7B) export copper directly or import it into vesicles to be released by fusion with the plasma membrane. Human copper content ranges from 3.1 to 5.1 mg/g wet weight [12–15]. Its spatial concentration varies, being higher in sections such as the locus coeruleus or substantia nigra [15–18]. This latter may be attributed to the reported ability of neuromelanin to bind and accumulate redox-active metals [19]. Also, the concentration of copper in extracellular liquid or glial cells appears to be higher than that found in neurons [20–22].

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder. Its main clinical symptoms are motor disturbances such as tremor, rigidity, and bradykinesia [23, 24]. The two main pathophysiological hallmarks of this disorder are the loss of nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinaceous inclusions designated as Lewy bodies [25, 26]. Although the etiology of this degeneration is still unknown, the incidence of PD in environmental exposure to metals and genetic disorders related to certain metal homeostasis support a role for transition metal accumulation in PD pathophysiology [27, 28]. An increase in cerebrospinal copper levels in PD patients has been reported [29, 30] and has been associated with oxidative stress and protein conformational changes. Wilson's disease, a genetic disorder due to a mutation in *ATP7B* gene produces a systemic accumulation of copper. The high rate of parkinsonism in Wilson's disease [31] suggests the existence of a selective neurotoxic mechanism in the brain for copper. Copper is also involved in the high incidence of parkinsonism observed in occupational exposure to copper [32, 33]. These data, along with the potential damaging properties that copper intrinsically presents, seem to suggest that an alteration in copper homeostasis may have neurological hazardous consequences.

In our latest study, we observed the in vitro ability of copper to increase the rate and magnitude of both $^{\cdot}OH$ and hydrogen peroxide formation in the presence of 6-hydroxydopamine (6-OHDA), this latter being a well-known dopaminergic neurotoxin widely used to generate experimental models of PD [34, 35]. In addition, we could also prove the ability of copper to increase the potential of 6-OHDA to cause oxidative damage in both lipids and proteins, using ex vivo samples from rat brain. Thus, the aim of the present study is to go further and explore in vivo both the accumulation and potential damage of copper when rats are chronically exposed to a daily dose, as well as its acute effect when directly injected in rat *striatum*, both alone and in combination with 6-OHDA.

Material and Methods

Chemicals

Ascorbic acid, antiserum to tyrosine hydroxilase (TH), bovine serum albumin (BSA), catalase, desipramine, 3,3′-diaminobenzidine, 5,5′-dithiobis-(2-nitrobenzoic acid), glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase, hidrogen peroxide (H_2O_2), NADPH, 6-OHDA hydrochloride, and thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Biotinylated horse anti-mouse IgG antibody and avidin-biotin-peroxidase complex ABC were purchased from Vector (Burlingame, CA, USA). Sodium dodecylsulfate, copper(II) chloride, TRIS base, and BHT were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Medetomidine was obtained from Orion Co. (Domtor®, Espoo, Finland) and ketamine from Merial Laboratorios S.A. (Imalgene 1000®, Barcelona, Spain) Water used for solutions was Milli-RiOs/Q-A10 grade (Millipore Cor., Bedford, MA, USA). All remaining chemicals were of analytical grade and purchased from Fluka Chemie AG (Buchs, Switzerland). Stock solutions of 6-OHDA were prepared in 1.0-mM KCl (pH 2.0) to prevent its autoxidation (Sullivan and Stern, 1981). All glassware was immersed overnight in 1-M HNO_3 followed by a thorough rinsing with Milli-RiOs/Q-A10 grade water to prevent metal contamination. For copper determination, nitric acid 69% hyperpur and hydrogen peroxide 33% (*w*/*v*) for analysis were purchased from AppliChem Panreac (Darmstadt, Germany). A stock standard solution of 1000 mg/L Cu in 2% HNO_3 was obtained from Perkin Elmer (Shelton, CT, USA).

Animal Treatment

Adult male Sprague-Dawley rats (225–250 g weight) from the Animal Breeding Unit of University of Santiago de Compostela were used to perform the studies. Animals were housed in polypropylene cages (two rats per cage; the first few days after surgery rats were housed individually) and maintained at 21 ± 1 °C on a 12-h light/12-h dark cycle with water and food ad libitum. All experiments were carried out in accordance with the European Directive 2010/63/EU and the Spanish RD/53/2013 for the protection of animals used for scientific purposes, and all efforts were made to minimize the number of animals and their suffering. The experimental design was approved by the corresponding committee at the

University of Santiago de Compostela. Dosages were adjusted according to animal's weight before each experiment.

For chronic intraperitoneal (IP) injection and subsequent studies, a total of 12 animals were used. Six rats were injected daily with copper(II) chloride in saline at a dose of 2 mg Cu^{2+}/kg for 30 days, whereas the remaining six rats were injected with the same volume of saline (NaCl 0.9%) over the same period.

For stereotaxical intrastriatal injection, a total of 38 animals were used, divided in four groups, each consisting of nine animals. Rats in group A ($n = 9$) were used as control group and received 3 μL of sterile saline in the right striatum. Rats in group B ($n = 9$) were intrastriatally injected with 10 μg of 6-OHDA in 3 μL of sterile saline containing 0.2% ascorbic acid. The lesion of the nigrostriatal pathway induced by intrastriatal injection of 6-OHDA is dose-dependent [34]. On the basis of our previous studies with this model, we found that the abovementioned dose is the most adequate in our hands to detect either increases or decreases in the lesion induced by additional treatments. Rats in group C ($n = 11$) received a first injection with 6-OHDA followed (5 min later) by a second injection of 0.88 mg of copper(II) chloride in 2.5 μL of sterile saline. Rats in group D ($n = 9$) were injected copper(II) chloride only. Thirty minutes prior to intrastriatal injection with 6-OHDA, saline, or copper(II), chloride rats were treated with the selective inhibitor for the norepinephrine transporter desipramine to prevent uptake of 6-OHDA by noradrenergic terminals.

Stereotaxic coordinates were 1.0 mm anterior to the bregma, 3.0 mm right of midline, and 5.5 mm ventral to the dura with tooth bar at − 3.3. The solution was injected with a 10-μl Hamilton syringe coupled to a motorized injector (Stoelting) at a rate of 0.5 μl/min, and the needle was kept in situ for 5 min after the injection allowing the solution to diffuse into the surrounding tissue. All surgery was performed under ketamine/medetomidine anesthesia (0.5 and 40 mg/kg). The survival of animals subjected to stereotaxic surgery was 100%.

Brain Samples

After 30 days, the daily IP injected animals were stunned with carbon dioxide and killed by decapitation. The brains were rapidly removed, rinsed in ice-cold saline, and sliced coronally (1 mm) with a tissue chopper set. To isolate the different regions (striatum, substantia nigra, and cortex), the individual 1-mm tissue slides were dissected on a precooled glass plate under a stereoscopic microscope (Leica M220, Heidelberg, Germany) and according to Paxinos and Watson [36]. Then, the tissue was frozen on dry ice and stored at − 80 °C until processed for determination of copper content and biochemical studies.

Each group of stereotaxycal injected rats was subdivided into five rats for biochemical studies, and the remainders were used for immunohistochemistry. The rats for biochemical studies were sacrificed—according to the above-cited procedure—72 h after intrastriatal injection, since it has been previously reported that the peak for oxidative stress is reached at this time [37]. Four weeks after lesion, the remaining rats from each group were anesthetized with an overdose of medetomidine/ketamine, transcardially perfused with 0.9% saline at 37 °C and then with cold 4% paraformaldehyde in 0.1-M phosphate-buffered saline (PBS), pH 7.4. Previous studies on the time course of the intrastriatal 6-OHDA lesion model have shown that the loss of dopaminergic neurons is very high after the first 2 weeks and that the process is stabilized or practically stabilized 4 weeks post-injection [34]. The brains were then removed, washed, and cryoprotected in the same buffer containing 30% sucrose and finally cut into 40-μm sections on a freezing microtome and stored in a cryoprotectant solution at − 20 °C until processing.

Sections used for TH immunohistochemistry were incubated for 1 h in 10% normal horse serum with 0.25% Triton X-100 in 20-mM potassium PBS containing 1% bovine serum albumin (KPBS-BSA) and then incubated overnight at 4 °C with a mouse monoclonal antiserum to TH 1:10000. After that, sections were incubated, first with the corresponding secondary antibody (biotinylated horse anti-mouse IgG antibody, 1:200) and then with avidin-biotin-peroxidase complex ABC (1:100). Finally, the labeling was revealed by treatment with 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine.

Protein Determination

The protein concentration in biological samples was determined according to the method of Markwell et al. [38], using BSA as the standard.

Sample Digestion for Copper Determination

Between 0.01 and 0.02 g of tissue from each segment were weighed in a digestion vessel and 2 mL of 69% HNO_3 and 0.5 mL of 33% *w*/*v* H_2O_2 were added. Afterwards, the vessels were closed and placed in a conventional microwave oven. After digestion, the sample was adjusted to its final volume (5 mL) by the addition of ultrapure water. Each digestion sample was duplicated.

Copper Determination

A portion of 20 μL of the digested sample was analyzed by electrothermal atomic absorption spectrometry (ETAAS) by triplicate. Measurements were carried out using a Perkin Elmer Model 1100B atomic absorption spectrometer, equipped with an HGA-700 graphite furnace atomizer and an AS-70 autosampler. The source of radiation was a copper hollow cathode lamp, operating at 15 mA, which provided a

324.8-nm line, with a spectral bandwidth of 0.7 nm. Deuterium background correction and pyrocoated L'vov graphite tubes were used. The signal was recorded as integrated absorbance with an integration time of 4 s.

Determination of TBARS and PTC

Lipid peroxidation (TBARS) and protein free-thiol content (PTC) were assessed spectrophotometrically according to a previously reported methodology [39], using thiobarbituric acid and 5,5′-dithiobis-(2-nitrobenzoic acid) as chemical dosimeters for the quantification of TBARS and PTC, respectively.

Determination of Enzyme Activities of GPx and CAT

GPx and CAT activities were measured spectrophotometrically by slight modifications introduced into the methodology previously published by Flohe and Gunzler [40] and Aebi [41], respectively. Briefly, GPx activity was measured by the decrease in absorption at 340 nm for 5 min after the addition of H_2O_2 (3 mM) to a pre-incubated mixture with glutathione reductase (0.5 IU), glutathione (2 mM), NADPH (3.4 mM), and the brain sample in Na_2PO_4/KH_2PO_4 buffer (isotonized with KCl, pH 7.4). Meanwhile, CAT activity was measured by the decrease in absorption at 240 nm after the addition of H_2O_2 (14 mM) to a pre-incubated mixture of brain sample and Na_2PO_4/KH_2PO_4 buffer (isotonized with KCl, pH 7.4).

Immunohistochemistry

Estimation of the total TH-immunoreactive (TH-ir) neurons in the substantia nigra compacta (SNc) was made with an unbiased stereological method (the optical fractionator) using an Olympus CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus) as previously described [42]. Briefly, uniform randomly chosen sections through the SNc—every fourth section—were analyzed for the total number of TH-ir cells by means of a stereological grid (fractionator), and the nigral volume was estimated according to Cavalieri's method [43]. Penetration by the antibody was determined by registration of the depth of each counted cell that appeared in focus within the counting frame. This analysis revealed incomplete penetration by the antibody, leaving 8 to 10 μm in the center poorly stained [44].

Statistical Analysis

For biochemical and copper content assessments, data are expressed as mean ± SEM. Statistical differences were tested using one-way ANOVA followed by Bonferroni's test for multiple comparisons. For immunohistological studies, statistical analysis was carried out with SigmaStat 3.0 from Jandel Scientific. Values are expressed as mean ± SEM and differences among means were analyzed using one-way ANOVA followed by a post hoc Holm-Sidak test. The normality of populations and homogeneity of variances were tested before each ANOVA. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Effects of Chronic Daily Administration of Copper on Copper Content in the Cortex, Striatum, and Substantia Nigra

Total copper content was assessed by ETAAS of the corresponding segments after 30 days of a daily copper(II) chloride IP administration (2 mg/kg) or the same volume of vehicle (control). As shown in Fig. 1, animals exposed to copper exhibited a significant increase in all frontal cortex (+ 72%), striatum (+ 59%), and substantia nigra (+ 68%).

Effects of Chronic Daily Administration of Copper on Lipid Peroxidation, Protein Oxidation, and Enzyme Activities in Cortex

Oxidative stress markers were assessed in the cortex of the same rats used to determine total copper content. Lipid peroxidation was evaluated by the determination of TBARS concentration, whereas protein oxidation was estimated by protein free-thiol content (PTC). Those animals, daily exposed to copper(II) chloride (2 mg/kg) for 30 days, exhibited significant oxidative damage, as shown by an increase in the indices of both lipid peroxidation (69% in TBARS) and free-thiol group oxidation (− 24% in PTC) (Fig. 2).

Enzyme activities of both GPx and CAT were also measured spectrophotometrically. In this case, the antioxidant enzyme activities showed a significant decrease for CAT (28%) and a slight but significant increase for GPx (6%) (Fig. 2).

Effects of Intrastriatal Copper Administration on DA-Terminals Degeneration when Administered Alone or in Combination with 6-OHDA

As shown in Fig. 3, four weeks post-injection, a total number of 11,546 ± 669 (mean ± SEM) dopaminergic neurons were counted in the ipsilateral SNc of sterile saline injected rats, which was not significantly different from contralateral SNc of the lesioned animals (12,771 ± 325). Treatment with the dopaminergic neurotoxin 6-OHDA induced a marked decrease in the number of TH-ir neurons (i.e., 6049 ± 475; about 50% decrease). However, the loss of TH-ir neurons was significantly higher when 6-OHDA was concomitantly administered with copper(II) chloride (3753 ± 191; about 70%

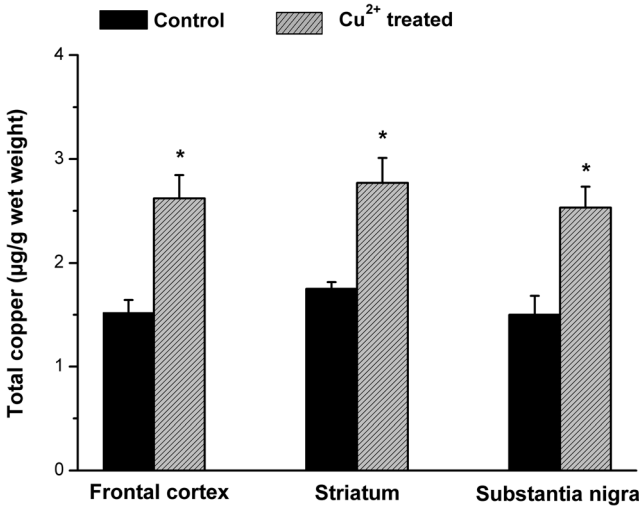

Fig. 1 Levels of total copper (μg/g wet weight) in different brain areas of rats treated intraperitoneally with saline (control) or copper(II) chloride (2 mg Cu/kg/day) for 30 days. Data are expressed as means ± SEM from five rats ($n = 5$). Significance of differences among groups was assessed by a one-way ANOVA followed by a Bonferroni's test. Asterisks denote values significantly different from the corresponding control; *$p < 0.05$

decreases). No significant differences were found between sterile saline and copper(II) chloride groups.

Effects of Intrastriatal Copper Administration on Oxidative Damage Markers: Lipid Peroxidation and Protein Oxidation

Once again, the concentration of TBARS was used as an index of lipid peroxidation and the thiol content as an index of protein oxidation, both measured in the striatum and substantia nigra, 72 h after ipsilateral intrastriatal injection.

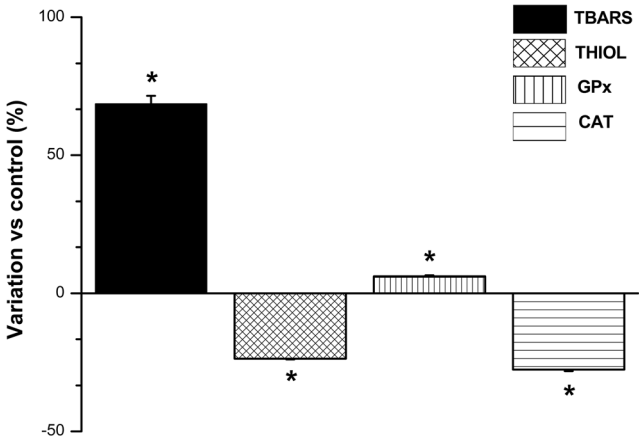

Fig. 2 The effect of copper(II) chloride administration (2 mg Cu/kg/day, IP, 30 days) on TBARS level, protein free-thiol content, catalase activity (CAT), and glutathione peroxidase activity (GPx). Results are expressed as a percentage of variation in relation to control. Data are expressed as means ± SEM from five rats ($n = 5$). Significance of differences among groups was assessed by a one-way ANOVA followed by a Bonferroni's test. Asterisks denote values significantly different from the corresponding control; *$p < 0.05$

In relation to the effects of copper on protein oxidation, a significant decrease in PTC was found in the ipsilateral striatum when 6-OHDA was injected (− 15%) (Fig. 4). However, PTC was significantly lower when 6-OHDA was concomitantly injected with copper(II) chloride (− 24%). No significant differences were found between sterile saline and copper(II) chloride groups. Also, a significant decrease in PTC was found in the ipsilateral substantia nigra when 6-OHDA was injected (− 26%). Again, PTC was significantly lower when 6-OHDA was concomitantly injected with copper(II) chloride (− 38%). In this case, the copper(II) chloride group also showed a significant difference (− 22%).

With regard to the effects of copper on lipid peroxidation, a significant increase in TBARS content was found in the ipsilateral striatum when 6-OHDA alone (19%) or copper(II) chloride alone (13%) were injected (Fig. 4). TBARS content was also significantly higher when 6-OHDA was concomitantly injected with copper(II) chloride (36%). Also, a significant increase in TBARS content was found in ipsilateral SNc when 6-OHDA alone (35%) or copper(II) chloride alone (30%) were injected. In a similar way, TBARS content was also significantly higher when 6-OHDA was concomitantly injected with copper(II) chloride (60%).

Effects of Intrastriatal Copper Administration on the Brain Activity of CAT and GPx

As shown in Fig. 5, a significant decrease in CAT activity was found in ipsilateral striatum when 6-OHDA was injected (− 21%). However, PTC was significantly lower when 6-OHDA was concomitantly injected with copper(II) chloride (− 44%). No significant differences were found between sterile saline and copper(II) chloride groups. In ipsilateral SNc, a significant decrease in CAT activity was found when 6-OHDA alone (− 20%) or copper(II) chloride alone (28%) was injected. TBARS content was also significantly higher when 6-OHDA was concomitantly injected with copper(II) chloride (32%).

A significant decrease in GPx activity was found in ipsilateral striatum when 6-OHDA alone (− 26%) or copper(II) chloride alone (43%) was injected (Fig. 5). TBARS content was also significantly higher when 6-OHDA was concomitantly injected with copper(II) chloride (60%). In ipsilateral SNc, a significant decrease in GPx activity was found in ipsilateral SNc when 6-OHDA alone (− 20%) or copper(II) chloride alone (26%) was injected. TBARS content was also significantly higher when 6-OHDA was concomitantly injected with copper(II) chloride (30%).

Discussion

The redox properties of copper enable it to play an essential role in many biological processes. However, as previously

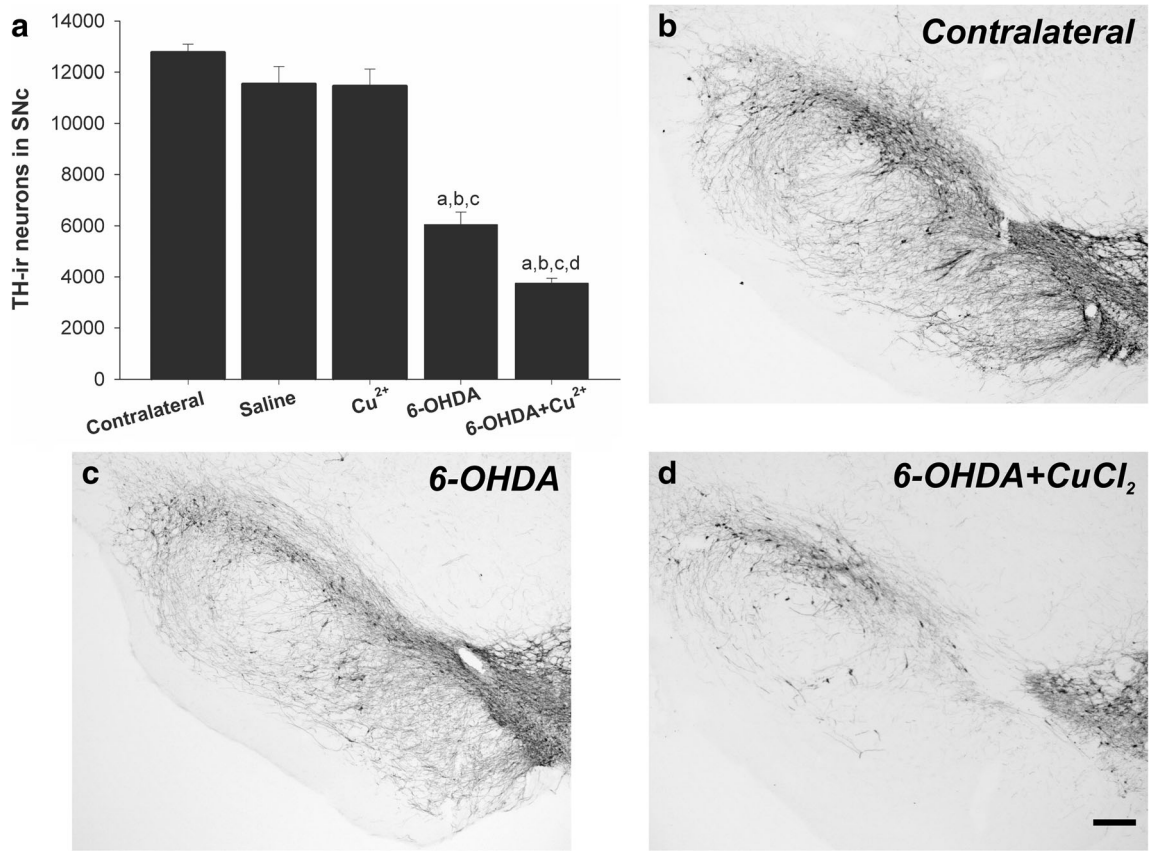

Fig. 3 Dopaminergic (TH-ir) neurons in the substantia nigra compacta (SNc) (**a**–**d**) of the contralateral non-injected side (**b**) and 4 weeks after injection of 6-OHDA (**c**), or 6-OHDA + $CuCl_2$ (**d**). Representative photomicrographs of the substantia nigra compacta of different groups of rats are shown in **b**–**d**. The estimated total number of dopaminergic neurons in the substantia nigra compacta of the different experimental groups is shown in **a**. Data are means ± SEM. Statistical significance at $p < 0.05$ (one-way ANOVA and Holm-Sidak post hoc test) was established in relation to the following controls: **a** contralateral non-injected side, **b** saline, **c** Cu^{2+}, **d** 6-OHDA. Scale bar 200 μm

reported [3, 4] and corroborated in our last study [45], its redox properties also involve copper in Fenton chemistry, allowing it to generate unstable radicals capable of attacking biological molecules and causing oxidative stress. In addition, both high metabolic brain activity and the low ratio of antioxidant defenses make copper dyshomeostasis a potential risk that could lead to neuronal damage and death [5–7]. It is a well-known fact that copper reaches brain cells by means of peripheral copper [46], and a variety of mechanisms regulating its cell content have been described [47]. Under physiological conditions, an excess of copper is chelated inside the cell in order to prevent its reactivity and then exported when it is above a certain concentration threshold [12–15]. However, when copper physiology is altered (i.e., copper over-exposure in Wilson's disease), a higher rate of parkinsonism incidence has been reported [31–33]. An increase in the cerebrospinal levels of copper has also been reported in PD patients [29, 30]. That is why the aim of the present study was to go further with our research on copper neurotoxicity by investigating the ability of chronic IP administration of copper to cause oxidative stress and its potential to enhance the intrastriatal damage induced by 6-OHDA. These objectives were approached by assessing its effect on lipid peroxidation and protein damage—as seen in ex vivo samples—together with potential changes in antioxidant enzyme defense and neuronal loss.

Our results clearly show that rats exposed to a daily dose of copper for 30 days (2 mg/kg, IP) causes a significant increase of its concentrations in cortex, striatum, and substantia nigra. Evidently, these data confirm the ability of copper to be absorbed, transported by blood, crossing the blood-brain barrier, and finally accumulated in the brain areas studied. In this sense, our results agree with previous reports showing the accumulation of copper in certain brain areas following IP administration [48]. To find out if this increase in brain levels of copper could be associated to increased reactivity and cause cell damage, we evaluated four biochemical indices related to oxidative stress: TBARS (lipid peroxidation), PTC (protein oxidation), and catalase and GPx activities (reduction in enzyme antioxidant defense). Thus, when cortex homogenates from the same rats were assayed, we found a significant increase in TBARS and a significant decrease in protein thiol content (PTC), both showing the ability of copper to cause in vivo oxidative damage to molecules and consequently oxidative stress. In addition, an increase in GPx activity was

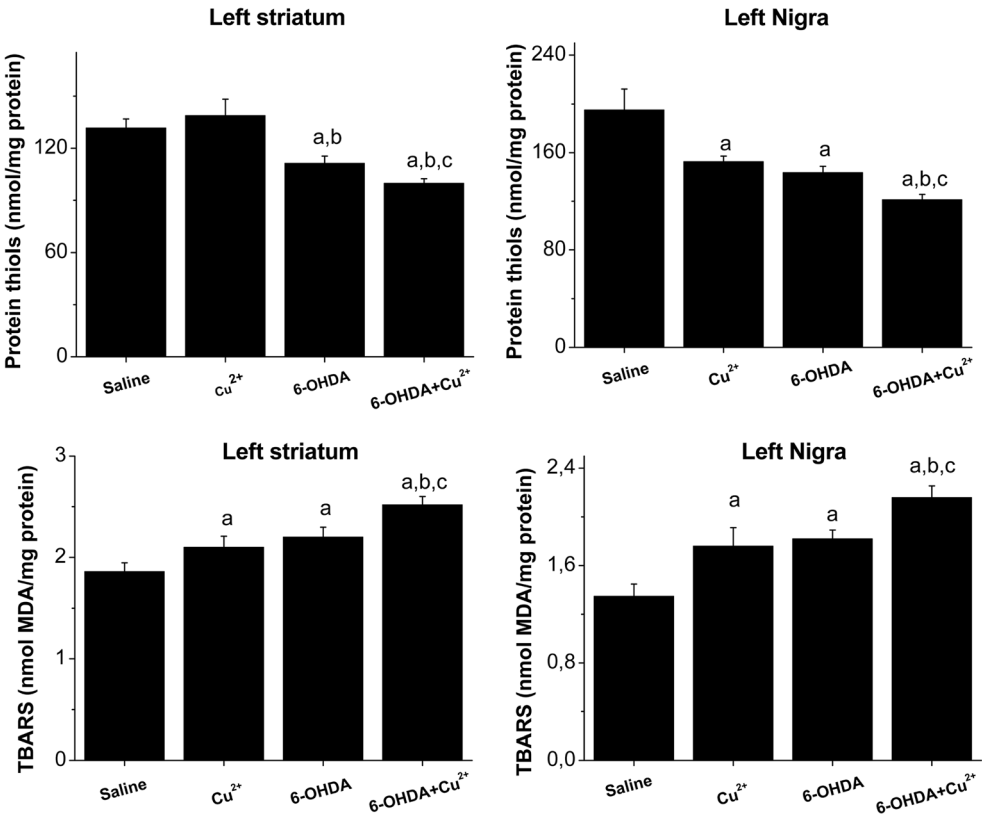

Fig. 4 Levels of protein free-thiol content (upper) and TBARS (lower) in ipsilateral striatum and SNc, 72 h after intrastriatal injection of 0.88 mg of copper(II) chloride in 2.5 μL of sterile saline. Data are expressed as means ± SEM from five rats ($n = 5$). Statistical significance at $p < 0.05$ (one-way ANOVA and Bonferroni test) was established in relation to the following controls: **a** saline, **b** $CuCl_2$, **c** 6-OHDA

observed, presumably as a consequence of an inductive effect caused by chronic oxidative stress affecting the expression of GPx. A similar observation was reported by our laboratory using other inducers of brain oxidative stress [49]. However,

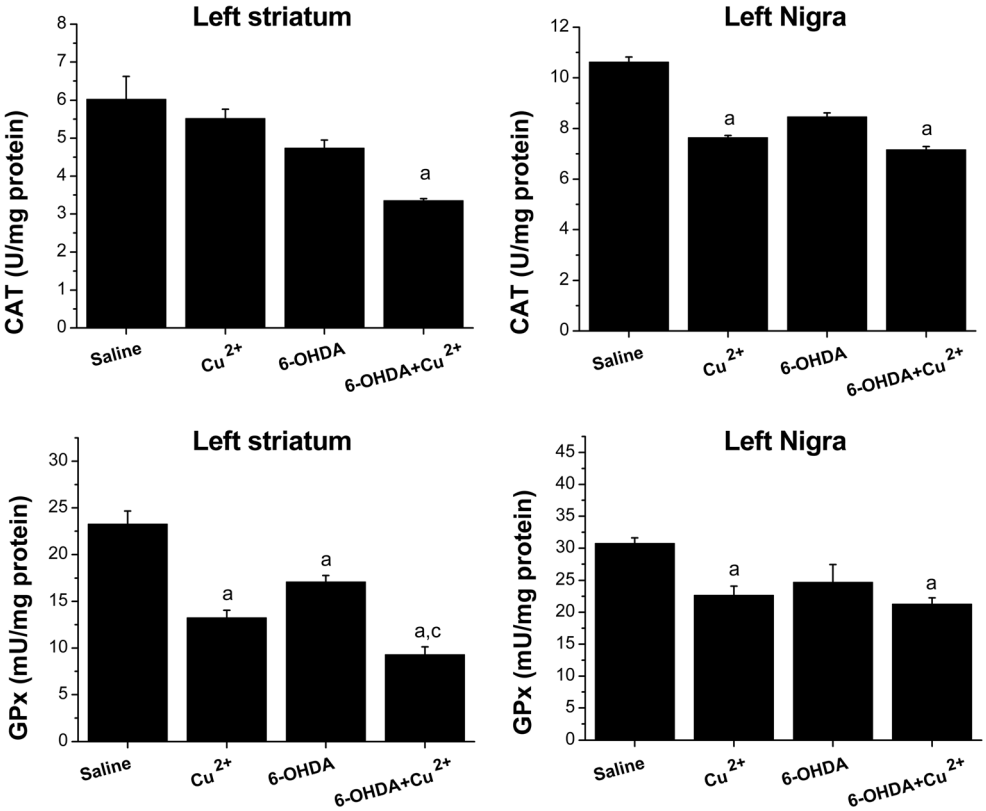

Fig. 5 Enzyme activity of CAT (upper) and GPx (lower) in ipsilateral striatum and SNc 72 h after intrastriatal injection of 0.88 mg of copper(II) chloride in 2.5 μL of sterile saline. Data are expressed means ± SEM from five rats ($n = 5$). Statistical significance at $p < 0.05$ (one-way ANOVA and Bonferroni's test) was established in relation to the following controls: **a** saline, **b** $CuCl_2$, **c** 6-OHDA

the in vivo effect caused by the chronic administration of copper on catalase activity in the cortex was a significant reduction of its activity in comparison with control. Taking into account the very slow kinetics of copper-catalyzed Fenton reaction in relationship to the consumption H_2O_2 [45], we hypothesized that the here reported ability of copper to decrease its activity could be a consequence of oxidative damage caused by a chronic oxidative environment. Although under our experimental conditions we observed that copper alone was not able to cause neuronal loss, our data clearly show that under an induced copper over-exposure, the copper homeostatic mechanisms in the brain are not enough to control its pro-oxidant activity, which could easily result in cell damage.

As demonstrated in our previous in vitro study [45], copper has the potential to enhance the capacity of 6-OHDA to generate H_2O_2 and $^{\cdot}OH$. Furthermore, we observed that copper can significantly increase the ex vivo oxidative damage caused by 6-OHDA in brain homogenates [45]. In the present study, this ability has been checked in vivo by quantifying the loss of dopaminergic neurons in the substantia nigra, when copper was concomitantly injected in the striatum with 6-OHDA. The marked loss of dopaminergic neurons after concomitant injection of copper and 6-OHDA clearly showed the in vivo potential of copper to enhance the oxidative damage induced by 6-OHDA alone. As previously reported [49–51], the neurotoxicity of 6-OHDA is mainly produced when internalized into dopaminergic cells through the DA transporter, where its oxidation leads to the formation of ROS ($^{\cdot}OH$ and H_2O_2); these latter affecting several cell components (proteins, phospholipids, etc.) and some biological systems, such as the mitochondrial electron transport system. Evidently, the increase observed in neuronal loss when copper is concomitantly injected with 6-OHDA suggests that copper enters DA cells by the copper transporter (Ctr1) and once inside is able to catalyze the autoxidation of 6-OHDA, and in this manner, enhances its hazardous properties to the point that a significant number of cells die in comparison to the action caused by 6-OHDA alone. Biochemical markers of oxidative stress were assessed in striatum and substantia nigra after injection in order to find earlier signs of biochemical damage previous to cell death. In this way, we could show how the neuronal loss observed with the combined action of copper and 6-OHDA was coherent with a significant rise in lipid peroxidation and protein oxidation together with a reduction in the antioxidant activity of catalase. The kinetics of oxidative damage of the brain induced by intrastriatal administration of 6-OHDA have been previously reported in an experimental model of PD in rats [37]. Although in this present study, we found a significant increase in the biochemical markers of oxidative stress when copper was intrastriatal injected alone, which is coherent with the results obtained with the IP administration of copper, copper per se was unable to induce dopaminergic degeneration, at least under our experimental conditions. However, the previously reported ability of intranigral administration of copper to cause dopaminergic degeneration [52] should apparently be attributed to the experimental conditions used.

Based on our results, we conclude that chronic copper administration can lead to an increase in its brain levels, which is very likely accompanied by brain oxidative stress state, this latter involving an increase in lipid peroxidation (TBARS) and oxidative damage to proteins (reduction in free-thiol content). These effects can also be accompanied by an increase in glutathione peroxidase activity and a reduction in catalase activity. We noticed that intrastriatal copper administration was unable to cause dopaminergic degeneration, but was capable of generating the same effects observed following IP administration of copper. However, copper can potentiate the neuronal loss caused by 6-OHDA in a rat model of PD. In this case, the enhancement caused by copper in the neurodegeneration induced by 6-OHDA was also associated with an increase in the assayed indices of oxidative stress, namely an increase in TBARS levels and a reduction in protein free-thiol content. Also, an increase in the activity of glutathione peroxidase and a reduction in the activity of catalase were observed. Consequently, although under our experimental conditions copper is unable to induce dopaminergic neurodegeneration per se, it is capable of increasing the degeneration provoked by other idiopathic cofactors, whose action is mediated through oxidative stress. Evidently, these findings add another potential etiological cofactor to the multifactorial nature of PD and support the current suggested need for the development of new multifunctional drugs capable of achieving a more effective treatment of this disorder.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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