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Research article

Neuroprotective effect of curcumin-I in copper-induced dopaminergic neurotoxicity in rats: A possible link with Parkinson's disease

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ABSTRACT

Numerous findings indicate an involvement of heavy metals in the neuropathology of several neurodegenerative disorders, especially Parkinson's disease (PD). Previous studies have demonstrated that Copper (Cu) exhibits a potent neurotoxic effect on dopaminergic neurons and triggers profound neurobehavioral alterations. Curcumin is a major component of *Curcuma longa* rhizomes and a powerful medicinal plant that exerts many pharmacological effects. However, the neuroprotective action of curcumin on Cu-induced dopaminergic neurotoxicity is yet to be investigated. The aim of the present study was to evaluate the impact of acute Cu-intoxication (10 mg/kg B.W. i.p) for 3 days on the dopaminergic system and locomotor performance as well as the possible therapeutic efficacy of curcumin I (30 mg/kg B.W.). Intoxicated rats showed a significant loss of Tyrosine Hydroxylase (TH) expression within substantia nigra pars compacta (SNc), ventral tegmental area (VTA) and the striatal outputs. This was correlated with a clear decrease in locomotor performance. Critically, curcumin-I co-treatment reversed these changes and showed a noticeable protective effect; both TH expression and locomotor performance was reinstated in intoxicated rats. These results demonstrate altered dopaminergic innervations following Cu intoxication and a new therapeutic potential of curcumin against Cu-induced dopaminergic neurotransmission failure. Curcumin may therefore prevent heavy metal related Parkinsonism.

1. Introduction

Copper (Cu) is an essential trace element that is widely involved in several biological processes and vital functions such as cell respiration, maturation of erythrocytes, and antioxidant defense. It is also a cofactor of several enzymatic cell machineries including superoxide dismutase (Cu, Zn-SOD), dopamine monooxygenase, and cytochrome oxidase [1,2]. Nevertheless, Cu is considered among the most toxic metals to living organisms [3]. Evidence suggests the involvement of Cu dyshomeostasis (excess or deficiency) in neurodegenerative diseases such as Parkinson's disease (PD) [4–6]; a chronic neurodegenerative pathology characterized primary motor dysfunction and classic clinical features including rigidity, bradykinesia, and walking difficulty. These features are the result of progressive death of dopaminergic neurons in the substantia nigra. In PD, Cu induces amyloid formation of α-synuclein (αSyn) [7,8] and improves oxidative damage of αSyn, which is associated with dopamine neuron death [9,8]. Moreover, Cu is a proposed risk factor and even a marker of PD [10]. A high concentration of Cu has been detected in both the cerebrospinal fluid and the substantia nigra of PD patients' brains [11]. Numerous studies have assumed that Cu-deficiency is linked to motor dysfunctions. However, Cu-deficiency in the rodent brain is also associated with neurological disorders including tremors, ataxia and hypokinesia, as well as reduced striatal dopamine levels, neuronal degeneration, diminution of antioxidant potential and altered mitochondrial morphology (for review see [12]). Recent findings support a neuroprotective role for Cu in PD and restoration of Cu-deficit in Parkinsonian brain may therefore represent a novel neuroprotective treatment of PD [12]. For instance, Cu sulfate pretreatment prevents mitochondrial electron transport chain damage and apoptosis against MPPþ-induced neurotoxicity [13] and the neuroprotective compound (CuII(atsm)) rescues nigral cell loss and improves dopamine metabolism in the MPTP model of PD [14].

For centuries, medicinal plants have been used to treat various pathologies and, today, bioactive molecules are isolated from plants in therapeutic research. Curcumin or diferuloyl-methane, also known as saffron in India, is the main yellow pigment of turmeric and is extracted from the rhizome of *Curcuma longa* L. [15]. It is a polyphenolic pigment widely known for its biological activities. Specifically, curcumin is a powerful antioxidant, a potent antiseptic and antibacterial, as well as a potent anti-inflammatory, antidepressant, antimutagenic, and an

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antitumor inhibiting cell proliferation [15,16]. Beneficial effects of curcumin have also been described against neurodegenerative diseases such as PD [15]. Nevertheless, the effect of curcumin against copper induced Parkinsonism has not yet been elucidated. Therefore, the aim of the present study was to assess the neuroprotective potential of curcumin-I against acute Cu-induced dopaminergic neurotoxicity and locomotor behavior in rats.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 200–250 g, aged four months, were obtained from the central animal-care facilities of Cadi Ayyad University, Marrakech (UCAM), Morocco. Rats were housed at constant room temperature (25 °C) on a 12-h dark–light cycle with free access to food. Rats were treated in compliance with the guidelines of the UCAM. All procedures were in accordance to the European decree, related to the ethical evaluation and authorization of projects using animals for experimental procedures, 1 February 2013, NOR: AGRG1238767A. Thus, all efforts were made to minimize the number of animals and suffering.

Animals were divided into 4 groups. Group-I: control rats (C) were injected i.p with physiological saline buffer (0.9% NaCl) for 3 consecutive days. Group-II: rats (Cu) were injected i.p. with Cu at a dose of 10 mg/kg BW for 3 days. Group-III: rats (Cur + Cu) received curcumin I (prepared in olive oil) at a dose of 30 mg/kg BW by oral gavage daily for 3 days. These rats were injected i.p. with Cu at a dose of 10 mg/kg BW 2 h after the last dose of curcumin consecutively for 3 days. Group-IV: rats (Cur) received only curcumin-I (prepared in olive oil) at a dose of 30 mg/kg B.W. by oral gavage daily for 3 days and were injected i.p. with physiological saline buffer (0.9% NaCl).

The experimental protocol and the doses of Cu and curcumin were based on our previous work and the literature [17–19]. For oral administration, curcumin was dissolved in olive oil and administered by oral gavage. For the purposes of intraperitoneal injections, Cu acetate was dissolved in physiological saline buffer (0.9% NaCl).

2.2. Chemicals

Cu-(II) acetate trihydrate was supplied by (Riedel-de Haen, Seelze, Germany; code No.25038, Lot No.83370). Curcumin-I was purchased from Alfa Aesar (Karlsruhe, Germany, code No. B21573).

2.3. Open-field test

The “open field” test was used to assess locomotor activity. The apparatus consists of 25 identical squares of 20 cm per side (100 cm × 100 cm × 40 cm) made out of wood. The animal is placed in the middle of the field and the ambulation (number of squares crossed by the animal) was recorded for 5 min to habituate animals. Rats were individually habituated to the open field for 10 min on 3 consecutive days before the study.

2.4. Immunohistochemistry

At the end of the experiment, animals were sacrificed 24 h after last Cu injection, between 10 and 12 a.m., for the immunohistochemical study. Rats were anesthetized intraperitonially with urethane (40 mg/kg i.p.) and perfused transcardiacally with chilled physiological saline (NaCl 0.9%) (Sigma-aldrich, St.Louis,MO, USA, Cas-No. 7647-14-5, lot-No. BCBH2237 V) and paraformaldehyde (4%) (Panreac Quimica SA, Barcelona, Spain, catalog No.141451.1211, lot No.0000078736) in phosphate buffered saline (PBS, 0.1 M, pH7.4) (Riedel-de Haen, Seelze, Germany). Brains were removed and post-fixed in the same fixative for 12 h at 4◦C, then dehydrated through a graded ethanol series (70–100%), passed through serial polyethylene glycol (Merck-Shuchardts, Hohenbrunn, Germany, Cas-No.25322-68-3) (PEG: 20–100%) solutions and embedded in pure PEG. Coronal sections of 20 μm thickness were cut by microtome according to the rat brain in stereotaxic coordinates, and collected in phosphate buffered saline (PBS). Sections were taken throughout the midbrain, through substantia nigra compacta (SNc), ventral tegmental area (VTA) (Bregma – 5.3 mm) and the dorsal striatum (Bregma 0.20 mm). The slices were selected and preincubated for 2 h in PBS with 0.3% triton and 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, USA, CAS-No.9048-46-8, Lot NO.078K0729) under agitation, the slices were then incubated overnight at 4 °C in a solution of monoclonal TH antibody (Santa Cruz, CA, USA; catalog No.SC-25269), diluted 1/1000 containing PBS (0.1 M, pH7.4), Triton (0.3%), and BSA (1%). The slices were then washed three times with PBS (0.1 M, pH7.4) for 5 min then incubated with the secondary antibody (rabbit anti-immunoglobulins, 1/500) (Vector Labs, Burlingame, CA, USA, Catalog NO.BA-1100, lot No.WO611) for 2 h at room temperature. After three washes, the slices were incubated for 1h30 min in PBS buffer containing Triton (0.3%) and the Avidin-biotine peroxidase complex (Kit ABC 1/500) (Vector Laboratories Burlingame, Californie, USA, Catalog No.PK-6101). TH was revealed following the enzymatic reaction of the peroxidase in presence of the 3,3-diaminobenzidine(0.03%) (Sigma-Aldrich; Oakville, Canada, CAS No.868272-85-9) and hydrogen peroxide (0.006%) in Tris buffer (0.05 M pH7.5). The sections were then collected, dehydrated and mounted in Eukit for optical microscopy observation. The specificity of the immunoreactive materials was tested by treating the slices to the same immunohistochemical procedure as described above and using the preimmune serum or omitting the primary antibodies. These tests showed that the primary antibodies used against TH display specific labeling [17,18].

2.5. Immunolabeling quantification

Quantification of TH-immunoreactivity (TH-IR) was performed according to the protocol published by Vilaplana and Lavialle [20]. The digitization and storage of images were achieved using a Zeiss-Axioskop 40 microscope (Carl Zeiss; Oberkochen, Germany) equipped with a Nikon digital camera. Images were digitized into 512 × 512 pixels with eight bits of gray resolution and were stored in TIFF format. Image processing and quantification were performed using Adobe Photoshop v.6.0 (Adobe Systems, San Jose, CA, USA). After conversion of each image to the binary mode, the percentages of black pixels were obtained using the image histogram option of Adobe Photoshop. This percentage corresponds to the TH-immunopositive area throughout the whole nucleus or the projections. Five sections from each animal of each group were randomly chosen for the quantification.

2.6. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA). Post hoc differences between group means were tested with the Tukey test. Data are reported as mean ± S.E.M. and p values < 0.05 were considered significant. Statistical analyses were performed using the computer software SPSS 10.0 for Windows (SPSS, IBM, Chicago, IL, USA).

3. Results

3.1. Effect of acute Cu-intoxication and curcumin-I on locomotor activity

Analysis of locomotor behavior revealed a significant ($p < 0.05$) reduction of crossed cases reflecting a loss of locomotor activity in Cu-intoxicated rats compared to their corresponding controls (Fig. 1A, B). Treatment with Curcumin-I significantly enhanced ($p < 0.05$) the locomotor performance in Cu-intoxicated rats (Fig. 1C) however, Curcumin-I itself (Fig. 1D) did not show any significant difference

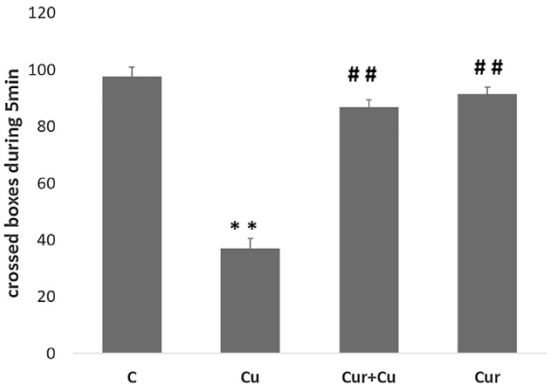

Fig. 1. Histogram displaying the number of crossed boxes during 5 mins observation in rats using the open-field test: C: control, Cu: Cu-treated, Cur + Cu: Curcumin I + Cu treated, Cur: curcumin treated. Data are shown as group mean values ± S.E.M. * $p < 0.05$ vs controls; and # $p < 0.05$ vs. Cu-group.

compared to control animals.

3.2. Effect of acute Cu-intoxication and curcumin-I on DAergic system within SNc, VTA and dorsal striatum

To assess whether the disturbed locomotor behavior is related to changes in the dopaminergic system, we used immunohistochemistry to examine the expression of TH within the midbrain DAergic nuclei (SNc and VTA) and the striatal outputs. The results demonstrate a significant ($p < 0.05$) loss of TH-immunoreactivity in Cu-intoxicated rats, within SNpc (Fig. 2B) and VTA (Fig. 3B) and their striatal outputs (fig. 4B). Neurons appear shrunken, the cell structures were not clear, and neurons and fibers show weak immunoreactivity compared to controls (Fig. 2A,B). Indeed, control rats showed clear cell structure, perikarya and neuronal processes were highly immunostained. Administration of curcumin-I to Cu-intoxicated rats protected against this reduction in neuronal DAergic innervations by increasing TH levels in the both the studied nuclei ($P < 0.05$) (Figs. 2 C, 3 C) and in dorsal striatum projections (fig. 4C), where neurons and fibers are obviously immunostained. By contrast, Curcumin-I alone did not induce a significant increase in TH-immunoreactivity compared to controls (Figs. 2 D, 3 D and 4 D).

4. Discussion

4.1. Cu-intoxication decreases TH expression and locomotor performance

Our data show that Cu administration causes a loss of TH-immunoreactivity within midbrain nuclei of the DAergic system, including SNc and VTA, as well as a decrease of TH-immunopositive fibers density in the dorsal striatum. This suggests a possible down-regulation of DA biosynthesis within those structures following Cu neurotoxicity. Such disturbances could be linked to an array of abnormal neurobehavioral symptoms outcomes including the locomotor deficiency observed in our Cu-intoxicated rats. Numerous reports support this finding. Indeed, Cu reduces brain levels of DA in common carp (Cyprinus carpio) after exposure to Cu sublethal levels [21]. Cu also induces damage of the DAergic innervations in the nigrostriatal system of rats [17]. Rats show a reduction of DA and its metabolites contents in striatum and an inhibition of TH mRNA expression in SNc [22]. In addition, Cu may act as a cytotoxic agent altering DAergic neurons within the globus pallidus and the basal ganglia, which may lead to Parkinsonism [23]. Cu also has a selective toxicity for neocortical neurons inducing neuronal apoptosis [24], a physiopathological process initiated through pathways involving the production of oxidative stress and free radicals [22]. This process is widely assumed to be one of the causes of neuronal death in many neurodegenerative diseases [25]. Cu exerts the same effects through disruption of the antioxidant power. That is, Cu was shown to repress the expression of Gpx1 (encode glutathione

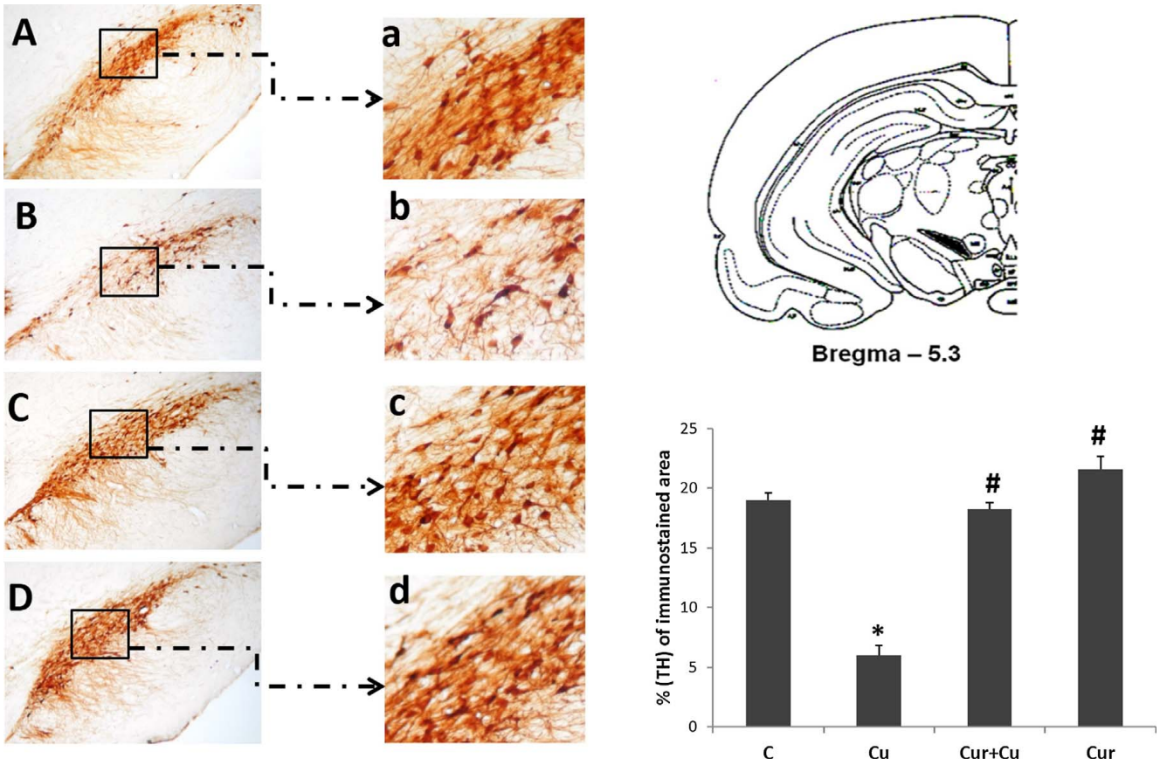

Fig. 2. Light micrographs of frontal sections through substantia nigra compacta (SNc) immunolabelled with antiserum against dopamine (TH) in control (A), Cu-treated (B), Cu + Cur (C), Cur (D). a,b,c,d: high magnification of (SNc) in control, Cu-treated, Cu + Cur treated, and Cur treated, respectively. Data are shown as group means ± S.E.M. * $p < 0.05$ vs controls; and # $p < 0.05$ vs. Cu-group.

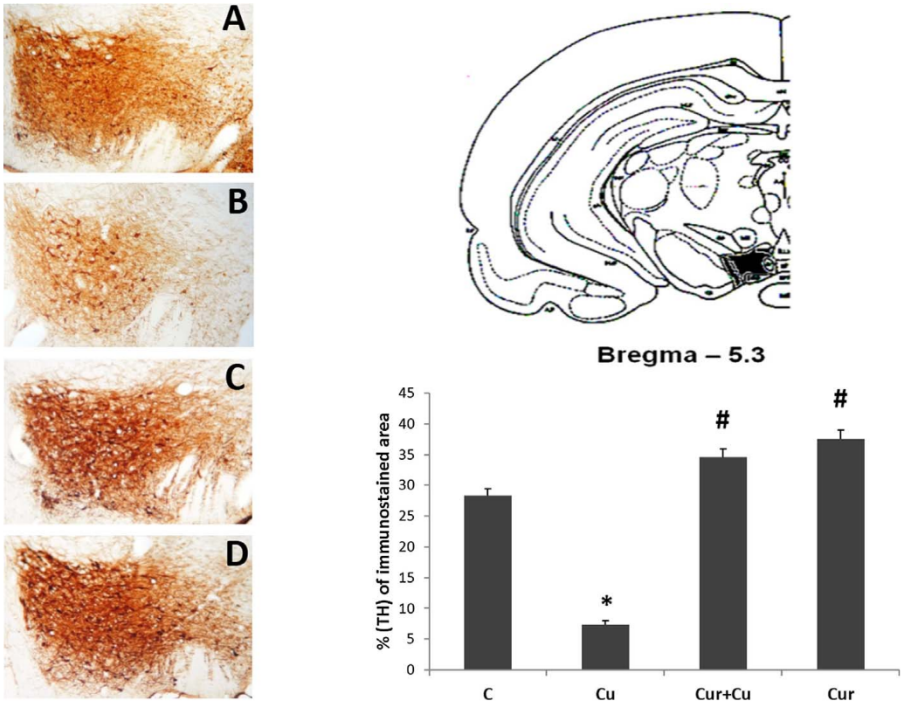

Fig. 3. Light micrographs of frontal sections through ventral tegmental area (VTA) immunolabelled with antiserum against dopamine (TH) in control (A), Cu-treated (B), Cu + Cur treated (C), Cur treated (D). Data are shown as group means ± S.E.M. * $p < 0.05$ vs controls; and # $p < 0.05$ vs. Cu-group.

peroxidase1) in PC12cells [22] and RCSN-3, a dopaminergic neuronal cell line [26]. Similarly, Cu decreased SOD activity in rats' midbrain [23]. As a result, this increased central ROS, the chemical species able to activate the caspase-3 activity [27], which lead to a cytotoxic effect of Cu by promoting neuronal apoptosis.

The nigrostriatal pathway is one of the major dopamine pathways in the brain and is critical for locomotor behavior. We observed a severe reduction in locomotor activity in Cu-intoxicated, which is consistent with the neuromodulatory role of dopamine within the nigro-cortico-striatal pathways in locomotor behavior [4]. The present data are also consistent with other results showing that Cu disturbs locomotor function in other species, including the ability of aquatic oligochaetes to avoid predators [28]. Elevated Cu levels also alter locomotor behavior in the adult carabid beetle *Pterostichus cupreus* L. [29] and chronic or acute Cu exposure reduced life-span and locomotor activity (i.e. climbing capabilities) in *Drosophila melanogaster* [4].

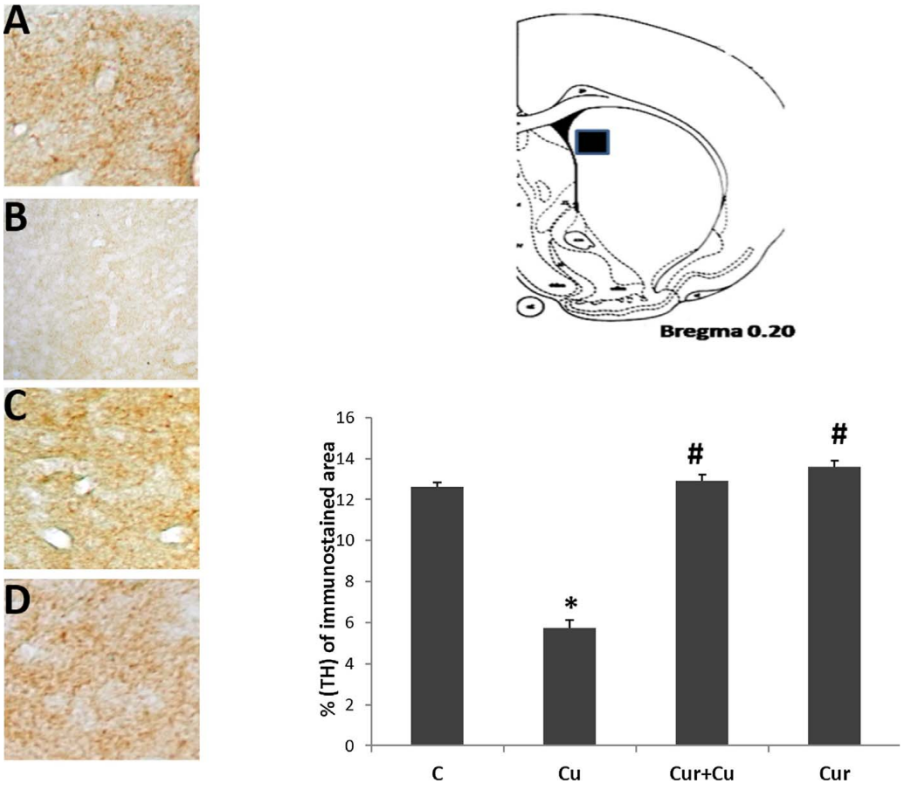

Fig. 4. Light micrographs showing TH-immunopositive fibers in the dorsal striatum of control (A), Cu-treated (B), Cu + Cur treated (C), Cur treated (D) rats. Data are shown as group means ± S.E.M. * $p < 0.05$ vs controls; and # $p < 0.05$ vs. Cu-group.

4.2. Curcumin-I repairs DAergic and locomotion disorders occurring in acute Cu-intoxicated rats

In the present study, we illustrated a neuroprotective potential of curcumin-I against Cu induced DAergic neurotoxicity. Curcumin-I induced a complete recovery of TH expression within SNc, VTA and dorsal striatum and enhanced locomotor activity in Cu-intoxicated rats. This result is consistent with a previous study showing that curcumin restores depleted dopamine levels in *Drosophila* PD model [30] and exhibits a beneficial effect against quinolinic acid induced motor deficit, biochemical and neurochemical abnormalities in rats [31]. Similarly, curcumin has an inhibitor effect on hippocampal damage in 6-OHDA induced PD in rat via improved neurofunction [32]. Numerous mechanisms may describe this neuroprotective effect of curcumin in the nigrostriatal system. Some studies reported an inhibitor effect of curcumin on MAO-b activity in mouse PD models [33], leading to increased amounts of monoamines stored and released from the nerve terminals. In addition, antioxidant mechanisms may be involved in the neuroprotective activity of curcumin. Several studies have shown that curcumin has a preventive potential against oxidative injury in the nigrostriatal dopaminergic system within PD animal models [34]. Furthermore, curcumin prevents histological injury, glutathione (GSH) depletion, maintains antioxidant enzyme status and lipid peroxidation by induction of enzymatic and non-enzymatic antioxidants, such as GSH, SOD and catalase [35,36]. It may also protect against ROS generation through its antioxidant effects [37]. The protective potential of curcumin may involve the protection and preferment of mitochondrial respiratory function [36,38]. Alternatively, curcumin may prevent neurotoxicity via its scavenging property of heavy metals due to its particular chemical structure [39] that allows it to directly bind to $Cu2+$ and other bivalent metals like $Fe2+$, which catalyzes formation of free radicals via the Fenton reactions [40].

5. Conclusion

This study reveals a clear neurotoxic effect of Cu on dopaminergic neurons and negative outcomes on locomotor performance. These deleterious effects were completely reversed by curcumin-I. As such, curcumin-I may qualify as a therapeutic agent against Cu neurotoxicity in general and DAergic system dysfunction in particular.

Conflict of interest

The authors declare that there are no conflicts of interest.

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