

# Minocycline exerts a neuroprotective action against 6-OHDA-induced neurotoxicity: *in vivo* and *in vitro* studies

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## Abstract

**Background:** Parkinson's disease (PD) is a chronic neurological disorder characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta*. The oxidative stress and inflammation, among other factors, are involved in the mechanisms of cell death in PD. We studied the neuroprotective properties of minocycline (Mino), focusing on its behavioral, neurochemical and immunohistochemical effects. Besides, Mino effects were also studied on an *in vitro* model of PD, as well as on the release of MPO in human neutrophils, and also its antioxidant activity.

**Methods:** We used *in vivo* (unilateral injection of 6-OHDA into the right striatum) and *in vitro* (SH-SY5Y cells) models of PD. For the *in vivo* model, male Wistar rats were divided into the following groups: sham-operated (SO), 6-OHDA-lesioned and untreated and 6-OHDA-lesioned and treated with Mino (10 and 25 mg/kg, p.o., 7 days). The animals were evaluated for behavioral, neurochemical and immunohistochemistry alterations. We also focused on Mino cytoprotective effects, as evaluated by its antioxidant and anti-inflammatory effects (nitrite determination and MPO release).

**Results:** Mino (10 and 25 mg/kg, p.o.) significantly reversed the decrease in striatal DA and DOPAC contents, the alteration in apomorphine-induced rotational behavior and the locomotor activity observed in 6-OHDA-lesioned rats, indicative of neuroprotection. Besides, it decreased the immunoreactivity for TNF-alpha in the hippocampus. In SH-SY5Y cells, Mino increased cells viability, as evaluated by the MTT assay, and significantly decreased the high nitrite levels observed

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in the cells after 6-OHDA-induced cytotoxicity. Mino presented anti-inflammatory and antioxidant effects as evaluated by inhibition of MPO release (an inflammatory marker) on human neutrophils and DPPH assay.

**Conclusion:** Minocycline exerts neuroprotective effects *in vivo* and *in vitro*, decreasing dopaminergic cell loss, through mechanisms that are a consequence of Mino anti-inflammatory and antioxidant properties, pointing out to its potential application for the treatment of neurodegenerative diseases as PD.

**Key Words:** Minocycline, neuroprotection, Parkinson's disease, inflammation, oxidative stress.

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta*. The impaired production and secretion of dopamine cause symptoms, including bradykinesia, tremor, rigidity and other motor and cognitive problems [1]. PD is a common neurodegenerative disorder, associated with substantial morbidity, increased mortality and high economic burden [2].

A major problem in PD is the complexity of its onset. Pathways including protein aggregation, defects in the ubiquitin-proteasome system, mitochondrial damage, and oxidative and nitrosative stresses can contribute to the loss of dopaminergic neurons [3]. While different mechanisms, including environmental toxins and genetic factors initiate neuronal damage in the *substantia nigra* and *striatum* of PD patients, there is evidence that the activation of neuroinflammatory cells aggravates this neurodegenerative process [4]. Furthermore, inflammation is a neuropathological feature of parkinsonian brains and of experimental models of the disease.

Besides, activated glial cells contribute to the neurodegenerative process, through the production of toxic molecules [5].

Evidences have indicated that, in both patients and experimental models of PD, neuroinflammation appears as a ubiquitous finding [6]. Furthermore, the immunohistochemical demonstration of reactive microglia and activated components suggests that chronic inflammation occurs in PD brain regions, and the involvement of neuroinflammatory processes in nigral degeneration has gained attention [7].

Post-mortem examination of human subjects exposed to MPTP revealed the presence of activated microglia, decades after drug exposure, suggesting that even a brief pathogenic insult can induce an inflammatory response [8]. The iNOS expression was shown to be up-regulated in the *substantia nigra* of PD patients, but not of age-matched controls [9], suggesting that glial activation and the resulting release of NO may contribute to the chronic neurodegeneration that characterizes PD. The neurotoxin 6-hydroxydopamine (6-OHDA), similarly to MPTP, is largely used as a model of PD. It is known to

cause inhibition of the mitochondrial complex I and oxidative stress, formed via DA oxidation [10, 11]. However, the exact mechanism by which 6-OHDA elicits its neurotoxic effects is not fully elucidated, although studies implicate a role for oxidative mediators [12, 13].

Minocycline (Mino) is a tetracycline exerting anti-inflammatory effects, distinct from its antimicrobial action [14]. The drug is known to protect against brain ischemia [15, 16] associated with reduced activation in microglia and inhibition of induction of the IL-1 $\beta$ -converting enzyme (ICE) m-RNA. This suggests that Mino may function by reducing cytotoxic properties of microglia, triggered either by ischemia or by excitotoxicity [17]. In addition, Mino inhibits MMP, superoxide production from neutrophils, and iNOS expression in human cartilages and murine macrophages [18, 19, 20].

The objectives of the present study were to investigate the neuroprotective action of Mino on a 6-OHDA-induced model of PD in rats. The focus was on behavioral alterations in untreated and Mino-treated 6-OHDA-lesioned animals, on measurements of striatal levels of DA and its metabolite DOPAC, as well as immunohistochemistry assays for TNF- $\alpha$  in the hippocampus. Besides, the effects of Mino on cell viability and nitrite concentrations in SH-SY5Y cells, on the release of MPO (an inflammation marker) from human-stimulated neutrophils, and the DPPH assay for antioxidant activities were also investigated.

## Materials and Methods

### Drugs and Reagents

6-Hydroxydopamine hydrochloride (6-OHDA), apomorphine and standard monoamines were purchased from Sigma, USA. Minocycline was from Ga-

lena Laboratories, São Paulo, Brazil. Ketamine was purchased from König Laboratories (Buenos Aires, Argentina) and xylazine from Vetbrands Laboratory (São Paulo, Brazil). All other reagents were of analytical grade.

### Animals

Adult male Wistar rats (260-280 g) from the Animal House of the Faculty of Medicine Estácio of Juazeiro do Norte (FMJ) were kept on a 12 h light/12 h dark cycle, with free access to water and standard food. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Health and Human Services Department. The protocol was approved by the Ethics Committee on Animal Experimentation, of the Faculty of Medicine Estácio of Juazeiro do Norte, Brazil.

### *In vivo* studies: experimental protocol and behavioral tests

The animals were anesthetized with ketamine (30 mg/kg, i.p.) and xylazine (25 mg/kg, i.p.), and received two unilateral stereotaxic injections of 12  $\mu$ g/ $\mu$ L 6-OHDA each, containing 0.2 mg/mL L-ascorbic acid, into the right striatum, according to the following coordinates starting from bregma [21]: AP 0.5/0.9, LL 2.5/2.7, DV 5.0/6.5. Sham-operated (SO) animals received vehicle, and were used as controls. The treatment with Mino (10 and 25 mg/kg, i.p.) started 1 h before the stereotaxic surgery (after the 6-OHDA-induced lesion), and continued daily for 7 days. Two weeks after the 6-OHDA injection, the behavior was assessed by monitoring body rotations induced by apomorphine (1 mg/kg, s.c.) when the number of net rotations (the number of 360° contralateral turns) was recorded for 60 min. The unilateral destruction of dopaminergic neurons causes a chemical imbalance of the brain content of this neurotransmitter. Because of this asymmetry, the administration of a dopamine agonist as apomor-

phine causes stimulation of intact dopamine neurons in the unaffected brain hemisphere. This is behaviorally manifested by locomotion in the direction of the unaffected hemisphere, making the animal to run in circles. The quantification of circling behavior is used to assess the efficacy of drugs potentially useful for the treatment of PD [22].

The animals were also submitted to the open field test, in order to evaluate their locomotor activity (usually affected in experimental models of PD), and at the next day were sacrificed, the striatal tissue collected and stored at  $-70^{\circ}\text{C}$  until use.

### Monoamine levels determination

For measurements of dopamine and its metabolite (3,4 dihydroxyphenylacetic acid - DOPAC), *striata* from both sides of 6-OHDA-lesioned (untreated or treated with Mino) and unlesioned sham-operated groups were used to prepare 10% homogenates. Homogenates were sonicated in 0.1 M  $\text{HClO}_4$ , for 30 s, centrifuged at  $4^{\circ}\text{C}$  for 15 min at 15,000 rpm, and the supernatants were filtered (0.2  $\mu\text{m}$ , Millipore). Twenty-microliter samples were then injected into a high-performance liquid chromatography (HPLC) column. The mobile phase was 0.163 M citric acid, pH 3.0, containing 0.02 mM EDTA with 0.69 mM sodium octanesulfonic acid (SOS), as an ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahydrofuran. The monoamines were electrochemically detected, using an amperometric detector (Shimadzu, Japan), by oxidation on a glassy carbon electrode at 0.85 V relative to the Ag–AgCl reference electrode. Their concentrations were determined by comparison with standards injected into the HPLC column at the day of experiment, and the values expressed as ng/mg tissue.

### Immunohistochemistry for TNF-alpha in the hippocampus

Hippocampal slices from SO, untreated 6-OHDA-lesioned and 6-OHDA-lesioned rats treated with Mino (10 and 25 mg/kg) were washed three times with 0.05 M Tris buffer (TB, pH 7.6). Then, the endogenous peroxidase was blocked with 3%  $\text{H}_2\text{O}_2$  in 10% methanol in TB, for 15 min. The slices were washed three times again with TB and pre-incubated with 5% normal goat serum (NGS), for 30 min in 0.3% Triton, followed by the incubation with the primary TNF-alpha antibody (diluted in 2% NGS/TB), overnight at  $4^{\circ}\text{C}$ , according to the manufacturer's instructions (Calbiochem, Nottingham, UK). Then, after three washings with TB, the slices were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG, 1:200, DAKO) for 2 h, washed three times again with TB, and revealed with 0.05% diaminobenzidine in 0.03%  $\text{H}_2\text{O}_2$ , for 10 min. After a final washing with distilled water, the slices were mounted for microscopy examination.

### In vitro studies with SH-SY5Y dopaminergic cells: cell culture

The SH-SY5Y cell line from human neuroblastoma has become a popular cell model for PD research, because this cell line possesses many characteristics of DAergic neurons. For example, these cells express tyrosine hydroxylase and dopamine-beta-hydroxylase, as well as the dopamine transporter [23]. The cells were cultivated in 25  $\text{cm}^2$  culture flasks (from Corning, USA) with a 50 mL volume, in 1:1 DMEM/F12 medium (Gibco, USA), containing 10% bovine fetal sera and 1% penicillin/streptomycin. The cells were then maintained in the oven at  $37^{\circ}\text{C}$  and a 5%  $\text{CO}_2$ /95%  $\text{O}_2$  atmosphere. The cell growth was followed by observation on an inverted microscope.

### MTT Assay in SH-SY5Y cells

Minocycline neuroprotection was evaluated by the MTT assay, based on the conversion of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) yellow salt to the blue formazan compound. This conversion only occurs in metabolically active and viable cells, by the action of the succinate dehydrogenase enzyme present in mitochondria. This permits the indirect quantification of the percentage of viable cells [24]. For the assay, monolayers of SH-SY5Y cells were plated in 96 wells, at a  $0.7 \times 10^5$  cells/mL density. The cells were cultured for 5 days and then Mino (at concentrations of 0.1, 1 and 10  $\mu\text{g/mL}$ , based on previous experiments) was added 1 h before 6-OHDA (12.5  $\mu\text{g/mL}$ ). After 24 h incubation in the oven, at 37°C and a 5%  $\text{CO}_2$ , 95%  $\text{O}_2$  atmosphere, the suspension of cells culture was centrifuged (1500 rpm/15 min) and the supernatant discarded. Then, 200  $\mu\text{L}$  of a 10% MTT solution (in 1:1 DMEM/F12 medium) were added to each well, followed by incubation for 3 h and centrifugation (3000 rpm/10 min). The supernatant was again discarded, and the formazan blue pellet resuspended; in 150  $\mu\text{L}$  DMSO and shaken for 10 min until complete dissolution. The quantification of the reduced MTT salt was carried out with a plaque reader at 595 nm.

### Nitrite determination in SH-SY5Y cells

The Griess reagent provides a simple and well characterized colorimetric assay for nitrites, and nitrates that have been reduced to nitrites, with a 100 nM detection limit. After cells incubation, the nitrite concentration was determined by the method of Green et al., 1981 [25]. For this, 100  $\mu\text{L}$  Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)-ethylenediamine hydrochloride/1%  $\text{H}_3\text{PO}_4$ /distilled water, 1:1:1:1) were added to 100  $\mu\text{L}$  cell culture supernatant, followed by 10 min incubation at room temperature. The standard curve was made with

concentrations of  $\text{NaNO}_2$ , ranging from 0.75 to 100  $\mu\text{M}$  under the same conditions. Blanks were prepared by the addition of 100  $\mu\text{L}$  Griess reagent to 100  $\mu\text{L}$  culture medium, and the absorbance determined with a plaque reader at 560 nm.

### Myeloperoxidase release from human neutrophils

Myeloperoxidase (MPO) is an enzyme stored in azurophilic granules of polymorphonuclear neutrophils and macrophages, and released into extracellular fluid in the setting of inflammatory process. It is involved in oxidative stress and inflammation, and thus considered as a marker for systemic inflammation. Following Lucisano and Mantovani, 1984 [26],  $2.5 \times 10^6$  human leukocytes were suspended in buffered Hank's balanced salt solution, containing calcium and magnesium. The preparations usually contained predominantly neutrophils ( $85.0 \pm 2.8\%$ ), and the cell viability, as determined by the Trypan blue test, was  $97.7 \pm 0.94\%$ . The cells were incubated with Mino (0.001 to 1  $\mu\text{g/mL}$ ) or indomethacin at 35.7  $\mu\text{g/mL}$  (Indo, as standard), for 15 min at 37°C. Human neutrophils were stimulated by the addition of phorbol myristate acetate (PMA, 0.1  $\mu\text{g/mL}$ ), for 15 min at 37°C. The suspension was centrifuged for 10 min at  $2000 \times g$  at 4°C. Aliquots (50  $\mu\text{L}$ ) of the supernatants were added to phosphate-buffered saline (100  $\mu\text{L}$ ), phosphate buffer (50  $\mu\text{L}$ , pH 7.0) and  $\text{H}_2\text{O}_2$  (0.012%). After 5 min at 37°C, TMB (1.5 mM, 20  $\mu\text{L}$ ) was added, and the reaction was stopped by 30  $\mu\text{L}$  sodium acetate (1.5 M, pH 3.0). The absorbance was determined using a spectrophotometer (620 nm)

### Diphenyl-2-picrylhydrazyl (DPPH)-scavenging activity

The stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) is used as an antioxidant assay, based on electron-transfer that produces a violet



solution in ethanol. This free radical is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. This assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry. Minocycline and alpha-tocopherol (as reference) were evaluated by the DPPH assay [27]. Briefly, an aliquot (0.1 mL) of Mino (1 to 50  $\mu\text{g}/\text{mL}$ ) or alpha-tocopherol (5 or 50  $\mu\text{g}/\text{mL}$ ) was mixed with 3.9 mL DPPH solution (0.3 mM). The mixture was vortexed for 1 min and then left standing at room temperature for 30 min. Finally, the absorbance of the resulting solution was read spectrophotometrically (Beckman Instruments Inc.) at 517 nm.

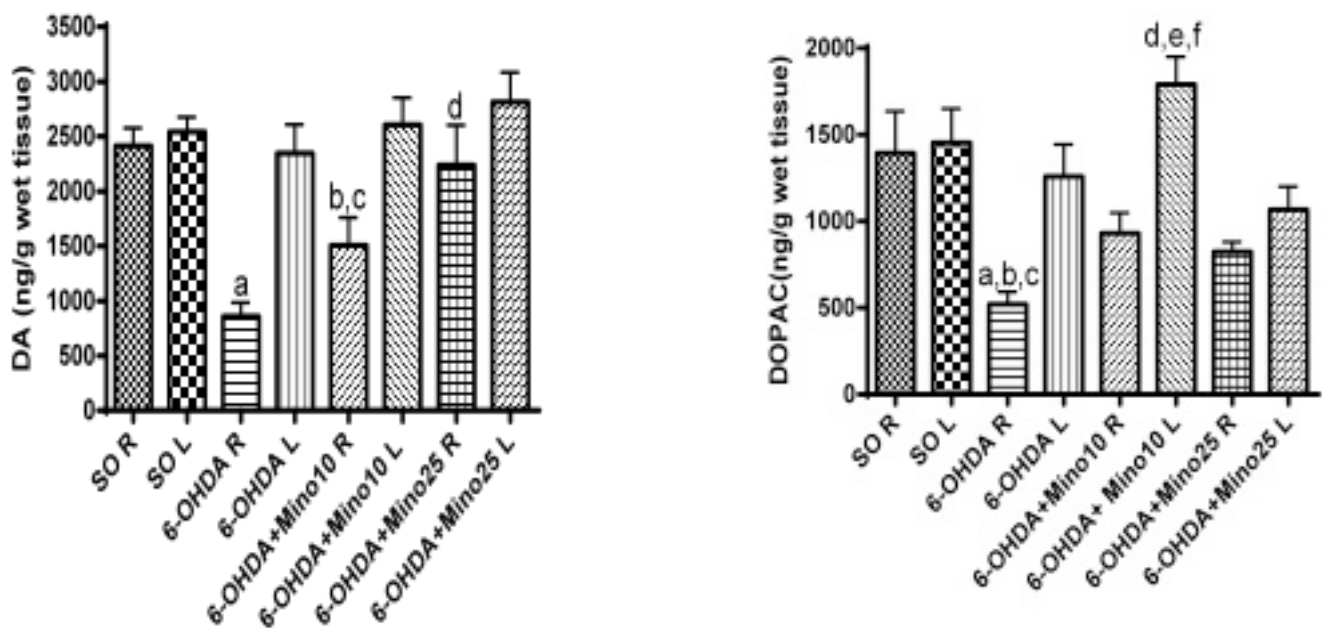
### Statistical Analyses

For monoamine contents, the data were analyzed by the Student's t test, when comparison was performed between right and left *striata* from the

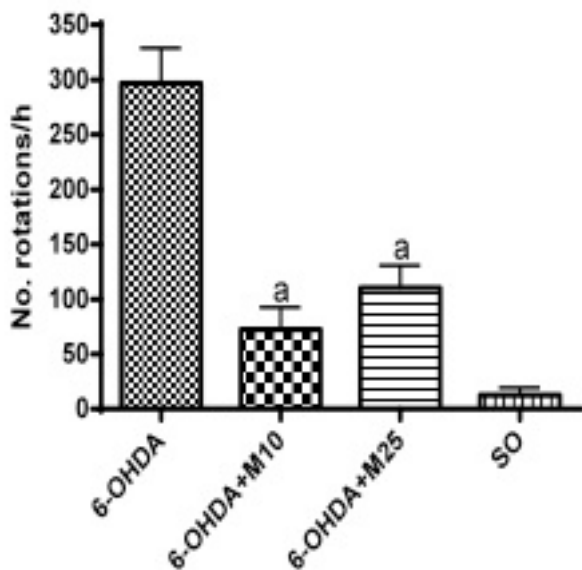
same group, and by One-way ANOVA followed by Newman-Keuls as a *post hoc* test, for comparing the same striatal side among groups. Identical procedure was used for all other statistical analyses.

## Results

*Effects of the minocycline (Mino) treatment on striatal DA and its metabolites in 6-OHDA-lesioned rats*  
The 6-OHDA-lesioned right striata showed a 63% decrease in DA concentrations, as compared to the unlesioned left striata of the same group. This decrease was lower in the 6-OHDA-lesioned right striata of Mino-treated rats, especially in the 6-OHDA+Mino25 group whose reduction was only 20%, as compared to its left side. The SO group showed no change in DA content (**Fig. 1A**) The percentage reductions of DOPAC levels in the right striata of the 6-OHDA-lesioned group were around



**Figure 1.** Minocycline (Mino: 10 and 25 mg/kg, i.p.) significantly reversed the DA (**A**) and DOPAC (**B**) contents in the lesioned right striatum (R), as related to its unlesioned left side (L) and mainly to the right side of the 6-OHDA-lesioned untreated group. The values are means  $\pm$  S.E.M. from 6 to 14 animals per group. SO = sham-operated group. (**A**): **a.**  $p < 0.001$  vs. 6-OHDA L; **b.**  $p < 0.05$  vs. 6-OHDA R; **c.**  $p < 0.05$  vs. 6-OHDA + Mino10 L; **d.**  $p < 0.001$  vs. 6-OHDA R. (**B**): **a.**  $p < 0.001$  vs. 6-OHDA L; **b.**  $p < 0.05$  vs. 6-OHDA + Mino10 R; **c.**  $p < 0.05$  vs. 6-OHDA + Mino25 R; **d.**  $p < 0.001$  vs. 6-OHDA + Mino10 R; **e.**  $p < 0.01$  vs. 6-OHDA L; **f.**  $p < 0.01$  vs. 6-OHDA + Mino25 L.

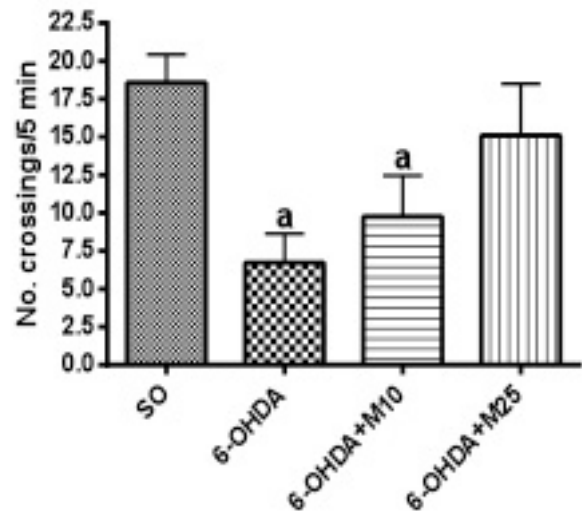


**Figure 2.** Minocycline (Mino: 10 and 25 mg/kg, i.p.) significantly reversed the increased apomorphine-induced rotational behavior, evaluated by the number of contralateral rotations for 1 h, as compared to the 6-OHDA-lesioned untreated group. As expected, very few rotations were seen in the sham-operated (SO) group. The values are means  $\pm$  S.E.M. from 17 to 22 animals per group. **a.**  $p < 0.001$  vs. 6-OHDA.

60%, in relation to its unlesioned left side, while these changes were lower after Mino treatments (50 and 23% decreases after the doses of 10 and 25 mg/kg, respectively). No significant alterations in DOPAC contents were observed in either side of the striata in the SO group (**Fig. 1B**).

### Apomorphine-induced rotational behavior in 6-OHDA-lesioned rats, without and with minocycline treatments

While 6-OHDA-lesioned and untreated rats presented high numbers of contralateral rotations (296.7 $\pm$ 32.12 turns/h), this effect was significantly reversed after Mino treatments (6-OHDA+Mino10: 72.6 $\pm$ 20.05 turns/h; 6-OHDA+Mino25: 110.5 $\pm$ 20.20 turns/h). On the other hand, almost no rotation was shown by the SO group (12.8 $\pm$ 6.53 turns/h) (**Fig. 2**).



**Figure 3.** Minocycline (Mino: 10 and 25 mg/kg, i.p.) partially reversed the decreased locomotor activity (number of crossings/5 min) observed in 6-OHDA-lesioned untreated rats. However, the locomotor activity was higher in the controls (sham-operated group = SO) but not statistically different from the 6-OHDA+Mino25 group. The values are means  $\pm$  S.E.M. from 7 to 10 animals per group. **a.**  $p < 0.05$  vs. SO.

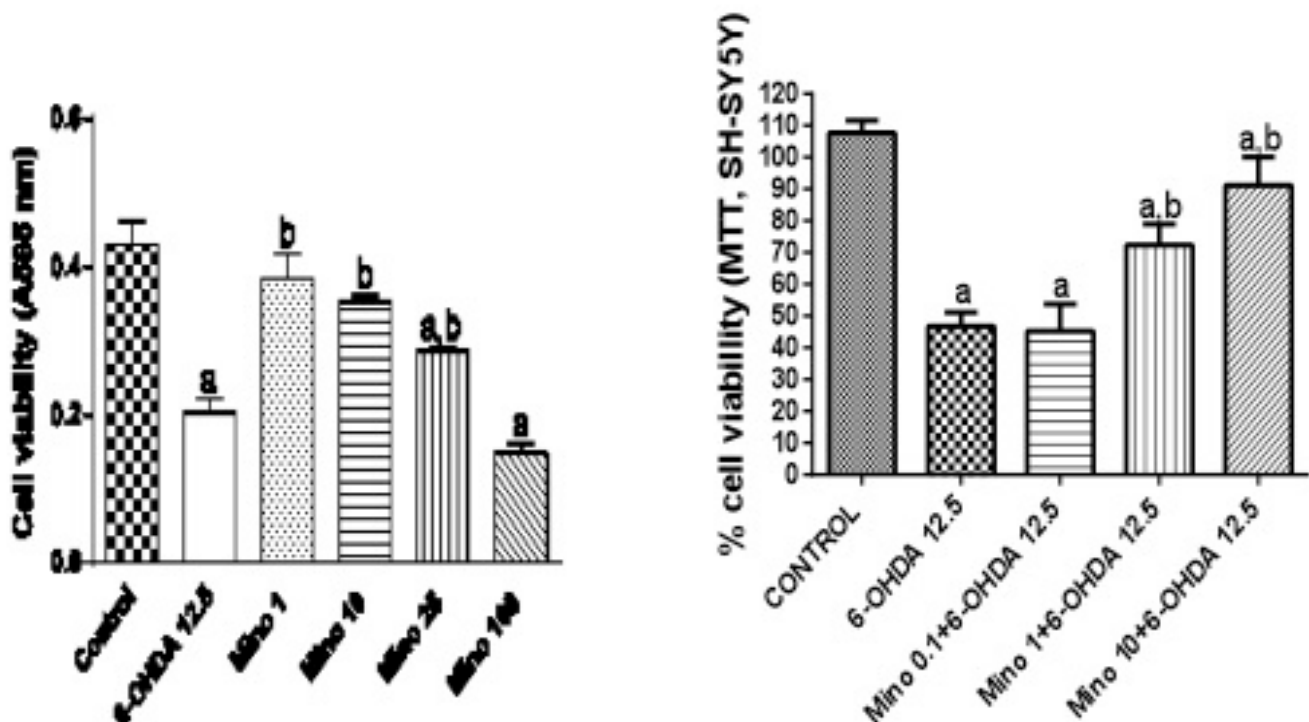
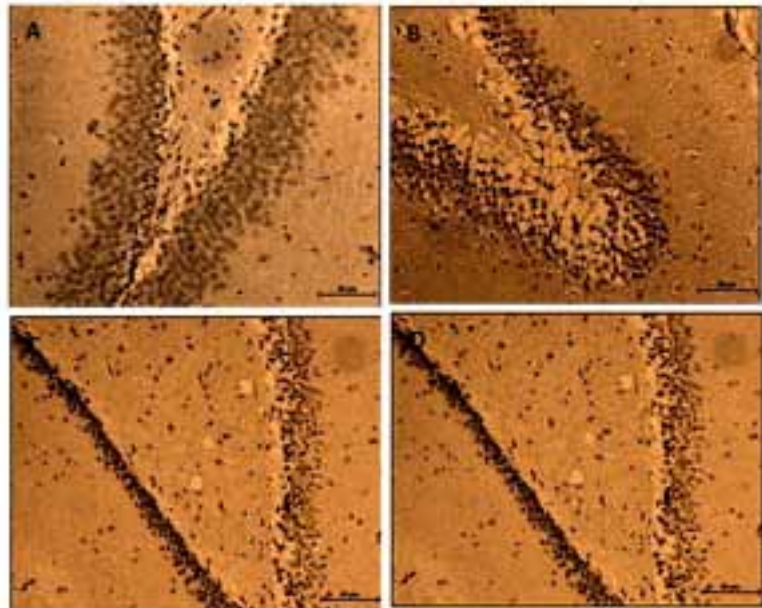
### Exploratory behavior in the open field test

The 6-OHDA-lesioned rats showed a significant decrease of 64% in locomotor activity (6.7 $\pm$ 1.92), compared to the SO group (18.6 $\pm$ 1.85), as evaluated by the number of crossings/5 min. The treatment of the 6-OHDA-lesioned group with the two doses of Mino reverted this effect, bringing values close to those of the SO group with the higher dose (6-OHDA+Mino25: 15.1 $\pm$ 3.38) (**Fig. 3**).

### Minocycline (Mino) decreases the immunoreactivity for TNF-alpha in the rat hippocampus

**Figure 4** shows representative photomicrographs of dentate gyrus where the untreated 6-OHDA-lesioned group presents a high immunoreactivity for TNF-alpha. This effect was in great part reversed by Mino treatments (6-OHDA+Mino 10 or 25). On the other hand, few immunopositive cells for TNF-alpha were seen in the SO group.

**Figure 4.** Minocycline (10 and 25 mg/kg) decreases the number of TNF-alpha immunopositive cells in the hippocampus (dentate gyrus), as related to the untreated 6-OHDA-lesioned group. **A:** SO; **B:** untreated 6-OHDA; **C:** 6-OHDA+Mino10; **D:** 6-OHDA+Mino25 (200X magnification).



**Figure 5.** Concentration-response curve of minocycline (Mino: 1 to 100  $\mu\text{g}/\text{mL}$ ) in SH-SY5Y cells, showing a cytotoxic effect at concentrations equal to or higher than 100  $\mu\text{g}/\text{mL}$  (A). 6-OHDA at the concentration of 12.5  $\mu\text{g}/\text{mL}$  decreased cell viability by 64%, as related to controls. The values are means  $\pm$  S.E.M. from 3 to 12 samples. a.  $p < 0.05$  to 0.001 vs. controls; b.  $p < 0.05$  to 0.01 vs. 6-OHDA 12.5. (B) Minocycline (Mino: 0.1, 1 and 10  $\mu\text{g}/\text{mL}$ ) significantly and dose-dependently protected SH-SY5Y cells from death, after their exposure to the 6-OHDA neurotoxin (12.5  $\mu\text{g}/\text{mL}$ ), as evaluated by the MTT assay. While 6-OHDA decreased by 66% the percentage of cell viability, Mino partially blocked the neurotoxin effect, bringing values closer to those of controls. a.  $p < 0.001$  vs. controls; b.  $p < 0.05$  vs. 6-OHDA 12.5.



## In vitro studies

### Effects of minocycline on cell viability, as evaluated by the MTT assay in SH-SY5Y cells

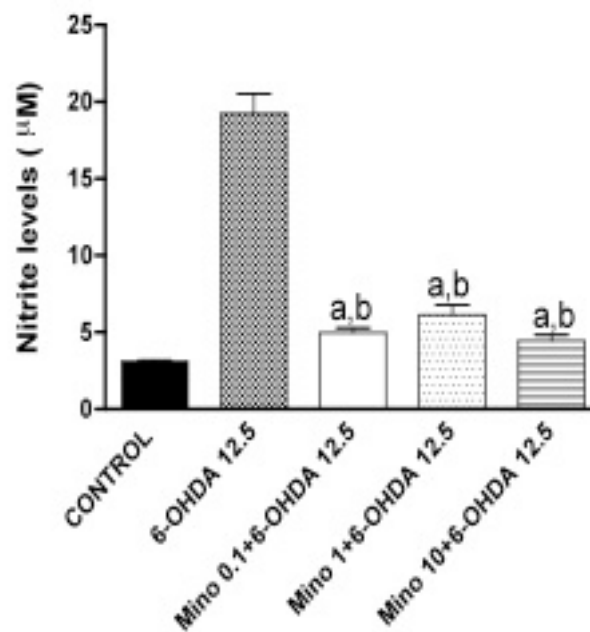
**Fig. 5A** shows the Mino concentration-response curve, demonstrating that it decreases cells viability at concentrations equal or higher than 100  $\mu\text{g}/\text{mL}$ , an indicative of cytotoxicity. A 57% decrease was observed in SH-SY5Y cells viability, after their exposure to the 6-OHDA neurotoxin (12.5  $\mu\text{M}$ ), as related to the untreated cells (controls). The cell treatment with Mino (0.1, 1 and 10  $\mu\text{g}/\text{mL}$ ) partially reversed the neurotoxin effect, improving cells viability which decreased by only 33 and 15% with Mino, at the concentrations of 1 and 10  $\mu\text{g}/\text{mL}$ , respectively. The lower dose (0.1  $\mu\text{g}/\text{mL}$ ) showed no cytoprotective effect (**Fig. 5B**).

### Effects of minocycline on nitrite concentrations in SH-SY5Y cells

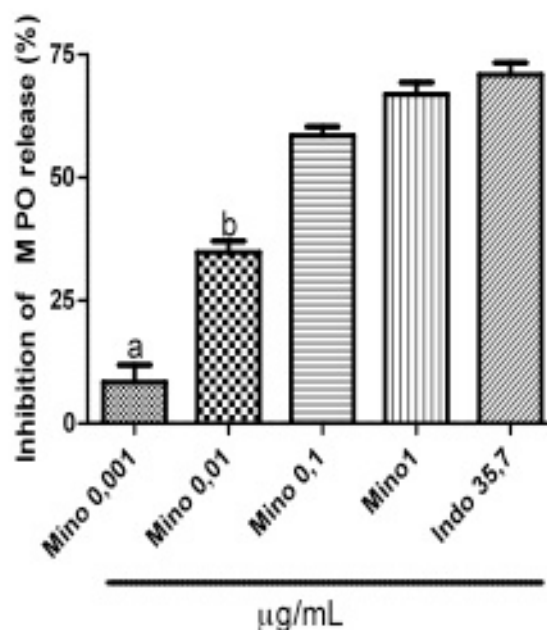
The SH-SY5Y cells exposure to 6-OHDA (12.5  $\mu\text{M}$ ) significantly increased the nitrite concentration by sixfold, as compared to the unexposed cells (controls). On the other hand, this neurotoxin effect was almost completely reversed after the addition of Mino, at concentrations of 0.1, 1 and 10  $\mu\text{g}/\text{mL}$ . Under these conditions, the nitrite concentrations increased only from 1.5- to 1.9-fold (**Fig. 6**).

### Minocycline inhibits MPO release in human neutrophils

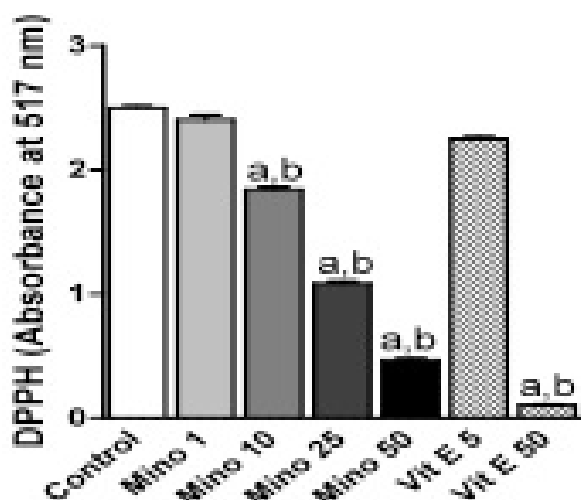
**Figure 7** shows a concentration-dependent effect of Mino on MPO release by human neutrophils. While only 8% inhibition was achieved in the presence of Mino, at 0.001  $\mu\text{g}/\text{mL}$ , the percentage inhibition increased to almost 70%, at the concen-



**Figure 6.** Minocycline (Mino: 0.1, 1 and 10  $\mu\text{g}/\text{mL}$ ) completely reversed the increased nitrite levels observed in SH-SY5Y cells, after their exposure to 6-OHDA, and the values (means  $\pm$  S.E.M. from 4 to 15 samples) were very close to those of controls. **a.**  $p < 0.05$  vs. controls; **b.**  $p < 0.001$  vs. 6-OHDA 12.5.



**Figure 7.** Minocycline ( $\mu\text{g}/\text{mL}$ ) inhibits the myeloperoxidase (MPO) release from human neutrophils, at 1  $\mu\text{g}/\text{mL}$  concentration, and this effect was similar to that observed in the presence of indomethacin (Indo, 35.7  $\mu\text{g}/\text{mL}$ ) used as reference. Assays were performed in at least quadruplicated samples. **a.** and **b.** vs. all other four groups,  $p < 0.0001$ .



**Figure 8.** Minocycline ( $\mu\text{g/mL}$ ) presents a radical scavenger activity, as evaluated by the DPPH assay. A significant effect was observed at a concentration of 50  $\mu\text{g/mL}$ , similar to that observed in the presence of vitamin E used as reference. Assays were performed at least in quadruplicated samples. a. and b.  $p < 0.0001$  vs. controls and Vitamin E 50.

tration of 1  $\mu\text{g/mL}$ . This value was similar to that observed in the presence of indomethacin (Indo, 35.7  $\mu\text{g/mL}$ ), used as reference.

### Scavenging activity of minocycline, as evaluated by the DPPH assay

Data on the antioxidant effect of Mino, at concentrations ranging from 1 to 50  $\mu\text{g/mL}$ , are shown in **Fig. 8**. In this assay, a significant effect of Mino was observed only with the concentration of 50  $\mu\text{g/mL}$ , comparable to that observed in the presence of vitamin E (used as reference), at a similar concentration.

## Discussion

Our data demonstrated that minocycline (Mino) can effectively protect striatal dopaminergic neurons from the effects of the 6-OHDA neurotoxin,

in rats which is a widely used model for Parkinson's disease. The neuroprotective effect of Mino (10 and 25  $\text{mg/kg}$ ) was observed after its intraperitoneal administration for 7 days. Although there are many studies showing the neuroprotective effects of Mino on various experimental models, such as cerebral ischemia [28, 29, 30, 31] among others, those on PD models are fewer [32, 33].

We showed, in the model of unilateral striatal 6-OHDA-lesioned rats, that the increased apomorphine-induced rotation turns were significantly decreased by Mino, indicative of a neuroprotective effect and less degeneration of dopaminergic neurons. The unilateral injection of 6-hydroxydopamine (6-OHDA) into the right *substantia nigra* or *striatum* is known to produce an extensive loss of dopaminergic cells. The imbalance in dopaminergic innervation between both sides results in a postural asymmetry, causing rotation away from the contralateral unlesioned side. Analyses of mitochondrial oxidative phosphorylation enzyme activities, in nigral tissue from 6-OHDA-lesioned rats, revealed a clear loss of complex I activity [34]. A deficit in mitochondrial complex I could conceivably contribute to cell death in parkinsonism, via free radical mechanisms, both directly by reactive oxygen species production and indirectly by the decreased ATP synthesis and energy failure.

Additionally, we showed that 6-OHDA significantly reduced DA contents in the lesioned right *striatum*, as compared to the unlesioned left *striatum* of the same experimental group. Mino treatments reversed the decreases in DA contents in the lesioned right *striatum*, compared to the 6-OHDA-lesioned right *striatum* of untreated rats, also suggesting that the drug presents a neuroprotective effect. A similar result was observed in DOPAC contents.

The toxicity of 6-OHDA seems to be related to the production of ROS, including superoxide radicals,

hydrogen peroxide and hydroxyl radicals [35, 36, 37]. The pronounced toxicity on DA neurons is due to the selective uptake of this toxin by the DA transporter (DAT) system, as well as the presence of monoamine oxidase (MAO) and low tolerance of the dopaminergic neuronal population to oxidative stress. Indeed, ROS and the oxidative stress have been pointed out as key factors in the pathogenic cascade, leading to loss of midbrain DA neurons in PD [38].

The intra-striatal administration of 6-OHDA has been found to induce a microglial reaction [39] that may participate in the progression or extension of neuronal loss caused by the neurotoxin. Minocycline was demonstrated to protect nigral cells from MPTP and 6-OHDA toxicities [40]. This antibiotic (45 mg/kg, i.p.) displayed beneficial effects against the striatal injection of 6-OHDA, and the subsequent nigral cell degeneration and microgliosis. Protection against this toxin has been also observed *in vitro* (41). The oral administration of Mino (60-120 mg/kg, p.o., or 11-45 mg/kg, i.p.) blocked MPTP-induced degeneration of dopamine neurons, as well as the loss of striatal dopamine and its metabolites, and mitigated the formation of nitrotyrosine. These effects were thought to be related to an inhibitory effect of Mino on microglial activation (42).

Almost all the beneficial effects of Mino are related to an inhibitory activity on inflammation processes and/or apoptotic cell death, both phenomena being intimately related to neuronal degeneration [43]. Interestingly, Mino also protected dopaminergic neurons from the activation of microglia, induced by an injection of lipopolysaccharide (LPS) within the *substantia nigra* [44]. Accordingly, several studies showed that Mino reduces the expression of inducible nitric oxide synthase and subsequent nitric oxide production, as well as caspase-1 activity/expression, and thereby prevents the formation of IL-1beta [45, 46].

Mino also prevents oxidative protein modifications and damage in disease models associated with inflammatory glial activation and oxidative stress and, although the drug is assumed to act by preventing the up-regulation of pro-inflammatory enzymes, it has been shown that Mino acts by a direct chemical interaction with ROS [47]. Others suggested that the neuroprotective effects of Mino might be associated with the mitigation of neuronal excitability, glutamate release and Ca<sup>2+</sup> overloading [48].

We showed that decreased locomotor activity observed in hemiparkinsonian rats was reversed after Mino treatment. A similar effect was seen in grooming behavior. Previously, we demonstrated that ischemia-induced behavior alterations were reversed by Mino, and in this case all the observed parameters (locomotor activity, rearing and grooming behaviors) were equally affected [49]. It has been found that Mino, administered to mice after traumatic brain injury (TBI), attenuated microglial activation and reduced the brain lesion volume, as well as TBI-induced hyperactivity [50].

Chronic neuroinflammation is a typical feature in neurodegenerative diseases, including PD, in which TNF-alpha expression appears to be upregulated representing a valuable target for intervention [51, 52, 53]. In addition, evidences indicate that TNF-alpha may play a role in the selective vulnerability of the nigrostriatal pathway associated to dopaminergic neurotoxicity [54]. Minocycline is known to protect cells, via an anti-inflammatory mechanism [55], and a recent study demonstrated that it decreased brain TNF-alpha levels, as evaluated by a hypothermic cardiac arrest model in rats [56].

We showed a decreased immunoreactivity for TNF-alpha in the hippocampus from 6-OHDA-lesioned rats after treatments with Mino, as related to the 6-OHDA-lesioned and untreated group. Neuro-pathological studies show that the hippocampus

is affected in PD, and hippocampal degeneration could be responsible for the patient's cognitive dysfunction [57]. Moreover, other data suggest interactions between the dopaminergic system and hippocampus, in synaptic plasticity, adaptive memory and motivated behavior [58], and that the declarative memory impairment in PD patients without dementia may be predicted by the rate of microstructural alterations in the hippocampal formation [59]. Our findings may certainly contribute to the drug neuroprotective action.

We also demonstrated that Mino protected SH-SY5Y cells against 6-OHDA cytotoxicity in a dose-dependent manner, as evaluated by the MTT assay. As a matter of fact, the drug seems to exhibit a toxic action, at concentrations equal to or higher than a 100 µg/ml. Furthermore, Mino significantly decreased nitrite contents of cells previously exposed to 6-OHDA, indicating an antioxidant action. SH-SY5Y cells are originated from human neuroblastoma and known to mimic many aspects of the dopaminergic neuronal death observed in PD, caused by mitochondrial complex I neurotoxins [23, 49, 50]. The SH-SY5Y cell line has become a popular model for PD, for possessing many characteristic of dopaminergic neurons. Thus, these cells express tyrosine hydroxylase and dopamine-β hydroxylase, as well as the dopamine transporter (DAT).

Although it is accepted that 6-OHDA induces cytotoxicity in different cell types, the mechanisms involved are controversial, and among them the generation of ROS is the most accepted one [42, 60, 61]. Thus, 6-OHDA can either undergo extracellular auto-oxidation or intracellular enzymatic oxidation through MAO B, yielding ROS, quinones and their degradation products [62]. It has been found that 6-OHDA induces mitochondrial fragmentation in SH-SY5Y cells [63]. These authors showed that 6-OHDA-induced mitochondrial fragmentation is an early event, preceding the collapse of the mem-

brane potential and cytochrome c release in SH-SY5Y cells.

Minocycline was shown to inhibit iNOS expression and NO release, and to increase neuronal survival, as well as to inhibit the production of TNF-α mediated by LPS in glial cells [64]. These authors used Mino concentrations ranging from 1 to 20 nM, and found a maximal neuroprotective effect without toxicity, at the concentration of 1 nM.

Minocycline was able to significantly decrease the myeloperoxidase release from human neutrophils, at very low concentrations (0.1 µg/mL), and its effect was similar to that of indomethacin used as reference. Myeloperoxidase (MPO) is a hemoprotein abundantly expressed in polymorphonuclear leukocytes (neutrophils) and secreted during their activation. This enzyme has been proposed as a biomarker of inflammation, under several pathological conditions [65, 66, 67]. In addition, Mino presented an antioxidant activity *in vitro*, as evaluated by the DPPH assay.

Increasing evidence [68] indicates that both oxidative stress and inflammation may play a fundamental role in the pathogenesis of PD, and minocycline was shown to prevent oxidative protein modifications and damage in disease models associated with inflammatory glial activation and oxidative stress [69]. Previously, we demonstrated a potent anti-inflammatory effect of Mino acutely administered in several models of inflammation [70]. In addition, Mino sub-chronically injected significantly reversed neurochemical and behavior alterations, in models of global ischemia and PD in rats [71]. Our data indicate that the anti-inflammatory and antioxidant effects, besides the inhibitions of iNOS and TNF-α among other properties, could stimulate translational data from experimental studies to randomized and well-designed clinical trials of neuroprotective agents as Mino, for new therapeutic



applications, especially those where inflammation plays a role [72]. We conclude that Mino may act as a dopaminergic agonist, and this effect together with the drug anti-inflammatory and antioxidant activities are probably responsible for its neuroprotective action.

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