J Neural Transm (2010) 117:1337–1351
DOI 10.1007/s00702-010-0464-x

BASIC NEUROSCIENCES, GENETICS AND IMMUNOLOGY - ORIGINAL ARTICLE

Proanthocyanidin-rich fraction from *Croton celtidifolius* Baill confers neuroprotection in the intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine rat model of Parkinson's disease

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Received: 24 March 2010 / Accepted: 16 August 2010 / Published online: 8 October 2010

Abstract We have recently demonstrated that rodents treated intranasally with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) suffered impairments in olfactory, cognitive and motor functions associated with time-dependent disruption of dopaminergic neurotransmission in different brain structures conceivably analogous to those observed during different stages of Parkinson's disease (PD). On the other hand, the proanthocyanidin-rich fraction (PRF) obtained from the bark of *Croton celtidifolius* Baill (Euphorbiaceae), a tree frequently found in the Atlantic forest in south Brazil, has been described to have several neurobiological activities including antioxidant and anti-inflammatory properties, which may be of interest in the treatment of PD. The present data indicated that the pre-treatment with PRF (10 mg/kg, i.p.) during five consecutive days was able to prevent mitochondrial complex-I inhibition in the striatum and olfactory bulb, as well as a decrease of the enzyme tyrosine hydroxylase expression in the olfactory bulb and substantia nigra of rats infused with a single intranasal administration of MPTP (1 mg/nostril). Moreover, pretreatment with PRF was found to attenuate the short-term social memory deficits, depressive-like behavior and reduction of locomotor activity observed at different periods after intranasal MPTP administration in rats. Altogether, the present findings provide strong evidence that PRF from *C. celtidifolius* may represent a promising therapeutic tool in PD, thus being able to prevent both motor and non-motor early symptoms of PD, together with its neuroprotective potential.

Keywords MPTP · Intranasal · Parkinson's disease · Catechin · Proanthocyanidin · *Croton celtidifolius* · Neuroprotection · Behavior · Rat

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder that affects approximately 1% of the population older than 50 years (Mayeux 2003), and it is characterized by a slow and progressive degeneration of nigrostriatal dopaminergic neurons (Dawson and Dawson 2003; Schapira 2008; Hirsch and Hunot 2009). Dopamine-replacement therapy has dominated the treatment of PD since the early 1960s; although the currently approved antiparkinsonian agents offer effective relief of the motor deficits, especially in the early/moderate stages

of the disease, they have not been found to alleviate the underlying dopaminergic neuron degeneration and drug efficacy is gradually lost (Allain et al. 2008). Despite extensive efforts and research, to date, there is no proven therapy to prevent cell death or to restore affected neurons to a normal state (Dawson and Dawson 2002). Preclinical studies on laboratory animals have provided several candidate neuroprotective drugs, but clinical end points are easily confounded by any symptomatic effect of the study intervention and thus do not provide an unequivocal measure of disease progression that can be used to determine if a drug has a neuroprotective effect (Schapira and Olanow 2004). Moreover, it can be hypothesized that the low clinical efficacy of several neuroprotective agents is due to a late diagnosis of PD. Frequently, new potential agents are tested when the patient already shows cardinal motor signs (i.e. bradykinesia, rest tremor and muscular rigidity). Unfortunately, the patients only fulfill these clinical criteria when 60–70% of the neurons of the substantia nigra (SN) are degenerated and the striatal dopamine content is reduced by 80% (Riederer and Wuketich 1976; Meissner et al. 2004).

Nowadays, there is considerable evidence showing that the neurodegenerative processes that lead to sporadic PD begin many years before the appearance of the characteristic motor symptoms, and additional neuronal fields and neurotransmitter systems are also involved in PD, including the anterior olfactory structures, dorsal motor nucleus of vagus, caudal raphe nuclei, locus coeruleus, the autonomic nervous system, hippocampus and the cerebral cortex (Braak et al. 2004). Accordingly, cholinergic, adrenergic and serotoninergic neurons are also lost, which seems to be responsible for the non-motor symptoms of PD encompassing olfactory and memory impairments, sleep abnormalities and depression, as well as gastrointestinal disturbance, which precedes the classical motor symptoms (Chaudhuri et al. 2006; Schapira et al. 2006). For instance, it has been suggested that areas in the central nervous system (CNS) processing olfactory information are affected at the early stages of PD, even before the development of its classical symptoms (Doty et al. 1988; Braak et al. 2004). Consequently, olfactory dysfunction might be an early indicator of PD, and the development of specific olfactory tests may represent an important tool in the clinical diagnosis of the early stages of this disease (Doty et al. 1995).

Consistent with this suggestion, we have recently proposed a new experimental model of PD consisting of a single intranasal (i.n.) administration of the proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in rats (Prediger et al. 2006, 2009) and mice (Prediger et al. 2010). Our previous reports demonstrated that rats treated intranasally with MPTP suffered impairments in olfactory, cognitive and motor functions (Prediger et al. 2006). Additionally, the i.n. administration of MPTP causes time-dependent loss of tyrosine hydroxylase (TH) in the olfactory bulb and SN of rats, resulting in significant dopamine depletion in the olfactory bulb, prefrontal cortex and striatum (Prediger et al. 2006), which are associated with alterations in the brain antioxidant status and lipid peroxidation (Franco et al. 2007), and apoptotic cell death mechanisms (Prediger et al. 2009).

Although the etiology of the neurodegenerative process found in PD is not completely understood, it is suggested that a state of oxidative imbalance is triggered by one or more factors, among which are brain aging, genetic predisposition, mitochondrial dysfunction, free radical production and environmental toxins (Langston 1996; Fukae et al. 2007; Henchcliffe and Beal 2008; Zhou et al. 2008). Currently, the most plausible explanation is that PD represents a multifactorial disease resulting from a combination of genetic and environmental factors (Langston 1996). Novel therapeutic neuroprotective strategies support the application of reactive oxygen species (ROS) scavengers, transition metal (e.g. iron and copper) chelators, nonsteroidal anti-inflammatory drugs, non-vitamin natural antioxidant polyphenols, anti-apoptotic drugs such as calcium channel and caspase inhibitors, and bioenergetics drugs in monotherapy or as part of an antioxidant cocktail formulation (Mandel and Youdim 2004).

In the last decade, our group has extensively studied the biological properties of the proanthocyanidin-rich fraction (PRF) obtained from the barks of *Croton celtidifolius* Baill (Euphorbiaceae), a tree frequently found in the Atlantic Forest in south Brazil and popularly known as "sangue-de-adave" (Smith et al. 1988). In previous reports, we described a series of biological activities of PRF, including antioxidant, anti-inflammatory and modulation of the superoxide dismutase enzyme activities (Nardi et al. 2003, 2007), as well as a pronounced anti-nociceptive effect in behavioral models of pain, with the involvement of capsaicin-sensitive C-fibers and a direct stimulation of dopamine D_2 receptors (DalBó et al. 2005, 2006). Recently, Moreira et al. (2010) have demonstrated that the PRF from *C. celtidifolius* possesses a wide spectrum of psychopharmacological properties in rats, and these effects could be attributed to the presence of various catechin and/or proanthocyanidin compounds. According to these biological activities previously described for the PRF from *C. celtidifolius*, we believe that this fraction might present neuroprotective properties against the neurodegenerative process found in PD. Therefore, the aim of the present study was to investigate the effects of the pretreatment with PRF from *C. celtidifolius* in the neurochemical and behavioral alterations induced by a single i.n. MPTP administration in rats.

Materials and methods

Plant material

The bark of *Croton celtidifolius* Baill was collected from the forest surrounding the city of Orleans (State of Santa Catarina, Brazil) and a voucher specimen (document number 31272) was identified and deposited at the Department of Botany of the Universidade Federal de Santa Catarina (UFSC, Florianópolis, Brazil). Air-dried powdered bark of *C. celtidifolius* was extracted by consecutive percolation with an aqueous solution containing 80% ethanol at room temperature. The solvent was removed by vacuum and the crude extract was dissolved in acetone to obtain a saturated solution. A large volume of water was then added, causing precipitate formation. This precipitate was subsequently removed by filtration and the solution was transferred to a rotavapor for acetone evaporation. The resultant aqueous solution was extracted with ethyl acetate, which generated the PRF after organic solvent removal. This method provided a yield of 0.56% of PRF from the original air-dried plant material.

Characterization of PRF

Phytochemical investigation performed by our group using column chromatography fractionation, following NMR spectral analysis of the isolated compounds, together with micellar electrokinetic chromatography (MEKC) analyses showed that the PRF fraction contains 27.4% catechin, 1.3% epicatechin, 6.4% gallocatechin, 16.7% catechin-(4$\alpha \rightarrow$ 8)-catechin, 4.9% gallocatechin-(4$\alpha \rightarrow$ 8)-catechin and 43.3% proanthocyanidin oligomers. Further thiolysis of the PRF following MECK demonstrated that proanthocyanidin oligomers were mainly composed of catechin and epicatechin units in the ratio of 1.5–1 (DalBó et al. 2008).

Animals

The subjects were adult male Wistar rats (4 months old, 300–350 g) and juvenile male Wistar rats (25–30 days old) provided by the animal facility of the Universidade Federal de Santa Catarina (UFSC, Florianópolis, Brazil). Juvenile rats were kept in groups of ten per cage and served as social stimuli for the adult rats in the social recognition task. Animals were maintained in a 12-h light–dark cycle (lights on at 6:00 a.m.) at constant room temperature (22 ± 2°C) and were housed in groups of five animals per cage with free access to food and water. All animals were allowed to adapt to the laboratory conditions for at least 1 week before the behavioral assessment. All procedures used in the present study complied with the guidelines on animal care of the local ethics committee on the use of animals (CEUA/UFSC), which follows the NIH publication "Principles of Laboratory Animal Care".

Intranasal administration of MPTP

MPTP HCl (Sigma Chemical Co., USA) was administered by i.n. route according to the procedure described by Dluzen and Kefalas (1996) and recently modified by Prediger et al. (2006, 2009). Briefly, rats were lightly anaesthetized with isoflurane 0.96% (0.75 CAM; Abbot Laboratórios do Brasil Ltda., RJ, Brazil) using a vaporizer system (SurgiVet Inc., WI, USA), and a 10-mm piece of PE-50 tubing was inserted through the nostrils. The tubing was connected to a peristaltic pump set at a flow rate of 12.5 µl/min. The MPTP HCl was dissolved in 0.9% NaCl (saline) at a concentration of 20 mg/ml, after which it was infused for 4 min (1 mg/nostril). The control solution consisted of saline. Animals were given a 1-min interval to regain normal respiratory function and then this procedure was repeated with infusions administered through the contralateral nostrils.

Experimental design

As summarized in Fig. 1, saline or PRF from *C. celtidifolius* (10 mg/kg) was administered by the intraperitoneal (i.p.) route during a period of five consecutive days. The present PRF dose was chosen based on previous studies (Nardi et al. 2003, 2007; DalBó et al. 2005; Moreira et al. 2010). On the third day of this pretreatment schedule, MPTP or vehicle (saline) were intranasally administered to half of the animals of the saline or PRF groups, completing a total of four experimental groups. During a period of 6–32 days after the i.n. administration of MPTP, independent groups of animals were submitted to a battery of behavioral tests (open field, social recognition, forced swimming and activity chamber). For the measurement of mitochondrial complex-I activity in the olfactory bulb and

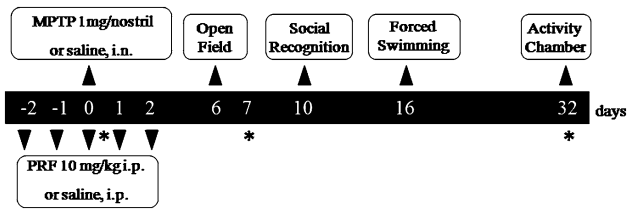

Fig. 1 Time course of behavioral and neurochemical tests following the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* and a single intranasal (i.n.) administration of MPTP (1 mg/nostril) in rats. *Asterisks* represents different times of killing after i.n. MPTP administration for the evaluation of mitochondrial complex-I activity (6 h) and the expression of the enzyme tyrosine hydroxylase (7 and 32 days)

striatum, and the expression of tyrosine hydroxylase (TH) in the olfactory bulb and SN, independent groups of animals were killed, respectively, at 6 h and 7 or 32 days after i.n. infusion of MPTP or saline (Fig. 1).

Behavioral tests

Open field

The locomotor and exploratory activities were examined with the open field arena (Archer 1973; Prediger et al. 2006). The apparatus, made of wood and covered with impermeable Formica, had a 100 cm × 100-cm white floor (divided by black lines into 25 squares of 20 cm × 20 cm) and 40 cm-high white walls. Each rat was placed in the center of the open field and allowed to freely explore the apparatus while the number of squares crossed and rearing was measured during 5 min. The experiments were conducted in a room under low illumination (10 lux) and the behavior of each animal was recorded by a video camera and monitored in an adjacent room.

Social recognition

Short-term social memory was assessed with the social recognition task described by Dantzer et al. (1987) and previously evaluated in our laboratory (Prediger et al. 2005). Adult rats were housed individually in plastic cages (42 cm × 34 cm × 17 cm) and used only after at least 7 days of habituation to their new environment. The test was scored by the same rater in an observation room, where the rats had been habituated for at least 1 h before the beginning of the test. All juveniles were isolated in individual cages for 20 min prior to the beginning of the experiment. The social recognition task consisted of two successive presentations (5 min each), separated by a short period of time, where the juvenile rat was placed in the home cage of the adult rat and the time spent by the adult in investigating the juvenile (nosing, sniffing, grooming or pawing) was recorded. At the end of the first presentation, the juvenile was removed and kept in an individual cage during the delay period and re-exposed to the same adult rat after 30 min. In this paradigm, if the delay period is less than 40 min, the adult rodents display recognition of this juvenile, as indicated by a significant reduction in the social investigation time during the second presentation (Dantzer et al. 1987; Prediger et al. 2005). However, when the same juvenile is re-exposed after a longer time (more than 60 min) after the first presentation, the adult rat no longer recognizes this juvenile, i.e. the social investigation time in the second presentation is similar to that observed during the first one. Thus, a 30-min interval between two presentations of the same conspecific juvenile was used to demonstrate possible MPTP-related deficits in the social recognition memory.

Forced swimming

The anhedonic depressive-like effect was assessed with the forced swimming test previously described by Porsolt et al. (1978). Rats were placed in individual glass cylinders (40 cm in height and 17 cm in diameter) containing water (water depth was 30 cm; 25 ± 1°C). Two swimming sessions were conducted (an initial 15-min pretest followed 24 h later by a 5-min test). The total duration of immobility was manually scored continuously during a 5-min period. A rat was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water.

Activity chamber

The general locomotor activity was examined in the activity chamber for 30 min (Moreira et al. 2010). The activity chamber consists of a wooden cage (40 cm × 12 cm × 20 cm) with steel grid floor and equipped with automatic movement detection using three pairs of infrared beans positioned 2 cm above the floor and evenly placed in the apparatus. The locomotion was automatically measured by a digital counter that recorded photocell bean interruptions.

Neurochemistry

Mitochondrial complex-I activity

Animals were killed by decapitation 6 h after i.n. infusion of MPTP. The brain was rapidly excised on a Petri dish placed on ice and the olfactory bulb and striatum were dissected, weighed and kept chilled until homogenization in 20 volumes (1:20, w/v) of phosphate buffer (PBS) (pH 7.4, 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA 0.1% BSA) with a Potter-Elvejhem homogenizer and centrifuged at 1,000.0×g for 10 min at 4°C, where the pellet was discarded and the supernatants were kept at −70°C until enzyme activity determination. Complex-I activity was measured by the rate of NADH-dependent ferricyanide reduction, and absorbance was monitored at 420 nm (1 mM^{-1} cm^{-1}) as described by Cassina and Radi (1996), with slight modifications, as detailed previously (Latini et al. 2005). The activities of the respiratory chain complexes were calculated as nmol/min/mg protein.

Western blotting analysis

Seven days after i.n. administration of MPTP, the animals were killed by decapitation and their brains were removed from the skull. The olfactory bulb was rapidly dissected on dry ice and stored at −70°C until determination of the expression of the enzyme TH. Tissues were gently homogenized in ice cold 10 mM HEPES (pH 7.4) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml pepstatin, 10 µg/ml leupeptin and 0.5 mM dithiothreitol. The homogenates were chilled on ice for 15 min and then vigorously shaken for 15 min in the presence of 0.1% Nonidet P-40. The homogenates were centrifuged at 10,000.0×*g* for 30 min, and the resulting supernatant collected was considered as the cytosolic fraction. This supernatant was stored at −70°C until use. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA).

Equivalent amounts of proteins were mixed in buffer (Tris 200 mM, glycerol 10%, SDS 2%, *β*-mercaptoethanol 2.75 mM and bromophenol blue 0.04%) and boiled for 5 min. Proteins (40 µg) were separated by SDS-PAGE in 10% gels. Proteins were detected immunologically following electrotransfer onto nitrocellulose membranes (Amersham-Pharmacia Biotechnology, USA). Protein and molecular weight markers (BioRad, Mississauga, Canada) were revealed by Ponceau Red staining. Membranes were blocked in PBS containing 5% powdered milk and 0.05% Tween-20 for 1 h at 25°C. Membranes were then incubated overnight at 4°C with anti-TH antibody (sc-7847; Santa Cruz Biotechnology, USA) in blocking solution and after that with horseradish peroxidase-conjugated anti-mouse IgG antibody for 1 h. Blots were visualized using the PerkinElmer ECL system.

Immunohistochemistry

At 32 days after i.n. administration of MPTP, the animals were intracardially perfused first with saline, then with 4% of the fixative solution formaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and kept overnight in the same solution. Serial sections (3 µM) were selected to include the midbrain. The immunostaining was assessed at three levels of the SNpc and identified according to the rat brain atlas of Paxinos and Watson (2005). Specifically, three alternate 4-µm sections of SNpc with an individual distance of ∼150 µm were obtained between coordinates 4.2 and 2.7 mm with respect to the interaural axis containing the SN ventral tegmental area complex. Immunohistochemistry was assessed using the rabbit anti-TH monoclonal antibody (1:400, MAB318, Millipore/Chemicon International Technology, USA), as previously described (Tadaiesky et al. 2008). After deparaffinization, the endogenous peroxidase was blocked for 20 min in 3% H_2O_2 in 100% methanol and the nonspecific binding was blocked by incubating sections for 1 h with 1% goat normal serum diluted in PBS. The slides were washed with PBS, and high temperature antigen retrieval was performed by immersing of the slides in a water bath at 95–98°C in 10 mM trisodium citrate buffer at pH 6.0, for 45 min. After overnight incubation at 4°C with primary antibody, the slides were washed and incubated for 2 h (RT) with goat anti-rabbit biotinylated secondary antibody (1:500, Vector Laboratories, Burlingame, CA, USA) followed by 1.5 h of incubation with the avidin–biotin complex (1:1,000, Vector Laboratories, Burlingame, CA, USA). The sections were washed in PBS, and the staining was visualized by reaction with 0.05% DAB (3,3′-diaminobenzidine, Sigma-Aldrich. St Lois, MO, USA); enhancement was done with 0.05% nickel ammonium sulfate, 0.05% cobalt chloride and 0.015% H_2O_2. Color development was stopped by rising the slides in distilled water for 5 min. Tissues from the four experimental groups were placed on the same slide and processed under the same conditions. For counting of individually stained neurons, the sections were placed in the microscope field with square grid (100 μm^2). The number of TH-immunoreactive neurons was estimated by counting positive cells in six microscope fields (×400 magnification) of three sections (18 fields per animal) encompassing rostral, medial and caudal levels of the SNpc, as previously described above. The TH-positive cells obtained were then plotted with respect to the area covering the square grid, and the results were expressed as TH-positive cells per mm^2.

Monoamine oxidase assay

To investigate whether the treatment with the PRF from *C. celtidifolius* may interfere with the generation of the toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) from MPTP, PRF from *C. celtidifolius* was tested for its in vitro inhibitory potential on rat MAO-A and MAO-B activities in brain mitochondrial homogenates by a fluorometric method using kynuramine as a substrate, as previously described (Matsumoto et al. 1985; Sant' Anna Gda et al. 2009). Briefly, assays were performed in duplicate in a final volume of 500 µl containing 0.5 mg of protein and incubated at 37°C for 30 min. Activities of the MAO-A and MAO-B isoforms were isolated pharmacologically by incorporating 250 nM selegiline (selective MAO-B inhibitor) or 250 nM clorgyline (selective MAO-A inhibitor) into the reaction mixture. The reaction mixture (containing mitochondrial fractions, PRF and inhibitors) was preincubated at 37°C for 5 min and the reaction was started by addition of 50 µL of kynuramine (90 µM for MAO-A and

60 μM for MAO-B). PRF was tested in a concentration range of 10–300 μg/ml and the IC_{50} values for both MAO isoforms were calculated.

Statistical analysis

Data are presented as mean ± SEM, and the statistical analysis was carried out using two-way analysis of variance (ANOVA) with pretreatment (saline vs. PRF) and treatment (saline vs. MPTP) as independent variables. The data from monoamine oxidase assay was carried out using one-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post hoc comparisons were performed using the Newman–Keuls test. The accepted level of significance for all tests was $P \leq 0.05$. All tests were performed using the Statistica® software package (Stat Soft Inc., USA).

Results

Effects of pretreatment with PRF from *C. celtidifolius* in the behavioral deficits induced by intranasal administration of MPTP in rats

Open field

The results of locomotor activity evaluated in the open field arena (for 5 min) 6 days after i.n. MPTP administration are summarized in Table 1. Two-way ANOVA (pretreatment vs. treatment) revealed a significant effect only for the pretreatment factor in the total squares crossed [$F(1,32) = 8.28$, $P \leq 0.05$]. However, it indicated no significant effects for the treatment factor and for the interaction factor between pretreatment and treatment ($P > 0.05$) in the number of squares crossed and in rearing. Posterior post hoc comparisons indicated that i.n. MPTP administration did not alter the total squares crossed and rearing in the open field arena at 6 days after treatment (Table 1).

Table 1 Effects of pretreatment (during 5 days) with the proanthocyanidin-rich fraction (PRF) of *Croton celtidifolius* on locomotor activity of rats evaluated in the open field arena (for 5 min), 6 days after i.n. infusion of MPTP

Pretreatment	Treatment	Crossings	Rearings
Saline	Saline	90.90 ± 7.08	13.70 ± 1.71
Saline	MPTP	111.77 ± 6.97	16.44 ± 1.37
PRF	Saline	67.37 ± 16.74	11.50 ± 3.56
PRF	MPTP	76.22 ± 9.45	10.22 ± 1.56

Data are expressed as mean ± SEM of 8–10 animals in each group

Social recognition

Figure 2 summarizes the effects of pretreatment with PRF from *C. celtidifolius* (10 mg/kg, i.p.) on the social recognition memory of MPTP-treated rats. Two-way ANOVA (pretreatment vs. treatment) revealed no significant effect for the treatment factor ($P > 0.05$) in the investigation time during de second presentation of the familiar juvenile. However, it indicated a significant effect for the pretreatment factor [$F(1,31) = 14.58$, $P \leq 0.001$] and for the interaction between pretreatment and treatment [$F(1,31) = 6.60$, $P \leq 0.05$] in the investigation time during the second presentation of the juvenile. Subsequent post hoc comparisons indicated that MPTP-treated group spent as much time investigating the juvenile rat during the second encounter as they did in the first exposure, reflecting a clear impairment of the juvenile's recognition ability. More importantly, Newman–Keuls test indicated that the pretreatment with PRF from *C. celtidifolius* prevented the social recognition deficits of MPTP-treated rats, since it promoted a significant reduction in the investigation time when the same juvenile was re-exposed after 30 min (Fig. 2).

Forced swimming

The effects of the pretreatment with PRF from *C. celtidifolius* (10 mg/kg, i.p.) on the depressive-like behavior evaluated in the forced swimming test at 16 days after i.n. MPTP administration are summarized in Fig. 3. Two-way ANOVA (pretreatment vs. treatment) revealed a significant effect for the pretreatment factor [$F(1,30) = 11.60$,

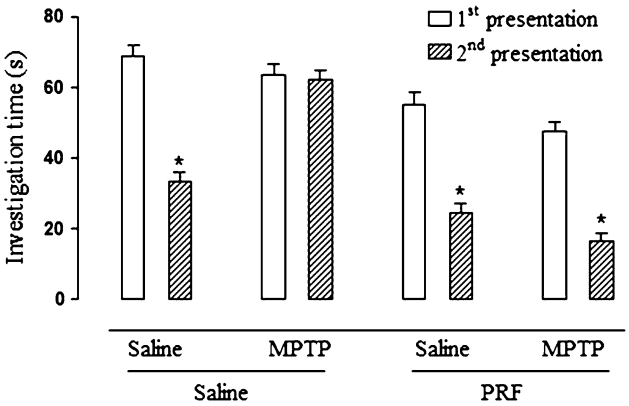

Fig. 2 Effects of the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on short-term social recognition memory of rats evaluated in the social recognition test, 10 days after i.n. infusion of MPTP (1 mg/nostril). Each value represents the mean ± SEM of seven to nine animals in each group. *$P \leq 0.05$ compared to the first trial of the same group (two-way ANOVA followed by Newman–Keuls test)

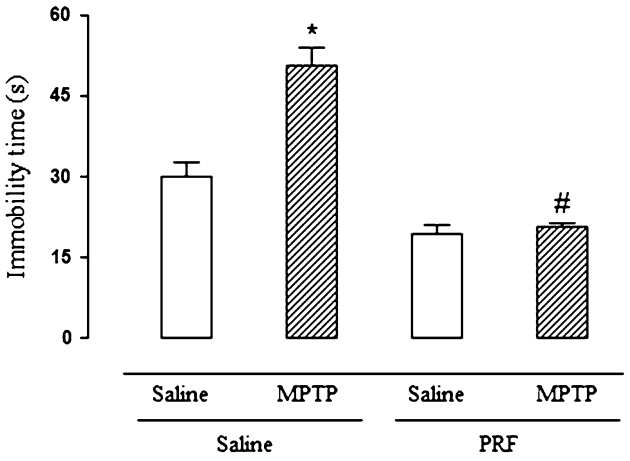

Fig. 3 Effects of the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on depressive-like behavior of rats evaluated in the forced swimming test, 16 days after i.n. infusion of MPTP (1 mg/nostril). Each value represents the mean ± SEM of seven to nine animals in each group. $^{*}P \leq 0.05$ compared to the saline/saline group; $^{\#}P < 0.05$ compared to the saline/MPTP group (two-way ANOVA followed by Newman–Keuls test)

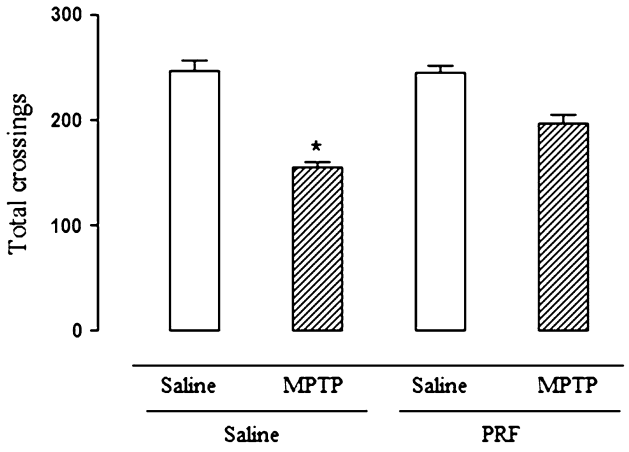

Fig. 4 Effects of the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on locomotor activity of rats evaluated in the activity chamber (for 30 min), 32 days after i.n. infusion of MPTP (1 mg/nostril). Each value represents the mean ± SEM of seven to nine animals in each group. $^{*}P \leq 0.05$ compared to the saline/saline group; $^{\#}P \leq 0.05$ compared to the saline/MPTP group (two-way ANOVA followed by Newman–Keuls test)

$P \leq 0.01$] in the immobility time. However, it indicated no significant effects for the treatment factor and for the interaction factor between pretreatment and treatment ($P > 0.05$) in this parameter. Subsequent post hoc comparisons indicated that MPTP-treated rats presented higher immobility time in comparison to control group ($P \leq 0.05$), suggesting a depressive-like profile. More importantly, the pretreatment with PRF from *C. celtidifolius* prevented the depressive-like behavior of MPTP-treated rats as indicated by a significant reduction in the immobility time (Fig. 3).

Activity chamber

Figure 4 summarizes the effects of the pretreatment with PRF from *C. celtidifolius* (10 mg/kg, i.p.) on locomotor activity of rats evaluated in the activity chamber (for 30 min) at 32 days after i.n. MPTP administration. Two-way ANOVA (pretreatment vs. treatment) revealed a significant effect for the treatment factor [$F(1,29) = 10.28$, $P \leq 0.05$] in the number of crossings. However, it indicated no significant effects for the pretreatment factor and for the interaction between pretreatment and treatment in this parameter. Subsequent Newman–Keuls test indicated that MPTP-treated rats displayed reduced locomotor activity in comparison to control-infused animals. The pretreatment with PRF from *C. celtidifolius* was able to prevent these motor impairments, increasing significantly the number of crossings of MPTP-treated rats (Fig. 4).

Effects of pretreatment with PRF from *C. celtidifolius* in the neurochemical alterations induced by intranasal administration of MPTP in rats

Mitochondrial complex-I activity

The effects of pretreatment with PRF from *C. celtidifolius* (10 mg/kg, i.p.) on the mitochondrial complex-I activity evaluated in the olfactory bulb and striatum at 6 h after i.n. MPTP administration are summarized in Fig. 5. In the striatum, two-way ANOVA (pretreatment vs. treatment) revealed no significant effect for the treatment factor ($P > 0.05$). However, it indicated significant effects for the pretreatment factor [$F(1,34) = 12.79$, $P \leq 0.01$] and for the interaction between pretreatment and treatment [$F(1,34) = 12.28$, $P \leq 0.01$] in the mitochondrial complex-I activity in the striatum (Fig. 5a). In the olfactory bulb, two-way ANOVA revealed no significant effects for the main factors (pretreatment and treatment) ($P > 0.05$). However, it indicated a significant effect for the interaction factor between pretreatment and treatment [$F(1,33) = 14.89$, $P \leq 0.05$] in the mitochondrial complex-I activity in the olfactory bulb (Fig. 5b).

Subsequent post hoc comparisons indicated that, as expected, i.n. MPTP treatment reduced significantly the mitochondrial complex-I activity in both the striatum and the olfactory bulb of rats. Of high importance, the pretreatment with PRF from *C. celtidifolius* was able to prevent the inhibition of mitochondrial complex-I activity

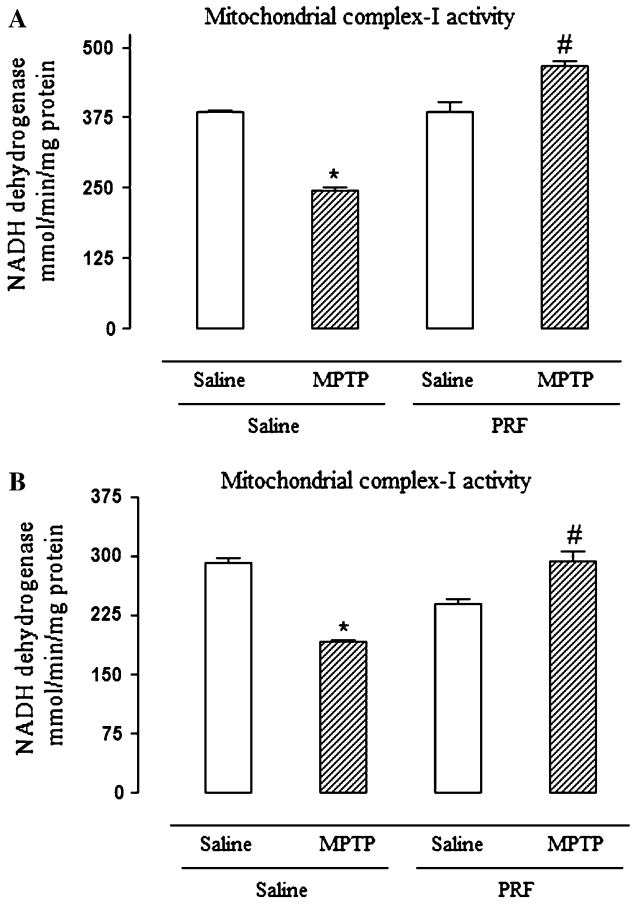

Fig. 5 Effects of the pretreatment (during three consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on mitochondrial complex-I activity of rats evaluated in the striatum (**a**) and olfactory bulb (**b**), 6 h after i.n. infusion of MPTP (1 mg/nostril). Each value represents the mean ± SEM of 7–9 animals in each group. $^{*}P \leq 0.05$ compared to the saline/saline group; $^{\#}P \leq 0.05$ compared to the saline/MPTP group (two-way ANOVA followed by Newman–Keuls test)

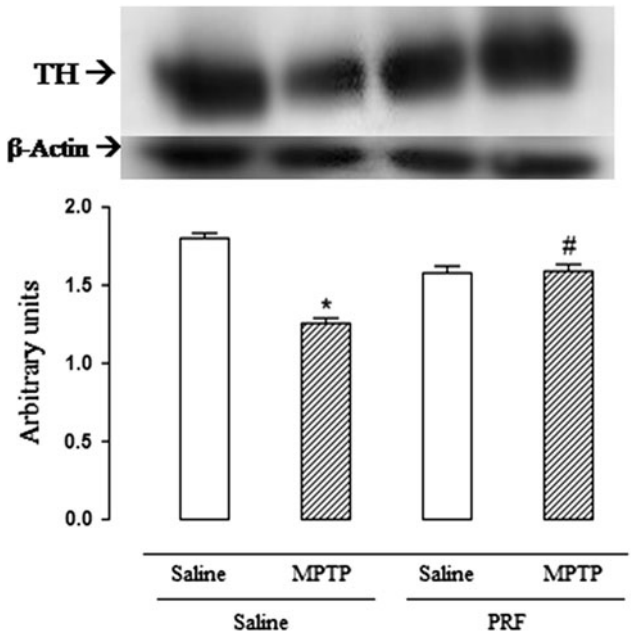

Fig. 6 Effects of the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on tyrosine hydroxylase (*TH*) expression in the olfactory bulb of rats evaluated through western blotting analysis at 7 days after i.n. infusion of MPTP (1 mg/nostril). Each value represents the mean ± SEM of four animals in each group. $^{*}P \leq 0.05$ compared to the saline/saline group; $^{\#}P \leq 0.05$ compared to the saline/MPTP group (two-way ANOVA followed by Newman–Keuls test)

induced by i.n. MPTP administration in these two brain structures (Fig. 5).

Western blotting analyses

With the purpose of determining the relationship between the short-term social recognition memory deficits induced by MPTP in rats and neurochemical alterations in dopaminergic neurotransmission, the expression of the enzyme TH in the olfactory bulb was measured at 7 days after i.n. administration of MPTP by western blot assays. Two-way ANOVA (pretreatment vs. treatment) revealed no significant effect for the pretreatment factor ($P > 0.05$) in the expression of TH in the olfactory bulb. However, it indicated significant effects for treatment factor [$F(1,12) = 12.57$, $P \leq 0.05$] and for the interaction factor between pretreatment and treatment [$F(1,12) = 13.91$, $P \leq 0.05$] in the expression of TH in the olfactory bulb. Post hoc comparisons indicated that the i.n. administration of MPTP promoted a significant reduction (about 25%) in the expression of the enzyme TH in the olfactory bulb. More importantly, the pretreatment with PRF from *C. celtidifolius* was able to prevent this reduction in the expression of TH in the olfactory bulb of MPTP-treated rats (Fig. 6).

Immunohistochemistry

With the purpose of determining the relationship between the motor impairments observed in the activity chamber at later periods after i.n. MPTP administration and dopaminergic cell death in the nigrostriatal pathway, the number of TH-positive cells in the SN was measured at 32 days after i.n. administration of MPTP by immunohistochemistry. Figure 7a shows representative photomicrographs of TH immunohistochemistry in the ventral mesencephalon containing SNpc. Two-way ANOVA (pretreatment vs. treatment) revealed no significant effect for the pretreatment factor ($P > 0.05$) on the TH immunostaining in the SNpc. However, it indicated significant effects for the treatment factor [$F(1,246) = 44.37$, $P \leq 0.0001$] and for the interaction between pretreatment and treatment [$F(1,246) = 10.79$, $P \leq 0.01$] on the TH immunostaining in the SNpc (Fig. 7b).

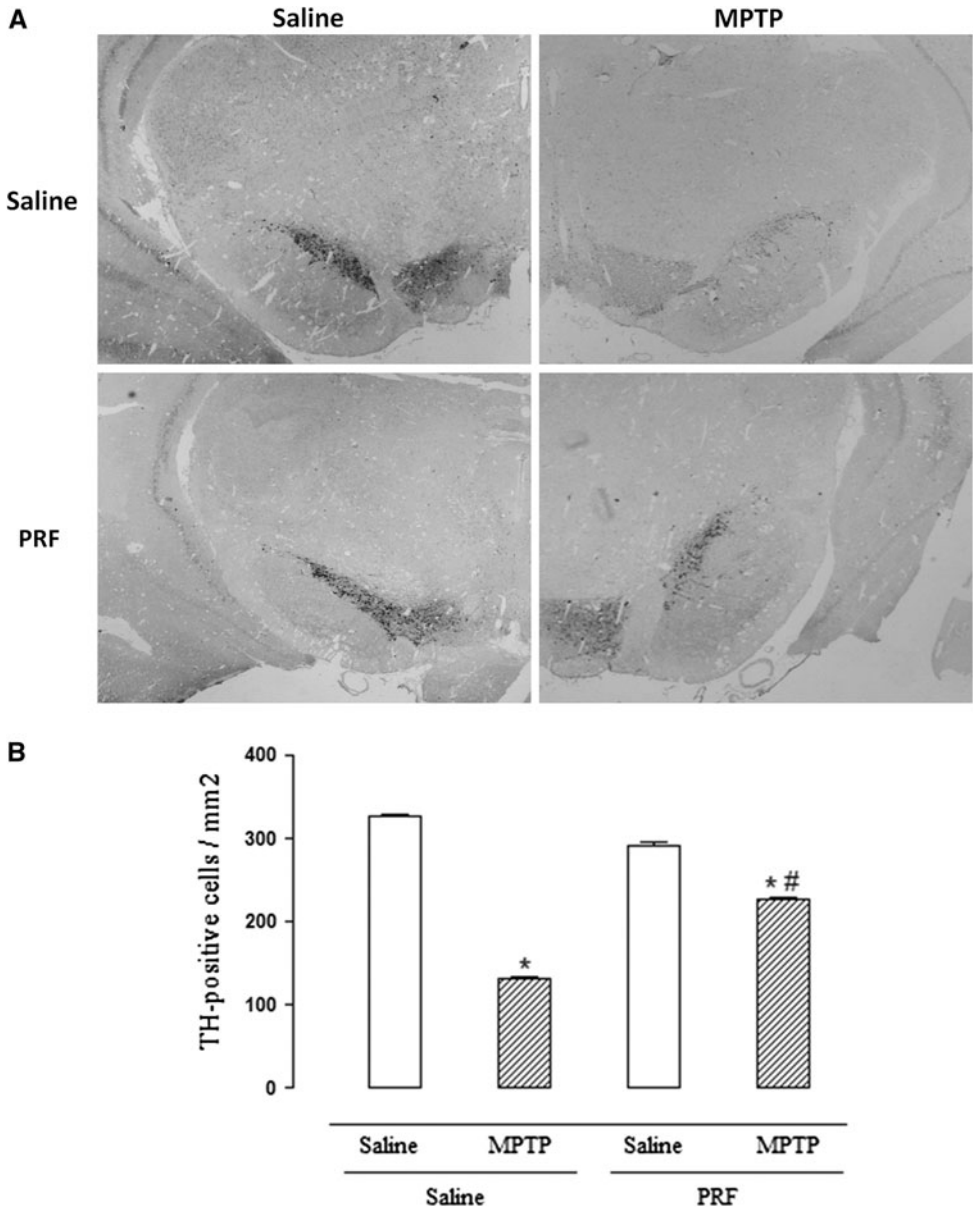

Fig. 7 Effects of the pretreatment (during five consecutive days) with the proanthocyanidin-rich fraction (*PRF*) (10 mg/kg; i.p.) from *C. celtidifolius* on tyrosine hydroxylase (TH)-positive cells in the substantia nigra part compacta (*SNpc*) of rats evaluated through immunohistochemistry at 32 days after i.n. infusion of MPTP (1 mg/nostril). **a** Representative images of TH immunostaining in the ventral mesencephalon containing SNpc. *Scale bar* 200 μm. **b** Graphic representation of the average TH immunostaining analysis. The values represent the mean ± SEM of TH-positive cells per mm^2 of four animals in each group. $^{*}P \leq 0.05$ compared to the saline/saline group; $^{\#}P \leq 0.05$ compared to the saline/MPTP group (two-way ANOVA followed by Newman–Keuls test)

Moreover, the counting of individual TH-stained neurons demonstrated that the i.n. administration of MPTP induced a significant reduction of TH immunostaining in the SNpc (55% lower) of rats and that the pretreatment with PRF from *C. celtidifolius* was able to attenuate significantly this reduction in the levels of TH in the SNpc of MPTP-treated rats (Fig. 7b).

Monoamine oxidase assay

As can be seen in Fig. 8, PRF from *C. celtidifolius* inhibited the activity of both MAO-A and MAO-B in the rat brain mitochondrial homogenates, with the respective IC_{50} values: MAO-A, 98.07 (84.26–114.10) μg/ml; and MAO-B, 79.70 (69.58–91.29) μg/ml.

Discussion

Therapeutic strategies that slow or stop the neurodegenerative process of PD are expected to have a major impact on the treatment of PD (Meissner et al. 2004). The current hypothesis about the mechanisms by which neurons come into necrotic or apoptotic process has led to believe that the therapeutic use of antioxidants may be beneficial in aging and neurodegenerative disorders (Di Matteo and Esposito 2003; Zhou et al. 2008). Following this line of evidence, the number of studies with natural compounds with potential and/or hypothetical neuroprotective properties, including polyphenolic compounds or polyphenols (Ramassamy 2006; Mandel et al. 2008), has increased considerably during the last few years. Additionally, research on new

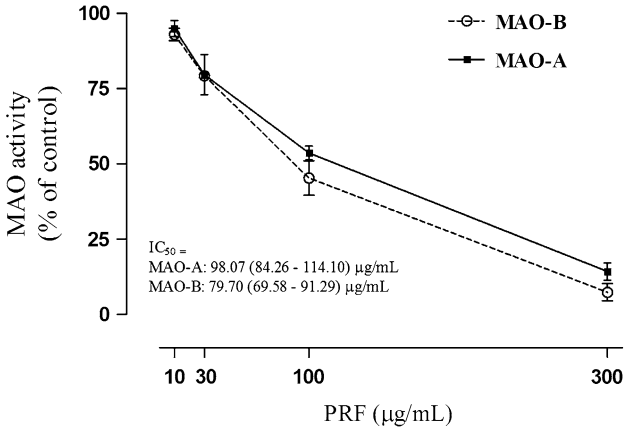

Fig. 8 Inhibition of rat brain mitochondrial MAO-A and MAO-B activity by PRF from *C. celtidifolius* in vitro. PRF was tested in a concentration range of 10–300 µg/ml. The values represent the mean ± SEM of three individuals experiments, performed in duplicate

molecules from natural sources offers a great opportunity to evaluate new chemical classes of drugs, as well as new and relevant mechanisms of action (Mahady 2001; Balunas and Kinghorn 2005).

Catechins are a group of natural flavonoids, which have recently been intensively studied because of their high concentrations in green tea (*Camellia sinensis*), a plant widely used in traditional Chinese and Japanese medicine (Crespy and Williamson 2004). Several actions on the CNS, such as anxiolytic and sedative (Adachi et al. 2006), and neuroprotective properties in animal models of Alzheimer's disease and PD (Levites et al. 2003; Mandel and Youdim 2004), are being assigned to various catechins, such as epigallocatechin-3-gallate (EGCG). Catechins and oligomeric proanthocyanidins (formed of catechin and epicatechin units) are the main molecules found in the proanthocyanidin-rich fraction (PRF) obtained from the bark of *C. celtidifolius*. As previously mentioned, several activities for PRF, such as anti-nociceptive, anti-inflammatory, antioxidant and modulation of superoxide dismutase activity (Nardi et al. 2003, 2007; DalBó et al. 2005, 2006), have already been characterized by our group. More recently, Moreira et al. (2010) demonstrated that the PRF from *C. celtidifolius* possesses a wide spectrum of psychopharmacological properties in rats. In the present study, we demonstrated that PRF prevented mitochondrial complex-I inhibition as well as a wide spectrum of behavioral impairments and dopaminergic cell death induced by i.n. infusion of MPTP (1 mg/nostril) in rats.

The development of new neuroprotective therapies in PD depends on the existence of representative animal models to facilitate the evaluation of new pharmacological agents and therapeutic strategies before they are applied in clinical trials (Gerlach et al. 2003). PD is one of the several human diseases that seem not to occur spontaneously in other animals. However, the characteristics of this disease can be mimicked in laboratory animals through the administration of different compounds, for example reserpine, 6-hydroxydopamine, MPTP and rotenone (Gerlach and Riederer 1996; Dawson 2000; Beal 2001). MPTP is a toxin with complex toxicokinetics and it has several stages, involving oxidative stress (Przedborski et al. 2004). Once in the brain, MPTP is rapidly converted to the toxic ion 1-methyl-4-phenyl-piperidinium (MPP^+) by the enzyme monoamine oxidase B (MAO-B), mainly in glial cells. Unlike its precursor MPTP, MPP^+ is a polar molecule and does not cross biological membranes freely. However, this toxin has a high binding affinity to the dopamine transporter (DAT), thus being captured into dopaminergic terminals (Beal 2001). Dopaminergic neurons accumulate MPP^+ in mitochondria to levels that inhibit complex-I of the electron transport chain, causing a severe depletion of ATP and increased production of reactive oxygen species (ROS), particularly superoxide. The production of ROS seems to be one of the primary events of the mechanism of MPP^+ neurotoxicity and, although not directly responsible for neuronal death, acts as an activator of several cellular cascades that cause the death of dopaminergic neurons (Hasegawa et al. 1997; Beal 2001; Przedborski et al. 2004). Franco et al. (2007) demonstrated that the i.n. infusion of MPTP caused time-dependent changes in oxidative stress markers in brain structures of rats inferred from a significant increase in the end products of lipid peroxidation (TBARS) and glutathione metabolism. These changes were observed in the olfactory bulb and striatum of rats, reaching a peak response within 6 h after the i.n. administration of MPTP (Franco et al. 2007). Based on this peak time, we evaluated the mitochondrial complex-I activity at 6 h after the i.n. infusion of MPTP in brain structures known to be affected in PD. As expected, MPTP was able to significantly inhibit the activity of complex-I in the olfactory bulb and striatum of the animals. Interestingly, pretreatment with PRF was able to prevent this inhibitory effect of MPTP, maintaining the activity of mitochondrial complex-I at normal levels in these two brain structures. This activity may be associated, at least in part, with PRF protection against oxidative stress in mitochondria. In fact, as mitochondrial oxidative stress is a key pathogenic mechanism in neurodegenerative diseases such as PD, current research is focusing on uncovering new antioxidants that are selective for the mitochondria (Zhao et al. 2005). With respect to catechins, a study with cerebellar neurons in culture by Schroeder et al. (2009) showed that after incubation with ^{3}H-EGCG, 90–95% of this catechin accumulated in the mitochondrial fraction, indicating that this molecule may have some selectivity for this cellular

structure. The same study also showed the neuroprotective action of catechins in models involving apoptosis induced by insults that cause mitochondrial oxidative stress, which agrees with our findings on the properties of PRF against MPTP neurotoxicity.

With regard to PD symptoms, an increasing number of studies have demonstrated that they are not restricted to motor impairments. In fact, PD seems to be a multidimensional disease and, besides motor deficits, it is associated with a number of cognitive and emotional disturbances that result in a loss in the quality of life of individuals (Chaudhuri et al. 2006). The symptoms observed in PD include depression (Cummings and Masterman 1999), anxiety (Richard et al. 2004), cognitive impairments (Goldman et al. 1998) and olfactory loss (Doty et al. 1988, 1995). Non-motor features of PD invariably do not respond to dopaminergic medication and probably form the major current challenge faced in the clinical management of PD (Chaudhuri et al. 2006). In this context, we have recently demonstrated that a single i.n. infusion of MPTP in rats produces diverse signs of PD, such as impairments in olfactory, cognitive and motor functions (Prediger et al. 2006).

According to Braak's hypothesis, the olfactory bulb is one of the first brain structures to display the pathology related to PD (Braak et al. 2004). Corroborating this hypothesis, approximately 90% of PD patients exhibit during the earliest phases of the disease olfactory dysfunction unresponsive to current treatments of PD (Doty et al. 1988). Moreover, dopamine seems to be necessary for olfactory memory, since its release increases during olfactory learning (Coopersmith et al. 1991), while antagonists of dopamine receptors (Weldon et al. 1991; Prediger et al. 2004, 2005) or treatments that reduce dopaminergic neurotransmission such as reserpine (Prediger et al. 2004, 2005) and MPTP (Dluzen and Kreutzberg 1993; Prediger et al. 2010) inhibit short-term olfactory memory. Corroborating our previous study performed in mice (Prediger et al. 2010), in the present study, rats infused with MPTP (1 mg/nostril) spent significantly more time investigating the juvenile rat during the second presentation than they did in the first encounter, suggesting an impaired ability to recognize the juvenile rat after a short time. Moreover, this short-term olfactory memory impairment induced by i.n. administration of MPTP was temporally correlated with a significant reduction in the expression of the enzyme TH in the olfactory bulb of rats. Of high importance, the present findings demonstrate that the pretreatment with PRF from *C. celtidifolius* (10 mg/kg, i.p.) during five consecutive days was able to prevent both the short-term social memory deficits and the decrease in the expression of TH in the olfactory bulb of MPTP-treated rats. Therefore, from these limited results, it appears that PRF from *C. celtidifolius* might be particularly useful to restore impaired olfactory and memory processes in PD.

Beyond the olfactory and cognitive symptoms, depressive disorders commonly occur in PD (Cummings and Masterman 1999), affecting approximately 40% of the patients during the early stages of the disease (Tolosa et al. 2007). Several studies suggest that the pathophysiology underlying mood disorders in PD may be different from the mechanisms that account for the behavioral symptoms observed in the general population (Lieberman 2006). Although the pathophysiology of psychiatric symptoms in PD is not fully understood, striatal, frontal and limbic dopaminergic, cholinergic, serotonergic, noradrenergic and GABAergic pathways are thought to be involved in their genesis (Schrag 2004). Tadaiesky et al. (2008) have recently demonstrated that a partial striatal lesion with 6-OHDA induces depressive-like symptoms (e.g. anhedonia and behavioral despair) in rats. In accordance with this previous literature, in the present study the i.n. infusion of MPTP caused a depressive-like behavior in rats, reflected by an increased immobility time in the forced swimming test (Porsolt et al. 1978). This depressive-like behavior might be correlated with dopamine changes at the striatal level (Tadaiesky et al. 2008). Indeed, although no significant differences in the serotonin levels were observed in any of the brain structures investigated after i.n. administration of MPTP in mice (Prediger et al. 2010), it must be conceded that, at this moment, we cannot rule out the serotonergic participation in the emotional alterations found in the MPTP-treated rats. In fact, there is pathophysiological evidence of serotonin alterations in patients with PD-associated depression (Schrag 2004), and a "serotonin hypothesis" has even been proposed for depression in PD (Mayeux 1990). Of high interest, the pretreatment with PRF from *C. celtidifolius* significantly prevented behavioral despair induced by i.n. MPTP, inferred from the fact that fraction-treated animals showed a similar level of immobility time as the control group. However, further research is needed to clarify the exact molecular mechanisms involved in the antidepressive-like effect induced by PRF from *C. celtidifolius* in MPTP-treated rats.

Additionally, in the present study we observed a later (32 days after i.n. MPTP administration) reduction in the locomotor activity of MPTP-treated rats evaluated in the activity chamber. This late decrease in locomotor activity of MPTP-treated rats was accompanied by a significant reduction (about 55%) in the expression of the enzyme TH in the SNpc. These results corroborate our previous findings using the i.n. rat model where a reduction in locomotor activity of MPTP-treated rats in the open field was only observed at 21 days after i.n. MPTP administration (Prediger et al. 2006). However, the later reduction in

locomotor activity of MPTP-treated rats contrasts with previous studies demonstrating that rats infused bilaterally with MPTP directly into SNpc do not present gross motor alterations (Da Cunha et al. 2001; Gevaerd et al. 2001; Miyoshi et al. 2002; Ferro et al. 2005). In fact, the recovery of motor performance appears to be almost universal following acute MPTP models (Gerlach and Riederer 1996). Given that even the gross neuroanatomical structures affected by i.n. administration of MPTP remain uncertain, at the present stage, it is not possible to determine the exact mechanism of such motor signs. Nevertheless, some speculative explanations can be given. The administration of MPTP by i.n. route may be actuating in a more diffuse way then when it is directly infused into SNpc. Thus, brain structures (e.g. olfactory bulb, amygdala and locus coeruleus) implicated in sensory-motivational responses may have been affected by i.n. MPTP, and the present reduction in the number of crossings in the activity cage may not be entirely related to motor abnormalities. In accordance with this view, the i.n. administration of the same MPTP dose did not alter the motor performance of rats when they were tested in the grid walking, rotarod and grasping stretch tests (data not published). More importantly, the administration of PRF from *C. celtidifolius* demonstrated once again its neuroprotective properties, preventing the reduction of the number of crossings in the activity cage and the decrease of the TH immunoreactivity in the SNpc induced by i.n. infusion of MPTP.

One possible mechanism by which PRF may exert protective effects against MPTP neurotoxicity as demonstrated in this study perhaps involves the catechol-type structure present in catechins (Levites et al. 2001). Compounds containing the catechol-type structure are potent antioxidants and chelators of ferric iron, such as (R-) and (S-) apomorphine (Gassen et al. 1996; Grinberg et al. 1997). Additionally, the catechol structure may also be correlated with an inhibitory activity in the uptake of dopamine, as previously demonstrated by Pan et al. (2003) in striatal synaptosomes extracted from mice treated with a single i.p. dose of green tea catechins. This inhibition is suggestive of the blockade of the uptake of MPP^+ (the toxic metabolite of MPTP), thus protecting dopaminergic neurons against MPTP toxicity (Weinreb et al. 2009). Moreover, it is well known that MAO-B inhibition causes slowing of dopamine turnover in the mammalian brain (Finberg et al. 1998) and limits the formation of hydrogen peroxide (Gökhan-Kelekçi et al. 2007). MAO-B inhibitors also reduce the generation of the MPP^+ from MPTP, protecting against the dopaminergic cell death in the SN (Heikkila et al. 1984). In this context, Wu and Zhu (1999) demonstrated the involvement of MAO inhibition ($IC_{50} = 36.45$ μg/ml) in the neuroprotective effects of *Ginkgo biloba* extract against MPTP-induced nigrostriatal dopaminergic toxicity in mice. Therefore, it must be conceded that, at this time, we cannot rule out the possibility that the treatment with PRF from *C. celtidifolius* may interfere with the MPP^+ production as well as it uptake and accumulation inside the dopamine neurons. The evaluation of the time course of MPP^+ kinetic in the rat brain after i.n. MPTP administration constitutes a very interesting field that requires additional research.

Despite this limitation, in the present study, we investigated whether the PRF from *C. celtidifolius* can inhibit the activity of MAO-A and/or MAO-B through in vitro experiments on rat brain mitochondrial homogenates. PRF was tested in a concentration range from 10 to 300 μg/ml, and the IC_{50} values obtained for MAO-A and MAO-B inhibition were, respectively, 98.07 and 79.70 μg/ml. On the other hand, it is important to remind that oxidative stress, depletion of brain antioxidants and the increase in MAO-B activity in reactive microglia are closely related risk factors in neurodegenerative diseases including PD (Mandel et al. 2008). Compounds that combine MAO-B inhibitory action with anti-inflammatory and antioxidant properties may therefore offer significant advantages in blocking the underlying pathophysiological process in PD (Youdim et al. 2004). The current results together with our previous findings (Nardi et al. 2007) may qualify PRF from *Croton celtidifolius* as a multifunctional agent that functions by limiting the formation of free radicals, reducing the activation of environmental pro-neurotoxins and by minimizing the generation of neurotoxic aldehydes. Consistent with this suggestion, catechins have extensive scavenging activity of free radicals and act as biological antioxidants (Mandel and Youdim 2004). Catechins can sequester the superoxide, hydroxyl and peroxyl radicals, nitric oxide, singlet oxygen and lipid peroxides, as well as peroxynitrite, by preventing nitration of tyrosine. Additionally, these compounds can chelate metal ions such as copper (II) and iron (III) to form inactive complexes and prevent the potential generation of toxic free radicals (Weinreb et al. 2009).

In conclusion, the present study provides pre-clinical data indicating that repeated systemic treatment with the PRF from *C. celtidifolius* may confer neuroprotection against the underlying dopaminergic neuron degeneration in the i.n. MPTP rat model of PD. However, further studies are needed to demonstrate whether these neuroprotective effects also extend to other animal models of PD, and the exact molecular mechanisms by which the PRF from *C. celtidifolius* can protect dopaminergic neurons from degenerating remains to be elucidated. Indeed, the present experimental data also suggest the potential of the PRF from *C. celtidifolius* in the management of non-motor symptoms (e.g. memory and psychiatric symptoms) of PD that do not improve with the current dopaminergic drugs.

Acknowledgments This study was supported by grants from the Brazilian institutions Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Apoio à Pesquisa do Estado de Santa Catarina (FAPESC). The authors have no financial or personal conflicts of interest related to this study.

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