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Basic nutritional investigation

Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice

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ABSTRACT

Objective: Parkinson's disease (PD) may be caused by the interaction of a number of factors, including genetics, toxins, oxidative stress, mitochondrial abnormalities, and aging. Studies have shown that consumption of an antioxidant-rich diet may reduce the incidence of neurodegenerative diseases. The aim of this study was to evaluate the role of the flavonoid hesperidin in an animal model of PD induced by 6-hidroxidopamine (6-OHDA).

Methods: Aged mice were treated with hesperidin (50 mg/kg) during 28 d after an intracerebroventricular injection of 6-OHDA. The enzymatic activities of superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase, the levels of glutathione, reactive oxygen species, total reactive antioxidant potential, dopamine and its levels of metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid, was analyzed in the striatum. The behavioral parameters (depressive-like, memory, and locomotor) were measured.

Results: This study demonstrated that hesperidin (50 mg/kg) treatment was effective in preventing memory impairment in the Morris water maze test, as well as, depressive-like behavior in the tail suspension test. Hesperidin attenuated the 6-OHDA-induced reduction in glutathione peroxidase and catalase activity, total reactive antioxidant potential and the dopamine and its metabolite levels in the striatum of aged mice. 6-OHDA increased reactive oxygen species levels and glutathione reductase activity in the striatum, and these alterations were mitigated by chronic administration of hesperidin.

Conclusion: This study demonstrated a protective effect of hesperidin on the neurotoxicity induced by 6-OHDA in aged mice, indicating that it could be useful as a therapy for the treatment of PD.

Introduction

Parkinson's disease (PD) is a neurologic disorder that is associated with dopamine (DA) depletion in the striatum and induction of a gradual dysfunction of the basal ganglia in the central nervous system [1]. The motor symptoms of PD include resting tremor, bradykinesia (slowed movements), rigidity (increased muscular tone), postural instability, and gait impairment, and these features are attributable to dopaminergic cell loss and the resultant dysfunction of the basal ganglia, a cluster of deep nuclei that participate in the initiation and execution of movements [2]. Non-motor symptoms, including memory impairments, impaired olfaction, disordered sleep, and neuropsychiatric manifestations (depression, hallucinations, and dementia), become prominent, and these features are probably due to the spread of pathology beyond the basal ganglia with the continued involvement of oxidative stress [2,3]. The administration of 6-hydroxydopamine (6-OHDA) into the striatum of mice produces a well-established model of PD. 6-OHDA selectively destroys the dopaminergic nigrostriatal pathway by inducing oxidative stress, which can lead to the induction of inflammation and ultimately cell death [1,4,5]. The unilateral intrastriatal injection of 6-OHDA induces pronounced behavioral alterations and biochemical deficits similar to PD. Additionally, 6-OHDA is a neurotoxin that rapidly undergoes non-enzymatic oxidation and produces hydrogen peroxide, superoxide, and hydroxyl radicals [6].

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The glutathione (GSH) system, which is responsible for removing free radicals and maintaining protein thiols in their appropriate redox state, is an important protective mechanism for minimizing oxidative stress [7]. Moreover, antioxidant enzymes, such as glutathione reductase (GR), glutathione-peroxidase (GPx), glutathione-*S*-transferse (GST), catalase (CAT) and superoxide dismutase (SOD), also are important mediators in the reduction of oxidative stress [8].

A variety of antioxidant compounds, such as flavonoids derived from natural products, have demonstrated neuroprotective activity in either in vitro or in vivo models of PD [5,9–11]. The bioflavonoid hesperidin is a specific flavonoid glycoside that is frequently found in oranges and lemons [12]. It has been reported to possess significant anti-inflammatory, antiviral, anticancer [13], and antidepressant-like properties in mice through the modulation of κ-opioid and 5-HT_{1A} serotoninergic receptors [14,15]. Several previous studies have demonstrated the antioxidant activity and radical scavenging properties of hesperidin in neurodegenerative diseases such as Huntington's [16], stroke [17], and Alzheimer's [18]. Furthermore, this compound has an important neuroprotective property related to diverse neuronal insults, such as ischemia [13] and oxidative-induced damage [19], as well as pathology related to Alzheimer's [18] and Huntington's [16] diseases.

Based on the aforementioned evidence, we sought to investigate the effects of oral hesperidin treatment (50 mg/kg) over 28 d in an in vivo mouse model of PD induced by the injection of 6-OHDA. We investigated the protective effect of hesperidin on behavior alterations (i.e., depression-like (are utilized to investigate depression and action of antidepressants to simulate the pathophysiology of depressive illness using animals models), cognitive, and locomotor tests), modifications in antioxidant enzymes (CAT, GPx, GR, SOD, and GST), as well as levels of reactive oxygen species (ROS), GSH, and the total reactive antioxidantpotential (TRAP), in the striatum of mice. Additionally, we evaluated the protective effects of hesperidin against neurochemical alterations of DA, and its levels of metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) induced by the striatal injection of 6-OHDA in the aged mice.

Materials and methods

Animals

Experiments were performed using aged female C57 BL/6 mice (25–35 g, 18 mo old). Animals were maintained at 22°C to 25°C with free access to water and food, under a 12-h light–dark cycle, with lights on at 0700. All manipulations were carried out during the light phase of the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 001/2013) of the Federal University of Pampa, Brazil.

Experimental design

Mice were randomly assigned to four groups (n = 10 per group): 1) sham/vehicle; 2) sham/6-OHDA; 3) hesperidin/vehicle, and 4) hesperidin/6-OHDA. The mice were subjected to stereotaxic surgical injections of 6-OHDA or vehicle. Seven d after the injections, treatment with oral hesperidin (50 mg/kg) was initiated for 28 d, and after behavioral testing, mice were sacrificed and the striatum was removed for biochemical assays (Fig. 1). All analyses (behavioral assessment and biochemical determinations) were performed in a blinded fashion.

Stereotaxic surgical injection of 6-OHDA

Surgery was performed under anesthesia with 10 mL/kg of 1% ketamine and 0.2% xylazine. 6-OHDA (5 μg in 2 μL of 0.9% NaCl with 0.2 μg/mL ascorbic acid) was injected slowly (0.5 μL/min) into the right striatum (0.9 mm anterior and 1.8 mm lateral from bregma, 3.0 mm ventral from the dura). After the injection, the syringe was maintained in the brain for an additional 3 min before it was slowly retracted. Controls were injected with the vehicle [19]. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Behavioral assessment

Tail suspension test

Antidepressant-like effects were measured using the tail suspension test (TST) [20], with minor alterations [14]. Each mouse was suspended by its tail using adhesive tape placed approximately 1 cm from the tip of the tail, and hung approximately 30 cm above a table. The animals were suspended for a period of 6 min, and the duration of immobility was scored manually during the last 4-min interval of the test (activity in the first 2 min was discarded because animals predominantly try to escape during this period). Mice were considered immobile only when they hung passively.

Morris water maze task

The Morris water maze task was used to check the memory capacity of the animals. The test was performed in a circular swimming pool similar to that described previously [21]. The pool consisted of black painted fiberglass, 97 cm in diameter and 60 cm in height. For the tests, the tank was filled with water maintained at 23°C + 2°C. The target platform (10 × 10 cm^2) was made of transparent Plexiglas and was submerged 1 to 1.5 cm beneath the surface of the water. Starting points for animals were marked on the outside of the pool as north (N), south (S), east (E), and west (W). Four distant visual cues (55 × 55 cm^2) were placed on the walls of the water maze room. They were all positioned such that their the lower edges were situated 30 cm above the upper edge of the water tank, and in the standard setting, the position of each symbol marked the midpoint of the perimeter of a quadrant (circle¼NE quadrant, square¼SE quadrant, cross¼SW quadrant, and diamond¼NW quadrant). The apparatus was

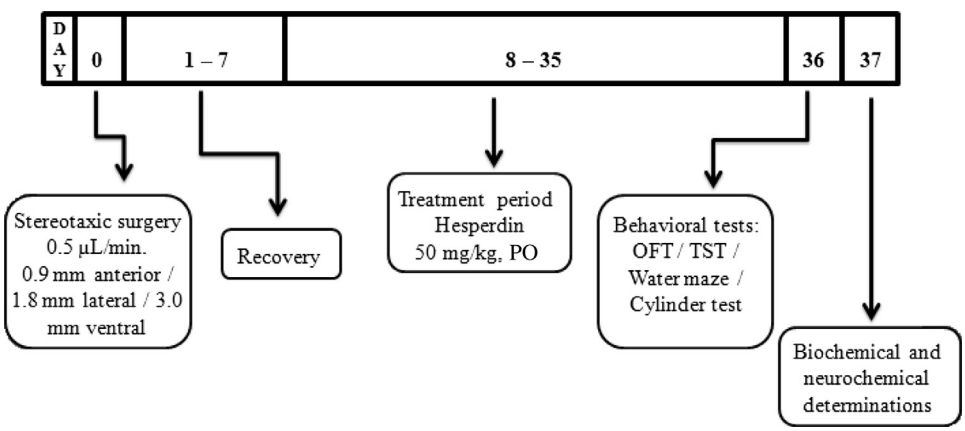

Fig. 1. Schematic representation of the experimental protocol describing the treatment periods with 6-OHDA and hesperidin. OFT, open field test; 6-OHDA, 6-hydroxydopamine; TST, tail suspension test.

located in a room with indirect incandescent illumination. Mice were submitted to a spatial reference memory version of the water maze by using a previously described protocol [22]. The training session consisted of 10 consecutive trials during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform. The platform was located in a constant position (middle of the southwest quadrant), equidistant from the center and the wall of the pool. If the animal did not find the platform during a period of 60 sec, it was gently guided to the platform. The animal was allowed to remain on the platform for 10 sec after escaping to it and was then removed from the tank for 20 sec before being placed at the next starting point in the tank. This procedure was repeated 10 times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. The test session was carried out 24 h later and consisted of a single probe trial wherein the platform was removed from the pool and each mouse was allowed to swim for 60 sec in the maze. The time spent in the correct quadrant (i.e., the quadrant in which the platform was located on the training session) was recorded and the percentage of the total time was analyzed.

Open field test

To verify the effects of treatments on the locomotor activity, the animals were submitted individually to a 5-min open field test (OFT; Insight model EP 154 C) 24 h after the last treatment of hesperidin. The parameters observed included the following: Distance (unit: Mm) and velocity (mm/sec) [23].

Cylinder test

We performed the cylinder test for behavioral evaluation at 1, 2, 3, and 4 wk after 6-OHDA injections. The cylinder test was used to assess the degree of forepaw asymmetry. Mice were placed in a transparent cylinder (diameter: 20 cm, height: 30 cm) for 3 min, with the number of forepaw contacts to the cylinder wall counted [23,24]. The score of cylinder test in this study was calculated as a contralateral bias: [(the number of contacts with the contralateral limb) − (the number of contacts with the ipsilateral limb)/(the number of total contacts) × 100] .

Tissue preparation

After behavioral tests, mice were sacrificed with a barbiturate overdose (intraperitoneal pentobarbital sodium 150 mg/kg). The striatum was removed and rapidly homogenized in 50 mM Tris-Cl, pH 7.4. The homogenate was centrifuged at 2400*g* for 15 min at 4°C, and a low-speed supernatant fraction (S1) was used for assays.

Biochemical determinations

GSH levels

GSH content was determined fluorometrically using ortho-phthalaldehyde (OPA) as the fluorophore [25]. S1 (100 μL) was incubated with 100 μL of OPA (0.1% in methanol) and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescence spectrophotometer at the excitation wavelength of 350 nm and at the emission wavelength of 420 nm. GSH levels were expressed as nmol/g of tissue.

ROS levels

To determine ROS levels, S1 (fresh preparation) was diluted (1:10) in 50 mM Tris-Hcl (pH 7.4) and incubated with 10 μL of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) at 37°C for 30 min. ROS levels were determined by a spectrofluorimetric method using the DCHF-DA assay, as previously described (2005). The DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. ROS levels were expressed in arbitrary units.

TRAP

The non-enzymatic antioxidant potential of the striatum was estimated by the TRAP [26]. The reaction is initiated by adding luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 4 mM), an external probe for monitoring radical production, as well as AAPH (2,2′-azobis–2-methylpropionamidine–dihydrochloride, 10 mM), a free radical source that produces peroxyl radical at a constant rate, in glycine buffer (0.1 M) pH 8.6 at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read in a liquid scintillation counter (Agilent Care Elipse) as counts/min. The sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (100 g of protein) in a TRAP protocol, and the area under the curve (AUC) was quantified. In the TAR protocol, results were expressed as the percentage of radical production (e.g., system counts considered to be 100% of radical production).

SOD activity

SOD activity was determined in the striatum according to the method previously described [27]. This method is based on the ability of SOD to inhibit autoxidation of epinephrine to adrenochrome. Briefly, the supernatant fraction (20–60 μL) was added to a medium containing glycine buffer (50 mM; pH 10.5) and epinephrine (1 mM). The kinetic analysis of SOD was started after the addition of epinephrine, and the color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 30°C, and results were expressed as units (U)/mg of protein.

CAT activity

CAT activity in the supernatant (S1) was assayed spectrophotometrically by a method previously proposed [28], which involves monitoring the disappearance of H_2O_2 in the presence of S1 at 240 nm. Enzymatic reaction was initiated by adding S1 and the substrate H_2O_2 (0.3 mM) in a medium containing 50 mM potassium phosphate buffer (pH 7.0). One unit of enzyme was defined as the amount of enzyme required for monitoring the disappearance of H_2O_2. The enzymatic activity was expressed as U/mg protein (1 U decomposes 1 μmol H_2O_2/min at pH 7.0 at 25°C).

GR activity

GR activity was determined spectrophotometrically as described previously [29]. In this assay, glutathione disulfide (GSSG) is reduced by GR at the expense of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. An aliquot of S1 was added in the system containing 0.15 M potassium phosphate buffer (pH 7.0), 1.5 mM EDTA, 0.15 Mm NADPH. After the basal reading, the substrate (GSSG 20 mM) was added. The enzymatic activity was expressed as nmol NADPH•min•mg^{-1} protein.

GPx activity

GPx activity in S1 was assayed spectrophotometrically by the method described previously [30], through the GSH/NADPH/GR system, by the dismutation of H_2O_2 at 340 nm. S1 was added to the medium containing the GSH/NADPH/GR system and the enzymatic reaction was initiated by adding H_2O_2 (4 mM). In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by the GR that is present in the assay media, at the expense of NADPH. The enzymatic activity was expressed as nmol NADPH•min•mg^{-1} protein.

GST activity

GST activity was assayed through the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), at 340 nm, as previously described [31]. An aliquot of S1 was added to a medium containing 0.1 M potassium phosphate buffer (pH 7.4). Then, 100 mM CDNB and GSH were added to the medium. CDNB was used as a substrate. The enzymatic activity was expressed as nmol CDNB conjugated•min•mg^{-1} protein.

DA, DOPAC, and HVA levels

Striatal tissues were homogenized with 300 μL of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (10 000*g* for 10 min at 4°C), the supernatant was filtered and then injected directly into a high-performance liquid chromatography system (Shimadzu; Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode versus Ag/AgCl reference electrode) was set at 700 mV. The analytical column was a TSKgel Super-ODS (4.6 mm I.D. 9 100 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate-sodium acetate buffer (pH 3.9) containing methanol (18%, v/v), disodium EDTA (4 mg/L), and sodium octanesulfonate (0.8 mM) [31].

Protein determination

Protein content was measured colorimetrically according to a previously described method [32,33], and bovine serum albumin (1 mg/mL) was used as the standard. The standard of bovine serum albumin was pipetted in different concentrations (5, 10, 25, 50 and 100 mg/mL).

Statistical analysis

Results were presented as the means ± SEM. First, we evaluated the normality of data using the D'Agostino and Pearson omnibus normality test. Comparisons between experimental and control groups were performed by two-way analysis of variance (ANOVA) followed by Newman–Keuls test for post hoc comparison when appropriate. Main effects of first-order interactions

are presented only when interaction was not significant. Comparisons between experimental and control groups were performed by one-way (hesperidin or 6-OHDA = independent variable) or two-way ANOVAs (6-OHDA × hesperidin = independent variables). A value of $P < 0.05$ was considered significant. All tests and plotting graphics were executed using the GraphPad Prism 5 software (San Diego, CA, USA).

Results

Behavioral assessment

Depressive-like behavior in the TST

A two-way ANOVA of depressive-like behavior in the TST revealed a significant 6-OHDA × hesperidin interaction ($F_{1,40} = 10.86$; $P = 0.002$), as well as main effects of hesperidin ($F_{1,40} = 89.68$; $P = 0.001$) and 6-OHDA ($F_{1,40} = 21.49$; $P = 0.001$). Post hoc comparisons demonstrated that animals in the 6-OHDA/vehicle group showed an increase in immobility time compared with animals in the sham/vehicle group, exhibiting 6-OHDA-induced depressive-like behavior in the TST. Daily, oral hesperidin treatment (50 mg/kg) protected against the increased time spent immobile in the TST caused by 6-OHDA and demonstrated an antidepressant-like activity on the sham/hesperidin group (Fig. 2).

Morris water maze task

The data presented in Figures 3A and 3B show that mice treated with 6-OHDA did not differ from the sham/vehicle group during training ($F_{1,40} = 0.75$; $P = 0.40$) in the escape latency for finding the platform, based on the training sessions when the mice were submitted to the spatial reference memory version of the water maze. The animals from all experimental groups were able to learn the task, given that their mean escape latencies improved throughout the training days.

A two-way ANOVA revealed that time quadrant was changed significantly by a 6-OHDA × hesperidin interaction ($F_{1,40} = 13.17$; $P = 0.001$), as well as main effects of hesperidin ($F_{1,40} = 16.80$; $P = 0.003$) and 6-OHDA ($F_{1,40} = 18.60$; $P = 0.002$). Post hoc comparisons demonstrated that animals in the 6-OHDA/hesperedin group showed an increase in the percentage time spent in the quadrant compared with that of animals in 6-OHDA/vehicle group. Oral daily administration of hesperidin (50 mg/kg) protected against impairments in water maze test performance caused by 6-OHDA administration (Fig. 3C).

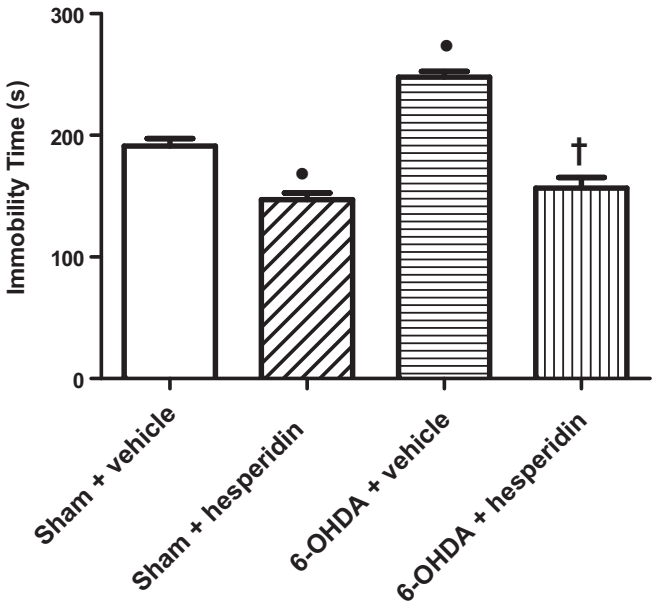

Fig. 2. Effect of daily oral hesperidin treatment (50 mg/kg) and the stereotaxic surgery injection of 6-OHDA on TST in aged mice. Values are mean ± SEM (n = 10/group). •$P < 0.05$ when comparing 6-OHDA/vehicle or sham/hesperidin with sham/vehicle. †$P < 0.05$ when comparing 6-OHDA/hesperidin with 6-OHDA/vehicle. 6-OHDA, 6-hydroxydopamine; TST, tail suspension test.

Locomotor activity in the OFT

A two-way ANOVA revealed that the total distance in OFT was not changed significantly by a 6-OHDA × hesperidin interaction ($F_{1,40} = 0.77$; $P = 0.38$), nor by main effects of hesperidin ($F_{1,40} = 2.78$; $P = 0.10$) or 6-OHDA ($F_{1,40} = 0.14$; $P = 0.70$) (data not shown).

Statistical analysis of the velocity performed in OFT was not significantly altered by a 6-OHDA × hesperidin interaction ($F_{1,40} = 0.25$; $P = 0.61$) or a main effect of 6-OHDA ($F_{1,40} = 0.92$; $P = 0.34$), although a main effect of hesperidin was found ($F_{1,40} = 4.15$; $P = 0.04$) (data not shown). With this test it is possible to observe that neither 6-OHDA nor hesperidin treatments caused sedative or excitatory effects on the animals.

Cylinder test

The scores of the cylinder test in the hesperidin group were ameliorated over time at 2, 3, and 4 wk after 6-OHDA injections (contralateral bias: 35% ± 5.6%, 31% ± 4%, and 20% ± 3%), compared with those in the control group (contralateral bias: 79% ± 7%, 65% ± 5%, and 64% ± 3%) (data not shown).

Biomarkers of oxidative stress and neurochemical alterations

GSH levels

A two-way ANOVA revealed that striatal GSH levels were changed significantly by 6-OHDA × hesperidin interaction ($F_{1,40} = 3.22$; $P = 0.04$), as well as by a main effect of hesperidin ($F_{1,40} = 6.65$; $P = 0.02$) and 6-OHDA ($F_{1,40} = 11.12$; $P = 0.004$). Post hoc comparisons demonstrated that 6-OHDA significantly decreased the striatal GSH levels. Oral administration of hesperidin (50 mg/kg daily) prevented the inhibition of GSH levels caused by 6-OHDA in striatum (Table 1).

ROS levels

A two-way ANOVA of striatal ROS levels demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1,40} = 5.35$; $P = 0.03$), including main effects of hesperidin ($F_{1,40} = 6.55$; $P = 0.02$) and 6-OHDA ($F_{1,40} = 7.47$; $P = 0.01$). Post hoc comparisons revealed that 6-OHDA significantly increased striatal ROS levels. Hesperidin treatment (50 mg/kg daily) protected against the elevated striatal ROS levels induced by injections of 6-OHDA (Table 1).

TRAP

A two-way ANOVA of striatal TRAP demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1,40} = 3.45$; $P = 0.04$). Furthermore, main effects of hesperidin ($F_{1,40} = 16.34$; $P = 0.005$) and 6-OHDA ($F_{1,40} = 37.10$; $P = 0.001$) were observed. Post hoc comparisons revealed that 6-OHDA significantly decreased striatal TRAP. Oral administration of hesperidin (50 mg/kg daily) prevented reductions of TRAP in striatum (Table 1).

SOD activity

A two-way ANOVA of striatal SOD activity demonstrated a main effect of 6-OHDA ($F_{1,40} = 6.39$; $P = 0.02$). Post hoc

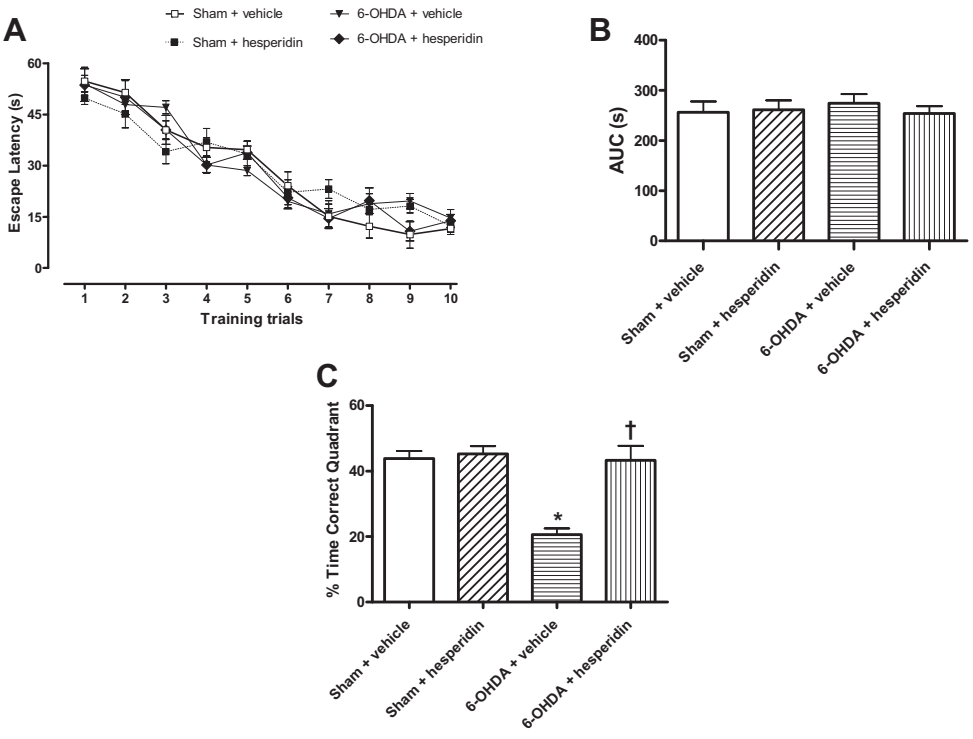

Fig. 3. Effects of daily oral hesperidin treatment (50 mg/kg) on the spatial learning and memory of mice evaluated in the Morris water maze task. Values are mean ± SEM (n = 10/group). Latency, in seconds (sec), for escape to a submerged platform (A) AUC of the latency in seconds (sec) (B) and percent time in the correct quadrant (C). The probe test session to evaluate the percent time in the correct quadrant was performed 24 h after the training trials. $^{*}P < 0.05$ when comparing 6-OHDA/vehicle with sham/vehicle. $^{\dagger}P < 0.05$ when comparing 6-OHDA/hesperidin with 6-OHDA/vehicle. AUC, area under curve; 6-OHDA, 6-hydroxydopamine.

comparisons revealed that 6-OHDA significantly increased the striatal SOD activity, but hesperidin treatment did not protect against the alterations in SOD activity (Table 2).

CAT activity

A two-way ANOVA of striatal CAT activity demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1.40}$ = 9,54; $P = 0.004$), as well as main effects of hesperidin ($F_{1.40} = 8.91$; $P = 0.001$) and 6-OHDA ($F_{1.40} = 7.21$; $P = 0.01$) (Table 2). Post hoc comparisons revealed that 6-OHDA significantly inhibited striatal CAT activity. Oral administration of hesperidin at the daily dose of 50 mg/kg prevented against the inhibition of striatal CAT activity (Table 2).

Table 1
Effect of hesperidin on GSH and ROS levels and TRAP in striatum of mice injected with 6-OHDA

Groups	GSH*	ROS†	TRAP‡
Sham/vehicle	6.2 ± 0.6	12.8 ± 1.3	99.6 ± 2.9
Sham/hesperidin	6.7 ± 0.8	9.0 ± 1.1	108.8 ± 6.0
6-OHDA/vehicle	3.0 ± 0.6§	3s9.2 ± 5.7§	62.4 ± 4.9§
6-OHDA/hesperidin	5.8 ± 0.6‖	19.6 ± 3.2¶	91.2 ± 3.7‖

GSH, glutathione; 6-OHDA, 6-hydroxydopamine; ROS, reactive oxygen species; TRAP, total reactive antioxidantpotential
Each value expressed as the mean ± SD (n = 10 mice/group)
* GSH levels were expressed as mol/g of tissue.
† ROS levels were expressed as arbitrary unit.
‡ Results were expressed as percentage of radical production.
§ $P < 0.05$ when compared 6-OHDA/vehicle with sham/vehicle.
‖ $P < 0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle.
¶ $P < 0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle and sham/vehicle.

GR activity

A two-way ANOVA of striatal GR activity demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1.40} = 28.43$; $P = 0.001$), including main effects of hesperidin ($F_{1.40} = 28.57$; $P = 0.001$) and 6-OHDA ($F_{1.40} = 12.35$; $P = 0.002$). Post hoc comparisons revealed that 6-OHDA significantly increased the striatal GR activity, and the daily administration of hesperidin (50 mg/kg) prevented against the increase of GR activity caused by 6-OHDA (Table 2).

GPx activity

A two-way ANOVA demonstrated that GPx activity in the striatum revealed a significant 6-OHDA × hesperidin interaction ($F_{1.40} = 8.47$; $P = 0.01$), as well as main effects of hesperidin ($F_{1.40} = 13.73$; $P = 0.001$) and 6-OHDA ($F_{1.40} = 10.08$; $P = 0.005$) (Table 2). Post hoc comparisons demonstrated that 6-OHDA significantly inhibited striatal GPx activity. Oral administration of hesperidin (50 mg/kg daily) prevented the 6-OHDA-induced inhibition of GPx activity.

GST activity

A two-way ANOVA of striatal GST levels did not show a significant 6-OHDA × hesperidin interaction ($F_{1.40} = 0.03$; $P = 0.85$), nor main effects of hesperidin ($F_{1.40} = 0.95$; $P = 0.33$) or 6-OHDA ($F_{1.40} = 0.38$; $P = 0.53$).

DA, DOPAC, and HVA levels

The statistical analysis of striatal DA levels revealed a significant 6-OHDA × hesperidin interaction ($F_{1.40} = 6.57$; $P = 0.02$), including main effects of 6-OHDA ($F_{1.40} = 84.64$; $P = 0.001$) and hesperidin ($F_{1.40} = 4.86$; $P = 0.04$). Post hoc comparisons

Table 2
Effect of hesperidin on SOD, CAT, GR, GPx and GST Activities in striatum of mice injected with 6-OHDA

Groups	SOD*	CAT†	GR‡	GPx§	GST‖
Sham/vehicle	6.0 ± 0.4	0.25 ± 0.04	47.7 ± 2.78	44.9 ± 2.1	21.6 ± 4.7
Sham/hesperidin	7.5 ± 0.6	0.27 ± 0.02	54.4 ± 2.26	47.5 ± 3.8	19.6 ± 1.5
6-OHDA/vehicle	9.2 ± 1.8¶	0.10 ± 0.02¶	76.6 ± 2.31¶	25.3 ± 1.6¶	23.6 ± 1.6
6-OHDA/hesperidin	10.2 ± 0.6¶	0.22 ± 0.02#	43.9 ± 2.55**	46.9 ± 4.3**	20.7 ± 1.2

CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; 6-OHDA, 6-hydroxydopamine; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; SOD, superoxide dismutase
Each value expressed as mean ± SD (n = 10 mice/group)
* SOD activity are expressed as (U)/mg protein.
† CAT activity are expressed as (U)/mg protein.
‡ GR activiy are expressed as nmol NADPH•min•mg^{-1} protein.
§ GPx activity are expressed as nmol NADPH•min•mg^{-1} protein.
‖ GST activity are expressed as nmol CDNB conjugated•min•mg^{-1} protein.
¶ $P < 0.05$ when compared 6-OHDA/vehicle or 6-OHDA/hesperidin with sham/vehicle.
# $P < 0.05$ when compared 6-OHDA/Hesperidin or 6-OHDA/vehicle with sham/vehicle.
** $P < 0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle.

demonstrated that 6-OHDA significantly decreased DA levels in the striatum. Oral administration of hesperidin (50 mg/kg daily) prevented the 6-OHDA-induced decrease of striatal DA levels (Table 3).

A two-way ANOVA of DOPAC levels in the striatum demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1,40} = 4.15$; $P = 0.04$) and yielded a main effect of 6-OHDA ($F_{1,40} = 49.43$; $P = 0.001$) (Table 3). Post hoc comparisons demonstrated that 6-OHDA significantly decreased striatal DOPAC levels, and the daily administration of hesperidin (50 mg/kg) prevented reductions in DOPAC levels caused by 6-OHDA.

A two-way ANOVA of striatal HVA levels demonstrated a significant 6-OHDA × hesperidin interaction ($F_{1,40} = 3.56$; $P = 0.05$) and yielded a main effect hesperidin ($F_{1,40} = 5.62$; $P = 0.03$), as well as 6-OHDA ($F_{1,40} = 39.78$; $P = 0.001$) (Table 3). Post hoc comparisons demonstrated that 6-OHDA significantly decreased striatal HVA compared with the sham/vehicle group. Oral administration of hesperidin (50 mg/kg daily) prevented the decrease of striatal HVA levels caused by 6-OHDA.

Discussion

This study demonstrated the potential protective effects of hesperidin against nigrostriatal dopaminergic neuronal toxicity induced by 6-OHDA injections in aged mice. Injections of 6-OHDA potentiated both the cognitive and depressive deficits accompanying oxidative damage with the loss of DA and its levels of metabolites DOPAC and HVA. Oral hesperidin treatment (50 mg/kg daily) attenuated behavioral alterations and protected the striatum of aged mice against oxidative stress, as well as against reductions of DA and its levels of levels of metabolites caused by 6-OHDA exposure, probably through its demonstrated modulation of antioxidant status.

Table 3
Effect of hesperidin on DA, DOPAC, and HVA levels in striatum of mice injected with 6-OHDA

Groups	DA*	DOPAC*	HVA*
Sham/vehicle	726.6 ± 17.5	40.6 ± 2.1	71.0 ± 3.9
Sham/hesperidin	715.5 ± 36.5	39.9 ± 1.2	72.8 ± 3.3
6-OHDA/vehicle	360.0 ± 24.2†	22.2 ± 1.9†	40.6 ± 4.4†
6-OHDA/hesperidin	508.6 ± 40.7‡	29.8 ± 2.4‡	56.4 ± 2.8‡

DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 6-OHDA, 6-hydroxydopamine
Each value expressed as mean ± SD (n = 10 mice/group)
* Levels are expressed as ng/g wet weight of striatum.
† $P < 0.05$ when compared with 6-OHDA/vehicle with sham/vehicle.
‡ $P < 0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle and sham/vehicle.

Regarding PD symptoms, an increasing number of studies have demonstrated that PD seems to be a multidimensional disease, and in addition to motor deficits, it is associated with a number of cognitive and depressive disturbances that result in a loss of quality of life of individuals with PD [34]. Depression is among the most common psychiatric conditions accompanying PD. Indeed, depending on the criteria measured, depression can affect 10% to 45% of patients with PD [35]. Furthermore, it has been shown that depression may largely precede the onset of motor symptoms of PD [35,36]. The findings discussed here indicated that infusions of the neurotoxin 6-OHDA were able to produce depressive-like behaviors, as assessed through the TST, similar to previous reports [37,38]. Alterations in the dopaminergic system suggest, therefore, that neurotransmitter systems play an important role in depressive-like behaviors in the current model tested, thereby further supporting the involvement of these neurotransmitter systems in PD-related depression [39]. Our results demonstrated that the 6-OHDA/hesperidin group demonstrated less immobility time than the 6-OHDA/vehicle group, showing that flavonoid had antidepressant-like effects in this model of 6-OHDA-induced PD in mice. Interestingly, we recently demonstrated that hesperidin treatment is also linked to the modulation of depression in mice [14,15].

In addition to the emotional deficits presented by patients with PD, the disease seems to produce cognitive deficits as well, particularly in procedural memory. The striatum has been the main area implicated in procedural learning dysfunctions [38,40], consistent with earlier studies reporting that PD models impair mouse performance in cognitive tests [41,42]. Therefore, we investigated putative spatial memory dysfunction in mice after 6-OHDA injections by using the Morris water maze task. Mice injected with 6-OHDA did not differ from the control group in their escape latencies for finding the platform, and all experimental groups were able to learn the task during training. Our data demonstrated that 6-OHDA injections induced long-term memory impairment in the 6-OHDA/vehicle group, as revealed by the reduction of percent of time spent in the correct quadrant in the Morris water maze task. This effect was revealed by the reduction of the percent of time spent in the correct quadrant in the Morris water maze task. It is important to emphasize that these treatments have not caused sedative effect or excitement in animals as shown in the OFT. Importantly, the percentages of

striatal DA, DOPAC, and HVA depletion, as well as the increase in oxidative stress observed in this study, were similar to previous studies showing deficits in memory tasks [41,43]. As for the cognitive deficits found in this test, our findings support the hypothesized involvement of striatal DA ant its levels of metabolites and antioxidant deficits induced by 6-OHDA in memory in this model of PD. In our study, hesperidin treatment at the dose of 50 mg/kg protected against cognitive impairment induced by 6-OHDA and preserved the spatial memory with mechanisms such as antioxidant and DA enhancement.

6-OHDA is a selective catecholamine neurotoxin and could easily undergo autoxidation to yield hydrogen peroxide and superoxide radicals, which then take part in a secondary metal-catalyzed Haber-Weiss reaction that produces hydroxyl free radicals [44]. In this study, 6-OHDA infusion caused an overproduction of free radicals that, in turn, caused oxidative damage to membrane lipids and protein levels, ultimately leading to a modification in the activity of antioxidant enzymes. This oxidative neuronal damage in 6-OHDA-treated rodents is consistent with previous reports [10,45]. As a result, the vehicle group of mice exposed to 6-OHDA exhibited inhibition of GPx and CAT activity and increases in GR and SOD. Additionally, our data confirms that the tested toxin initiated an oxidative cascade of events in the striatum, through the formation of ROS, and decreased levels of GSH and TRAP. The increase of SOD and GR activities it is suggested that as adaptive mechanism to compensate for increased RS levels, as well as decreased levels of GSH and TRAP. To meet this rise, increased SOD activity as a counterbalance mechanism, resulting in a large production of H_2O_2. Although the hesperidin treatment did not modify SOD activity, it may have acted directly on O_2– and also modulated the activities of CAT, GPx, and GR in the absence of a decrease in H_2O_2 levels. Thus the protective effect of hesperidin may be due to its action against oxidative stress, brought on by O_2–, and the modulation of the antioxidant enzymes with the exception of SOD. The increase in GR activity in the 6-OHDA/hesperidin group indicates hesperidin acts to maintain antioxidant defenses in part by maintaining GSH levels. In agreement with these data, previous studies have reported increased GR activity in different encephalic structures after prooxidative insults [46,47]. This work is in accordance with previous results [23,48] in studies on other neurodegenerative diseases. Indeed, across different biological systems, hesperidin has been demonstrated to act as an antioxidant that stabilizes biomembranes and thus prevents cell membrane damage in other neurodegenerative diseases, such as Alzheimer's and Huntington's [16,18].

The measurement of monoamine neurotransmitters in the basal ganglia serves as an important method to determine whether a particular drug has a therapeutic effect on dopaminergic neurons. The activities of dopaminergic neurons can thus be inferred by determining the DA, DOPAC, and HVA levels in the brain or in the cerebrospinal fluid [6]. To this end, we performed high-pressure liquid chromatography with electrochemical detection (HPLC ECD) to detect striatal monoamine neurotransmitters, and we found that DA, DOPAC, and HVA levels were significantly decreased following 6-OHDA exposure, which is similar to previous reports [9,10,49,50]. We can infer from the this study that oral administration of hesperidin (50 mg/kg) daily for 4 wk may attenuate the 6-OHDA-induced catecholamine neurotoxicity, thereby maintaining the concentration of DA and its levels of metabolites at normal or close to normal levels. Therefore, in this PD model induced by 6-OHDA, hesperidin appears to act via antioxidant and DA-enhancing mechanisms that rescue the compromised cells in striatum.

The present study demonstrated that treatment with hesperidin (50 mg/kg) daily for 28 d was effective in attenuating the following impairments resulting from 6-OHDA exposure in mice:

1. depressive-like behavior inferred from TST;
2. impairment of cognitive performance based on the Morris water maze task;
3. increased ROS levels;
4. decreased GSH levels and TRAP;
5. inhibition of GPx and CAT activity and rises in GR activity; and
6. decreased DA, DOPAC and HVA levels.

In view of our results, we have provided the first data indicating that hesperidin acts as a protective agent through the analysis of behavioral, neurochemical, and biochemical parameters in aging mice submitted to an experimental model of PD induced by 6-OHDA. These results provide new insights into experimental models of PD, indicating that hesperidin may represent a new therapeutic tool for the treatment of PD. Conversely, further research is needed to clarify the precise molecular mechanisms involved in the protective effects induced by hesperidin in the 6-OHDA model of PD.

Conclusion

This study demonstrated a protective effect of hesperidin on the neurotoxicity induced by 6-OHDA in aged mice, increasing the DA levels, enzymatic, and non-enzymatic activity, decreasing the ROS and improving the behavioral parameters. To our knowledge, we have provided the first set of preclinical data indicating that hesperidin could be useful as a therapy for the treatment of PD.

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