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Original article

PPAR-γ agonist pioglitazone reduces microglial proliferation and NF-κB activation in the substantia nigra in the 6-hydroxydopamine model of Parkinson's disease

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ABSTRACT

Background: Peroxisome proliferator-activated receptor γ (PPAR-γ) agonists have received much attention in research because of their neuroprotective and anti-inflammatory effects that reduce cell death and halt the progression of neurodegeneration. Thus, this study observed the pioglitazone effects on the main inflammatory markers after 6-hydroxydopamine (6-OHDA) lesion.
Methods: The effects of a 5-day administration of the PPAR-γ agonist pioglitazone (30 mg/kg) in male Wistar rats that received bilateral intranigral infusions of 6-OHDA. After surgery, the rats were evaluated in the open-field test on days 1,7,14, and 21. Immediately after the behavioral tests on day 21, the rats were euthanized, and the substantia nigra was removed to analyze the expression of nuclear factor κB (NF-κB) and IκB by western blot. To immunohistochemical, animals were *intracardially* perfused, with brain removal that was frozen and sectioned, being selected slices of the SNc region to detect tyrosine hydroxylase (TH) immunoreactivity, microglia activation (Iba-1) and NF-κB translocation in the nucleus.
Results: Pioglitazone protected rats against hypolocomotion and 6-OHDA-induced dopaminergic neurodegeneration on day 7. Decreases in the microglial activation and the NF-κB expression were observed, and the p65 activation was inhibited.
Conclusions: These results suggest that pioglitazone may be a potential adjuvant for the treatment of Parkinson`s disease because of its effects on pathological markers of the progression of neurodegeneration.

Introduction

Parkinson's disease (PD) is a slowly progressing neurodegenerative disease that affects approximately 1% of the population over 60 years of age, and the chance of developing Parkinson's disease advances with age [1,2]. It is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compact (SNc), which promotes the loss of nerve terminals in the basal ganglia [3–5].

Chronic inflammation is a main neuropathological marker of PD, with an increase in the microglial and the astrocytic activation in the substantia nigra that favors the progression of neurodegeneration [6–8]. When signs of damage occur, microglial cells undergo rapid changes by increasing the expression of surface molecules and releasing proinflammatory mediators. This can activate astrocytes and stimulate the up-regulation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling pathways. Thus, contributing to further increases in the inflammatory process [9–12].

The peroxisome proliferator-activated receptors (PPARs) are a superfamily of nuclear receptors that are known as transcription factors that depend on linkages that are involved in regulating energetic metabolism, homeostasis, cell differentiation, and inflammation [13–15]. The PPAR-γ agonist pioglitazone is used in the treatment of type 2 diabetes. Pioglitazone exerts anti-inflammatory effects by reducing cytokine production and oxidative stress in models of neurodegenerative disease [14,16,17].

The 6-hydroxydopamine (6-OHDA) model of Parkinson`s disease mimics the extensive inflammatory process in the SNc

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that is seen in parkinsonian patients. We evaluated the potential neuroprotective effects of pioglitazone on motor activity and the microglial and NF-κB activation in rats after exposure to 6-OHDA.

Materials and methods

Animals

Male Wistar rats, weighing 280-320 g at the beginning of the experiments, were used. The animals were randomly housed in groups of five in polypropylene cages with wood shavings as bedding and were maintained in a temperature-controlled room (22±2 °C) with a 12 h/12 h light/dark cycle. The animals had free access to water and food.

All of the experiments were performed in accordance with the guidelines of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

6-Hydroxydopamine (6 μg; Sigma, St. Louis, MO, USA) was prepared in artificial cerebrospinal fluid (CSF), supplemented with 0.2% ascorbic acid. Pioglitazone (30 mg/kg, Actos™, Abbott, IL, USA) or vehicle (1% Carboxymethylcellulose - CMC) were administered *orally* by gavage, once daily for 5 days. Anesthesia was induced by the *intraperitoneal* administration of Equithesin (0.3 mg/kg, *ip*) associated to atropine sulfate (0.4 mg/kg). After surgery, was administered sodium bicarbonate (0.8 mg/kg, *ip*) and the antibiotic penicillin G-procaine (20,000 U) *intramuscularly*.

Experimental design

Animals received bilateral intranigral infusions of 6-OHDA or artificial CSF supplemented with 0.2% ascorbic acid (Sham group). After surgery, animals were subdivided into treated groups: sham + vehicle, sham + pioglitazone, 6-OHDA + vehicle, and 6-OHDA + pioglitazone (n = 8–10/experiment). Pioglitazone (30 mg/kg) or vehicle, were administered *orally* by gavage, once for 5 days [18]. Pioglitazone treatment was initiated after the 1st exposures of the animals to the open-field test.

One, 7, 14, and 21 days after surgery, the animals were subjected to tests that evaluated their motor function. Immediately after testing on day 21, animals were deeply anaesthetized and *intracardially* perfused for histological assessment, or decapitated for removal of the substantia nigra.

Stereotaxic surgery

Animals were anesthetized with Equithesin (0.3 mg/kg) and received atropine sulfate before surgery. After surgery, sodium bicarbonate and penicillin G-procaine were administered. Bilateral infusions were performed using a 27-gauge stainless-steel needle according to the following coordinates: antero/posterior, -5.0 mm from bregma; medial/lateral ± 2.1 mm from midline; dorsal/ventral, -8.0 mm from skull [19]. One μl of 6-OHDA or artificial CSF was administered per hemisphere at a flow rate of 0.33 μl/min, using an electronic pump (Harvard Apparatus, Holliston, MA, USA). After each infusion, the needle was kept in the injection place for an additional 2 min to avoid reflux.

Open-field test

The apparatus consisted of a circular arena (97 cm diameter, 42 cm heigh) with a white floor that was divided into 19 quadrants, which was in a room with controlled lighting maintained at 40 lx during the experiment. The animals were placed in the center of the arena and allowed to freely explore the apparatus for 5 min. Two motor parameters were evaluated: locomotion (amount of quadrants that the animal passes with the four paws) and rearing frequencies (number of times that animal remained supported on the hind paws). The open field was cleaned with a 5% ethanol/water solution before the behavioral testing to eliminate possible bias caused by odors left by the previous rats. In the open-field test 10–12 animals per group were used.

Western blot

Animals were decapitated 21 days after surgery, and brains were rapidly dissected in ice-cold PBS. SNc was removed and stored at −80 °C until processed for analysis. Samples were manually homogenized in ice-cold lysis buffer that contained 50 mM NaCl, 50 mM HEPES, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and a EDTA-free complete protease inhibitor mixture (Roche, USA). Then, samples were centrifuged at 12,000 x g for 10 min at 4 °C, and supernatant was collected to determine the protein concentrations by Bradford method (Bio-Rad, Germany). Proteins were subjected to a 12% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). Membranes were blocked in 5% slim milk in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature, and incubated overnight with the desired primary antibody diluted in a blocking solution at 4 °C. The following antibodies were used: mouse monoclonal anti-β-actin (A5441, Sigma, St Louis, MO, USA), rabbit polyclonal anti-NF-κB p65 (sc-372, Santa Cruz, CA, USA), and rabbit polyclonal anti-IκBα (sc-371, Santa Cruz, CA, USA). Following primary antibody incubation, membranes were with TBS-T and incubated with a HRP- conjugated secondary antibody (Sigma, St. Louis, MO, USA) in blocking solution for 1 h at room temperature. Finally, the membranes were extensively washed with TBS-T, and immune complexes were detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, São Paulo, Brazil). Protein levels were quantified by densitometry, using Image J 1.47 software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

After the last behavioral test, 21 days after surgery, animals were deeply anesthetized with chloral hydrate (400 mg/kg) and intracardially perfused with 0.01 M PBS (pH 7.4) using a peristaltic pump (Insight, Ribeirão Preto, SP, Brazil) followed by fixative solution (4% paraformaldehyde in 0.1 M PBS, pH 7.4). Brains were removed, leaved in paraformaldehyde for 24 h at 4 °C, and then, immersed in 30% sucrose for cryoprotection. After, brains were snap-frozen in dry-ice, and stored at -80 °C until sectioning. Frozen tissue was sliced into coronal semi-serial 40 mm thick sections of the SNc using a cryostat (Leica Lm 1850, Germany), and brain slices spaced 200 mm apart were collected from bregma -4.68 to -6.36 mm [20] in 5 wells, totalizing 6–8 slices per well. The sections were stored at -20 °C until processing. Each well was used for a different marker.

Briefly, tissue slices were washed with 0.1 M PBS, incubated with 0.3% H_2O_2 for 10 min at room temperature, washed again, and incubated with blocking buffer for 30 min. Brain slices were incubated with mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (MAB318, clone LNC1, 1:2000; Millipore, Temecula, CA, USA) or with goat polyclonal anti-Iba-1 (1:500; Abcam, Cambridge, MA, USA) diluted in blocking buffer containing 0.3% Triton X-100 overnight at 4 °C. For Iba-1 immunohistochemistry reaction, before blocking endogenous peroxidase, tissue slices were incubated with 0.1 M citrate buffer (pH 6.0) at 50 °C for 30 min for antigen retrieval.

Slices were washed in PBS and incubated with the biotin-conjugated goat anti-mouse antibody (B0529, 1:300; Sigma, St. Louis, MO, USA) for 2 h at 4 °C. The antibody complex was detected by incubation of the ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, followed by a reaction with 3,3′-diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA). Tissue were dehydrated in aqueous graded ethanol solutions from 70% to 100%, cleared in xylene and mounted with Entellan New (Merck). Slices were photographed with a DP71 Olympus Optical digital camera using a BX50 Olympus microscope (Olympus Optical, Center Valley, PA, USA), and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). Analysis of TH-immunoreactivity in the SNc of each brain sections was based on optical density, and data are expressed as the percentage of TH-immunoreactive (IR) relative to the sham group. TH-IR evaluation was performed using 4–5 brains (6–8 slices) per group.

Iba-1$^+$ cells were quantified, and data expressed as activated and total microglia. Morphological classification of activated microglia was performed according to established criteria [20]. Data were expressed as the percentage of Iba-1-immunoreactive (IR) cells relative to the sham group. To observe the microglia response were used 4–5 brains (6–8 slices) per groups.

For NF-κB immunohistochemistry, brain slices were incubated in blocking buffer for 30 min and incubated with polyclonal rabbit anti-NF-κB p65 (sc-372, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer containing 0.7% Triton X-100 overnight at 4 °C. Slices were washed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300, Thermo Fischer, Waltham, MA, USA) for 2 h at 4 °C. Slices were placed on gelatin-coated slides and mounted with a 50% glycerol solution supplemented with DAPI 4′,6′-Diamidine-2′-phenylindole (1μg/ml). Slices were examined in a confocal microscope (A1RSiMP Nikon, Japan). To western blot analysis were used samples of 5–7 animals per groups.

Quantifications of NF-κB-p65 positive nuclei was performed using NIS-Elements Viewer 4.2 (Nikon Instruments, Melville, NY, USA). Data were expressed in porcentage of p65$^+$-nuclei/Total nuclei, relative to the sham group. To evaluated the NF-κB activation in the nucleus were used 6–8 slices of 4–5 brains each group.

Statistical analysis

The data from the open-field test were analyzed using a two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. The immunohistochemical data was analyzed using a one-way ANOVA followed by Tukey's test. Western blot data were analyzed using one-way repeated-measures analysis of variance (ANOVA) followed by the Tukey *post hoc* test. The results are expressed as mean ± standard error of the mean (SEM), and the level of significance was set at $p < 0.05$.

Results

Behavioral effects of pioglitazone in 6-OHDA-lesioned rats

The ANOVA revealed significant effects of treatment ($F_{3,27} = 5.253$, $p = 0.0055$) and day ($F_{3,81} = 51.10$, $p<0.0001$) on locomotion frequency and a significant treatment X day interaction ($F_{9,81} = 6.682$, $p <0.0001$). The 6-OHDA groups exhibited a decrease in locomotion frequency compared with the sham + vehicle group, 1 day after surgery ($p < 0.001$; Fig. 1). Seven days after surgery the 6-OHDA + vehicle group exhibited a decrease in locomotion frequency compared with the sham + vehicle group ($p < 0.05$). No difference in locomotion frequency was observed in the 6-OHDA+pioglitazone group compared with the sham+vehicle group ($p > 0.05$). Moreover, the 6-OHDA+pioglitazone group exhibited a significant increase in locomotion frequency compared with the 6-OHDA+vehicle group ($p < 0.001$) at this point in time. No difference in locomotion frequency was observed between the 6-OHDA+vehicle group and the sham+vehicle group on day 14 and after surgery ($p > 0.05$). Importantly, a significant increase in locomotion frequency was observed in the 6-OHDA + pioglitazone

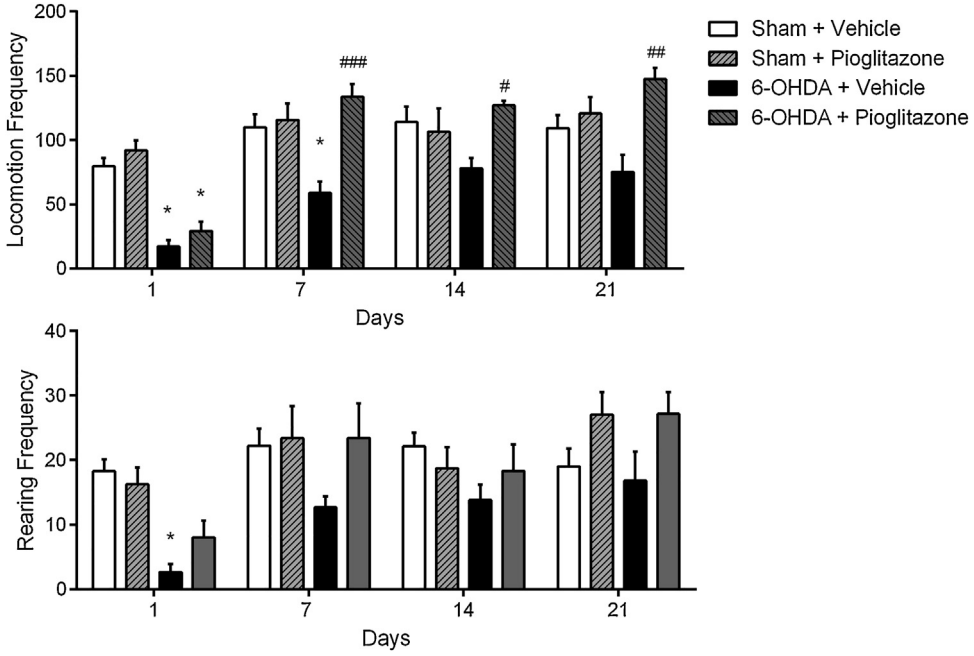

Fig. 1. Effects of pioglitazone in the open-field test in rats after intranigral infusion of 6-OHDA. (A) Locomotion frequency. (B) Rearing frequency. Rats that were subjected to 6-OHDA lesions exhibited hypolocomotion 1 day after surgery. After 7 days, hypolocomotion was still observed in the 6-OHDA+vehicle group compared with the sham +vehicle group. The 6-OHDA+pioglitazone group exhibited recovery of motor activity. The data are expressed as mean ± SEM (n = 10-12/group). *$p < 0.05$, compared with sham+vehicle group; #$p < 0.05$, ##$p < 0.001$, ###$p < 0.0001$, compared with 6-OHDA+vehicle group (two-way ANOVA followed by Bonferroni test).

group compared to 6-OHDA + vehicle group on day 14 ($p < 0.05$) and day 21 ($p < 0.001$).

To rearing frequency, ANOVA no demonstrated significant effects of treatment ($F_{3,27} = 2.959$, $p = 0.0501$), but there were significance on day ($F_{3,81} = 14.81$, $p < 0.0001$) and treatment X day interaction ($F_{9,81} = 2.2290$, $p < 0.0241$). The 6-OHDA + vehicle group showed a decrease in rearing frequency compared with the sham + vehicle on day 1 after surgery ($p < 0.01$). No difference in this parameter was observed between the 6-OHDA+vehicle group and the sham+vehicle group on days 7, 14, and 21 (all $p > 0.05$).

Effects of pioglitazone on tyrosine hydroxylase immunoreactivity

The neuroprotective effect of pioglitazone on dopaminergic neurons was examined by evaluating TH immunoreactivity by immunohistochemistry (Fig.2). The ANOVA revealed a significant effect of treatment ($F_{3,83} = 12.86$, $p < 0.0001$). Intranigral 6-OHDA administration (Fig. 2b) caused a 42% loss of TH-IR neurons in the SNc compared with the sham group ($p < 0.0001$). No difference in TH immunoreactivity was observed between the 6-OHDA+pioglitazone group and the sham+vehicle group.

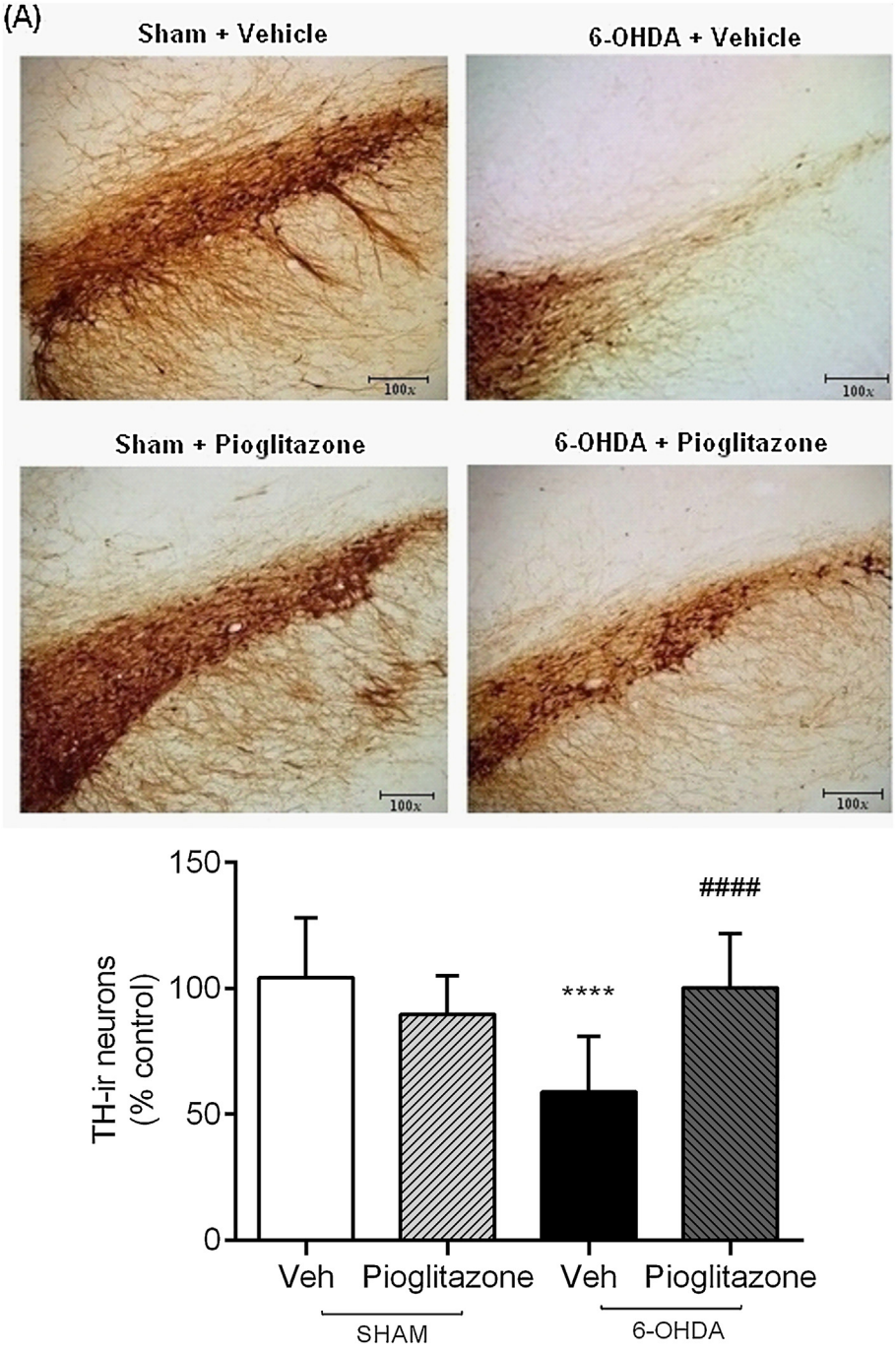

Fig. 2. Immunohistochemical analysis of the SNc in rats 21 days after surgery. (A) Photomicrograph of representative sections showing TH-ir neurons (100x magnification). (B) Quantification of TH-ir neurons. The 6-OHDA+vehicle group exhibited a 42% reduction of TH-ir neurons compared with the sham+vehicle group. No difference in TH immunoreactivity was observed between the 6-OHDA+pioglitazone group and sham+vehicle group. A 37% difference was observed between 6-OHDA groups. The data are expressed as mean ± SEM ($n = 4$-5/group). ****$p < 0.0001$, compared with sham+vehicle group; ####$p < 0.0001$, compared with 6-OHDA+vehicle group (one-way ANOVA followed by Tukey's test).

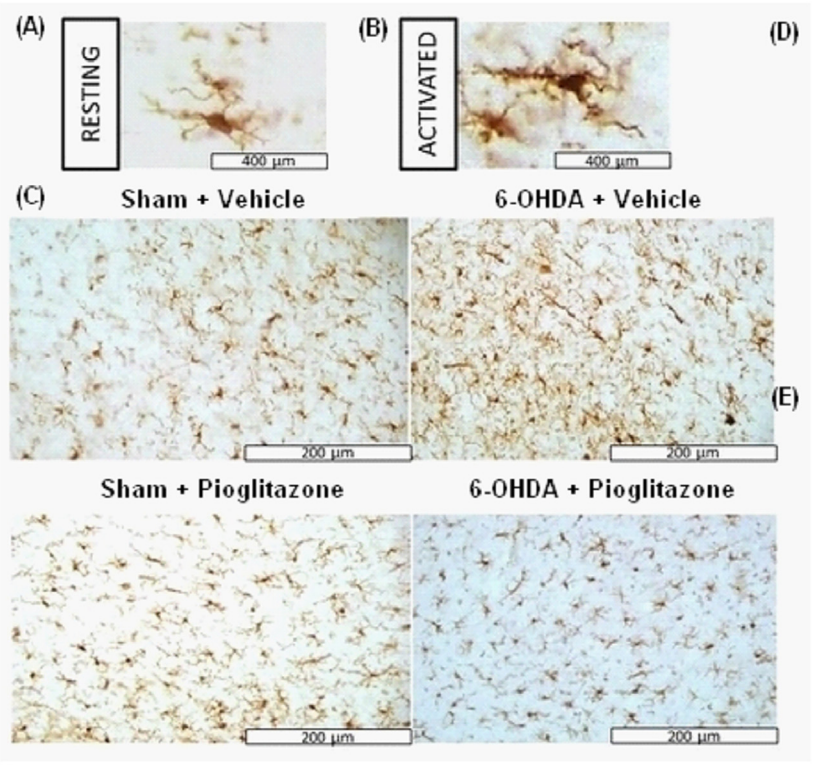

Fig. 3. Immunohistochemical analysis of IBA-1 in the SNc in rats 21 days after surgery. (A) Photomicrograph of resting microglia and (B) activated microglia. (C) Photomicrograph of Iba-1-IR microglial cells (200x magnification). (D) Percentage (%) of total microglial cells. The 6-OHDA+vehicle group exhibited a 79% increase in microglial cells compared with the sham+vehicle group, whereas the 6-OHDA+pioglitazone group exhibited a 29% increase. (E) Percentage (%) of activated microglial cells. Activated microglial cells increased by 50% in the 6-OHDA+vehicle group compared with the sham+vehicle group. No difference in activated microglial cells was observed between the 6-OHDA+pioglitazone group and sham+vehicle group. The data are expressed as mean ± SEM (n = 4-5/group). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{****}p < 0.0001$, compared with sham+vehicle group; $\#p < 0.05$, $\#\#\#\#p < 0.0001$, compared with 6-OHDA+vehicle group (one-way ANOVA followed by Tukey's test).

However, a significant 37% difference was observed in the 6-OHDA +pioglitazone group compared with the 6-OHDA+vehicle group ($p <$ 0.0001). These results indicate that pioglitazone protected against neuronal death.

Microglial activation after pioglitazone treatment

The total number of microglia cells, ANOVA revealed a significant effect of treatment ($F_{3,225} = 80.79$, $p <$ 0.0001). Both 6-OHDA groups exhibited an increase in the total number of microglial cells compared with the sham+vehicle group (Fig. 3). The 6-OHDA + vehicle group exhibited a 79% increase in the total number of microglial cells compared with sham + vehicle group ($p <$ 0.0001), while the 6-OHDA + pioglitazone group exhibited a 29% increase (p = 0.001; Fig. 3b). The 6-OHDA + pioglitazone group exhibited a difference of 50% in the total number of microglia compared with the 6-OHDA + vehicle group ($p <$ 0.0001). The sham + pioglitazone group exhibited a 41% decrease in the total number of microglia compared with the sham + vehicle group ($p <$ 0.0001).

Moreover, to the number of activated microglia cells the ANOVA demonstrated significant effect of treatment ($F_{3,214} = 9.412$, $p <$ 0.0001), occurring an increase of 50% in the 6-OHDA+vehicle group compared with the sham+vehicle group ($p <$ 0.001; Fig. 3c). No difference in the number of activated microglial cells was observed between the 6-OHDA + pioglitazone group and sham + vehicle group ($p >$ 0.05). In addition, a 50% difference was observed between the 6-OHDA+pioglitazone group and 6-OHDA +vehicle group.

Effects of pioglitazone on NF-κB and IκB expression

NF-κB expression in the SNc increased in the 6-OHDA + vehicle group compared with the sham + vehicle and 6-OHDA + pioglitazone groups group ($F_{3,20} = 3.900$, p= 0.0241; Fig. 4b). Moreover, no difference in the NF-κB expression was observed between the sham + vehicle group and the 6-OHDA + pioglitazone group.

IκB inhibitory protein expression in the substantia nigra increased in the 6-OHDA-lesioned groups compared with the sham + vehicle and sham + pioglitazone groups (both $p <$ 0.05; Fig. 4c).

NF-κB activation

The ANOVA showed a significant effect in the treatment ($F_{3,34} = 71.91$, $p =$ 0.0001). The 6-OHDA+vehicle group exhibited an increase in the number of p65-positive cells compared with the sham+vehicle group ($p <$ 0.0001), indicating that NF-κB activation increased because of greater reactivity in the nucleus (Fig. 5b). The 6-OHDA + pioglitazone group did not present a difference from the sham group in the p65+ number of cells. On the other hand, compared with the 6-OHDA + vehicle group there was a significant reduction in the p65+ number of cells in the group 6-OHDA that was treated with pioglitazone.

Discussion

The present study showed that pioglitazone protected against dopaminergic neuron loss, reflected for a prevention of the TH-IR

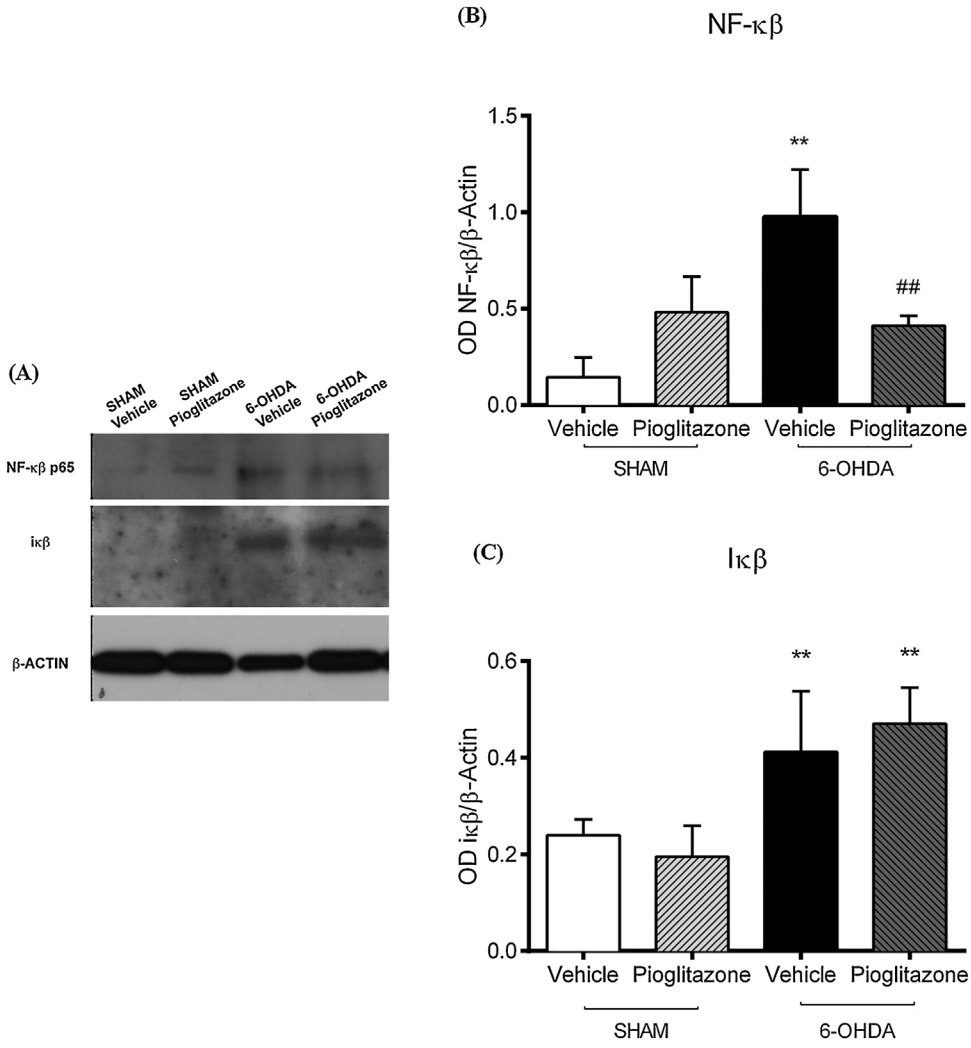

Fig. 4. NF-κB and IκB expression in brain lysates from the SN determined by Western blot in rats 21 days after surgery. (A) Western blot of protein extracts. (B) Quantification of NF-κB expression. (C) IκB expression determined by densitometry. The data were normalized to the densitometry of β-actin bands. The data are expressed as mean ± SEM (n = 5-6/group). $^{**}p < 0.001$, compared with sham+vehicle group; $\#\#p < 0.001$, compared with 6-OHDA+vehicle group (Student's t-test).

neurons loss in the SNc. Moreover, pioglitazone reduced microglial activation and decreased the total number of microglial cells in the SNc according the Iba-1 immunoreactivity. The pioglitazone treatment also decreased NF-κB expression, being confirmed NF-kB inhibition by immunohistochemistry with the quantification of p65-positive cells. Thus, the whole of these results, suggest that the diminution of NF-kB activity may has been responsible for lower microglia activity, and consequently, contributed with the inflammation reduction. With this, the dopaminergic neuronal death and 6-OHDA-induced motor impairment may have been also influenced for NF-kB modulation by pioglitazone.

Different of the other studies that beginning treatment few days before of the toxin infusion demonstrating the pioglitazone preventive effect in the animal models of PD [16,21,22], our study was focused in observe the treatment effects after the toxin infusion. In our schedule, it was able to observe that pioglitazone was able to reduce or inhibit the neurotoxicity of the 6-OHDA.

Intranigral injection of 6-OHDA caused hypolocomotion in rats after surgery. Such motor dysfunction was reported in other studies that used this model of Parkinson's disease [23,24]. Even initiating the treatment after 24 h from toxin infusion, pioglitazone was able to recover this motor deficit 1 week after surgery.

In the 6-OHDA group was observed a significant reduction of TH-IR neurons (Fig. 2) in the SNc 21 days after surgery. This effect has been reported in several studies [25–27]. Rats treated with pioglitazone did not exhibit significant reduction of TH-IR neurons, indicating that pioglitazone protected against 6-OHDA-induced neuronal death. Many studies demonstrated similar results with MPTP model, showing that pioglitazone partially prevented striatal dopamine loss [16,18,28]. Moreover, Sadeghian and colleges [22], showed that pioglitazone (20 mg/kg) was able to protect against 6-OHDA induced loss of dopaminergic neurons in the SNc.

In addition, not all studies have found positive results with pioglitazone. Despite Laloux and collaborators [21] demonstrated that pioglitazone was able to reduce weight loss and presented neuroprotective effect on the nigrostriatal pathway with MPTP model, in the 6-OHDA model did not find beneficial effects with treatment on motor impairment or dopaminergic neuronal loss, even using a lower concentration of toxin (3 μg), and a higher pioglitazone dose (50 mg/kg), than those we used (6 μg – 6-OHDA; 30 mg/kg – pioglitazone).

The present study showed that 6-OHDA activated and recruited microglial cells (Fig. 3b, c), which is well described in other studies. The extensive activation of microglial cells are observed in both patients and animal models of Parkinson's disease, being secreted high levels of inflammatory mediators not only by microglia, but by neurons and astrocytes too [29,30]. With 6-OHDA administration

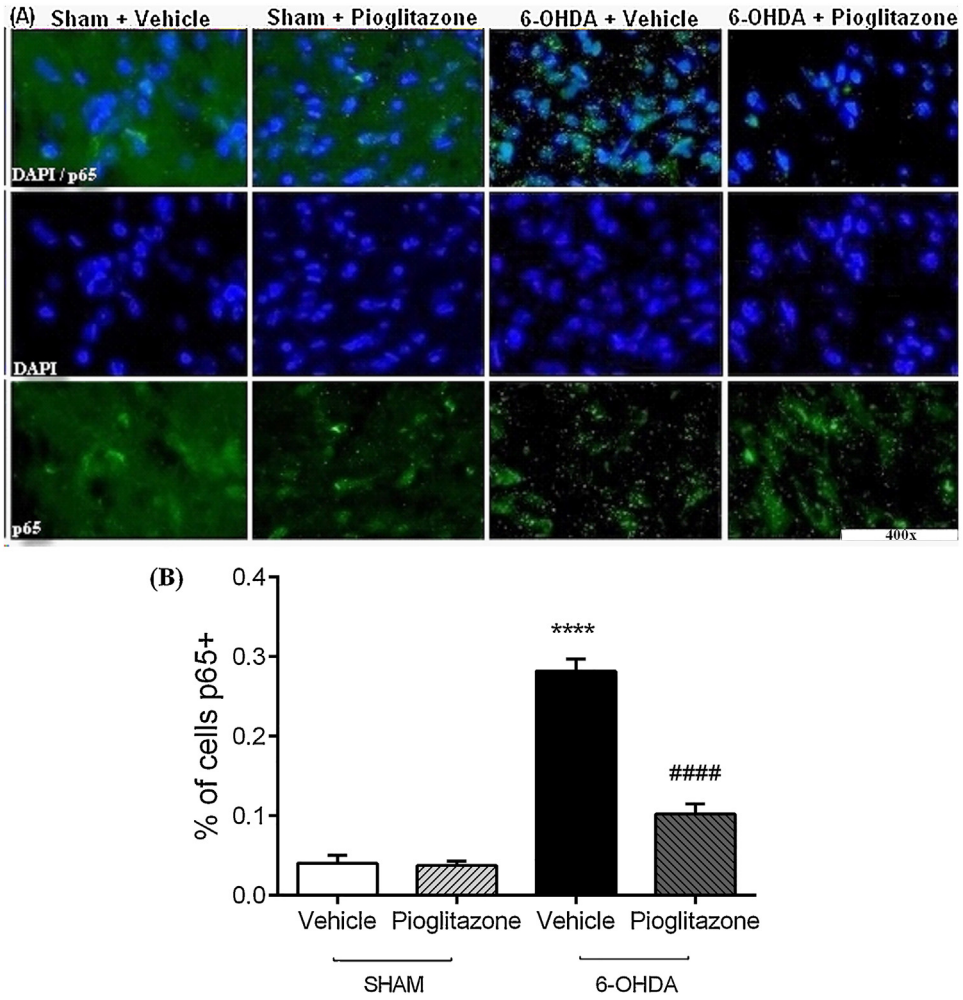

Fig. 5. Percentage (%) of cells with p65 colocalization in the SNc in rats 21 days after surgery. (A) Immunohistochemistry of DAPI (blue) and p65 (green) fluorescence (400x magnification). (B) Quantification of the percentage of p65-positive cells. The 6-OHDA+vehicle group exhibited an increase of p65+ cells. The 6-OHDA treated with pioglitazone not showed difference from the sham group, however compared with 6-OHDA+vehicle present a significant reduction of p65+ cells. The data are expressed as mean ± SEM (n = 4-5/group). ****$p < 0.0001$, compared with sham+vehicle group; #### $p < 0.0001$ compared with 6-OHDA + vehicle group (one-way ANOVA followed by Tukey's test).

in the SNc, He and colleges [31], reported an increased the number of activated microglia by 32% after 3 days, and by 55% after 1 week. In other study with non-human primates, over 2 years, Barcia and collaborator [32] administered daily intravenous doses of MPTP. One year after the last dose of MPTP, markers of microglial response were still found.

The present data showed that 6-OHDA-lesioned rats and sham group treated with pioglitazone exhibited a reduction of the activation and proliferation of microglia in the SNc (Fig. 3). Present results corroborate other studies that reported similar effects of pioglitazone treatment with regard to reducing the microglial activation in both the 6-OHDA and MPTP models of Parkinson's disease [16,22,33]. The protective effects of pioglitazone against microglial activation are related to a reduction of the proinflammatory cytokines production through the inhibition of NF-κB and possible stimulation of the production antiinflammatory cytokines [16,34].

The increase in proinflammatory cytokines that are produced by microglia stimulates the up-regulation of NF-κB signaling pathways, which plays a key role in regulating inflammation and oxidative stress [35]. In the present study, animals that received 6-OHDA injections in the SNc exhibited an increase in the NF-κB expression (Fig. 4b) with high p65 activation, reflected by the translocation of p65 to the nucleus (Fig. 5). Many authors have also demonstrated NF-κB activation using the 6-OHDA model [36–39]. When not activated, NF-κB remains in the cytoplasm and is linked to the inhibitory protein IκB. The degradation of IκB upon its phosphorylation by IκB kinase (IKK) in serine and tyrosine residues leads to the activation and nuclear translocation of NF-κB [40]. More importantly, pioglitazone reduced the NF-κB expression in 6-OHDA lesioned rats.

Many proinflammatory cytokines can promote the activation of IKK and subsequent phosphorylation of IκB [42]. This process releases NF-κB, which is then translocated to the nucleus where it binds to κB sites of genes that control the transcription of genes that are involved in the immune response [40,42–44].

The results obtained with our study corroborate previous research performed for Dehmer and collaborators [16]. These authors observed protective effects of pioglitazone which probably act through PPAR-γ activation, and the IκB induction besides blockage of NF-κB activation in lesioned mice with 5 x 30 mg/kg MPTP.

Pioglitazone was also recently shown to prevent the translocation and activation of NF-κB in a mouse model of nephrotoxicity [45]. PPAR-γ activation induces the up-regulation of IκB protein, thus confining p65/p50 to the cytosol and inhibiting its nuclear translocation [46, 48].

In the present data we did not observe a difference in IκB levels between the 6-OHDA-lesioned animals that were treated with vehicle or pioglitazone as was detected by [16]. This suggests that other mechanisms may underlie the reduction of NF-κB activation. Such mechanisms might be associated with the inhibition of NF-κB activation and a direct interaction with PPAR-γ, which may cause the translocation of p65 from the nucleus to the cytoplasm or induce degradation of the p65/p50 complex. There might also be competition between NF-κB and PPAR-γ through coativator proteins that are essential to the initiation of transcription [46]. Additionally, PPAR-γ may compete for promoter regions in the DNA, where NF-κB would bind to and then block its function [12, 47].

We found that pioglitazone reduced the activation of NF-κB in the 6-OHDA model (Fig. 4) and presented a strong trend toward reducing the NF-κB expression, suggesting that a reduction of the inflammatory process may be unleashed by NF-κB. Other effects of pioglitazone, such as reduces caspase-3 activation and protects against the depletion of dopamine and its metabolites in the striatum also has been described [29].

In conclusion, pioglitazone attenuated motor impairment in 6-OHDA-lesioned rats. This PPAR-γ agonist protected dopaminergic neurons in the SNc against neuronal death, reduced microglial activation, and reduced NF-κB activation. Several studies suggest potent neuroprotective effects of pioglitazone against various mechanisms that are involved in the progression of neurodegeneration in Parkinson's disease. The present results indicate that pioglitazone has the potential to be used clinically as an adjuvant for the treatment of Parkinson's disease.

Conflict of interest

There is no conflict of interest.

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