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Oral administration of carvacrol/β-cyclodextrin complex protects against 6-hydroxydopamine-induced dopaminergic denervation

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ABSTRACT

Carvacrol (CARV) presents valuable biological properties such as anti-inflammatory and antioxidant activities. However, pharmacological uses of CARV are largely limited due to disadvantages related to solubility, bioavailability, preparation and storage processes. The complexation of monoterpenes with β-cyclodextrin (β-CD) increases their stability, solubility and oral bioavailability. Here, the protective effect of oral treatment with CARV/β-CD complex (25 μg/kg/day) against dopaminergic (DA) denervation induced by unilateral intranigral injection of 6-hydroxydopamine (6-OHDA - 10 μg per rat) was analyzed, in order to evaluate a putative application in the development of neuroprotective therapies for Parkinson's disease (PD). Pretreatment with CARV/β-CD for 15 days prevented the loss of DA neurons induced by 6-OHDA in adult Wistar rats. This effect may occur through CARV anti-inflammatory and antioxidant properties, as the pretreatment with CARV/β-CD inhibited the release of IL-1β and TNF-α; besides, CARV prevented the increase of mitochondrial superoxide production induced by 6-OHDA in cultured SH-SY5Y cells. Importantly, hepatotoxicity or alterations in blood cell profile were not observed with oral administration of CARV/β-CD. Therefore, this study showed a potential pharmacological application of CARV/β-CD in PD using a non-invasive route of drug delivery, i.e., oral administration.

1. Introduction

Carvacrol (CARV) is a phenolic monoterpene found in many aromatic plants, including oregano and thyme (Suntres et al., 2015). CARV presents biological properties of potential pharmacological interest, such as anti-inflammatory, antioxidant, analgesic and antimicrobial activities (Guimaraes et al., 2015; Silva et al., 2016; Suntres et al., 2015). CARV easily crosses the blood-brain barrier (BBB) and modulates central neurotransmitters and neuromodulators, such as dopamine (Baluchnejadmojarad et al., 2014; Lins et al., 2018; Mechan et al., 2011; Trabace et al., 2011; Zotti et al., 2013). Previous studies have demonstrated neuroprotective effects of CARV in different animal models, and these effects seem to be particularly related to antioxidant and anti-inflammatory actions (Baluchnejadmojarad et al., 2014; Deng et al., 2013; Lins et al., 2018; Yu et al., 2012). However, such applications in pharmaceutical industry are limited due to CARV instability, volatility and low water solubility (Guimaraes et al., 2015; Marques, 2010; Silva et al., 2016).

Cyclodextrins (CDs) are cyclic oligosaccharides commonly used as complexing agents in pharmaceutical industry. CDs are non-toxic, water-soluble compounds widely available in human diet, and they improve solubility, stability, oral bioavailability and pharmacological action of bioactive molecules (Kurkov and Loftsson, 2013; Marques, 2010). Previous works have demonstrated that complexation of CARV with β-CD improved CARV therapeutic effects in experimental models (Guimaraes et al., 2015; Silva et al., 2016). Therefore, the encapsulation of CARV in β-CD (CARV/β-CD) represents an alternative with biotechnological value for the development of new drugs.

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Abbreviations

6-OHDA	6-hydroxydopamine;
ALT	alanine aminotransferase
AP	antero-posterior
AST	aspartate aminotransferase
ALY	atypical lymphocytes
BAS	basophils
CARV	carvacrol
CARV/β-CD	carvacrol/β-cyclodextrin complex
CSF	cerebrospinal fluid
CBC	complete blood count
CDs	cyclodextrins
DA	dopaminergic
DV	dorso-ventral
EOS	eosinophils
HCT	hematocrit
HBG	hemoglobin
LIC	large immature cells
LYM	lymphocytes
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MPV	mean platelet volume
ML	medio-lateral
MON	monocytes
NeuN	neuronal nuclear antigen
PFA	paraformaldehyde
PD	Parkinson's disease
PCT	plateletcrit
PLT	platelet count
PCT	platelet distribution width
ROS	reactive oxygen species
RBC	red blood cell
RDWcv	red cell distribution width - coefficient of variation
RDWsd	red cell distribution width - standard deviation
NEU	segmented neutrophils
SN	substantia nigra
TH	tyrosine hydroxylase
WBC	white blood cells

Inflammation plays an important role in age-related neurodegenerative disorders, such as Parkinson's disease (PD) (Costantini et al., 2018; JordanMcKenzie, 2018; Qin et al., 2016). PD is a progressive neurodegenerative disease characterized by motor and locomotor deficits, resulting from progressive damage of the nigrostriatal dopaminergic (DA) pathway (Lima et al., 2012). In previous studies, it was shown that CARV modulates neurotransmitter/neuromodulatory pathways, including DA and GABAergic systems (Guimaraes et al., 2012; Melo et al., 2010, 2011). Neuroprotective actions of CARV were demonstrated in a focal cerebral ischemia/reperfusion injury model, where a PI3K/Akt-mediated antiapoptotic effect was observed (Yu et al., 2012). It was also observed that CARV protected against ethanol-mediated hippocampal neuronal impairment through ERK1/2, Bcl-2, Bax and caspase-3 modulation (Wang et al., 2017). In two different experimental models, CARV was protective against PD-related deficits: it protected against reserpine-induced DA cell death and motor impairment (Lins et al., 2018) and it also protected against 6-OHDA-induced DA denervation in the nigrostriatal axis (Dati et al., 2017). Most neuroprotective effects of CARV have been attributed to its antioxidant and anti-inflammatory actions, as well as its ability to modulate TRPM7 and other ion channels (Oz et al., 2015).

In the present work, we demonstrated neuroprotective effects of CARV/β-CD oral administration against DA denervation and release of proinflammatory cytokines induced by 6-OHDA injected in the substantia nigra (SN) of Wistar rats. We also demonstrated that CARV reduced mitochondrial superoxide production induced by 6-OHDA *in vitro*. The oral administration of CARV/β-CD did not induce toxicity in Wistar rats, since no alterations in blood cells profile and serum aminotransferases activities were found. Therefore, CARV/β-CD oral administration shows a neuroprotective activity in the nigrostriatal axis, indicating a potential pharmacological application in PD and related disorders.

2. Methods

2.1. Ethics statement

All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, 2011) for animal care. Our research protocol was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul - Brazil (CEUA-UFRGS) under the project number #32235.

2.2. Animals

Male Wistar rats (60-days-old) were obtained from our breeding colony. They were caged in groups of four animals with free access to water and standard commercial food (Chow Nuvilab CR-1 type; Curitiba, PR, Brazil), maintained in a 12-h light-dark cycle in a temperature-controlled colony room (21 °C). Animals were handled for 7 days before the procedures to reduce stress caused by subsequent weighting, manipulation and oral gavage administration.

2.3. CARV/β-CD and 6-OHDA preparation

The CARV/β-CD complex was prepared as previously described (Guimaraes et al., 2015). Each rat received 25 µg/kg of CARV/β-CD by gavage (Guimaraes et al., 2015). 6-OHDA containing ascorbic acid as stabilizer (H116 - Sigma-Aldrich®; St. Louis, USA) was prepared at 10 mM 6-OHDA and 0,01% (w/v) ascorbic acid in sterile saline, preventing from heat and light exposure. Each rat received 10 µg of 6-OHDA via intranigral injection (Gasparotto et al., 2017).

2.4. Experimental design

The rats were randomly distributed into four groups (n = 8 per group) as follows:

Group 1: Control; animals received vehicle (saline) by gavage for 15 days. On the 15th day, an intranigral injection of saline was administered.

Group 2: CARV/β-CD; animals received CARV/β-CD by gavage for 15 days. On the 15th day, an intranigral injection of saline was administered.

Group 3: 6-OHDA; animals received vehicle by gavage for 15 days. On the 15th day, an intranigral injection of 6-OHDA was administered.

Group 4: CARV/β-CD + 6-OHDA; animals received CARV/β-CD by gavage for 15 days. On the 15th day, an intranigral injection of 6-OHDA was administered.

Fifteen days after 6-OHDA injection, all the animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via an intraperitoneal injection (i.p.). Blood was collected from cardiac puncture and cerebrospinal fluid (CSF) was collected from *cisterna magna* with a syringe for cytokines analysis. The animals were perfused via the vascular system for immunofluorescence microscopy assessment of SN.

2.5. Surgical procedure

The animals were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) for surgical procedure. On a stereotaxic apparatus (Insight-EFF 338, SP, BRA) the anesthetized rats were immobilized by locking with ear and nose bars. The fur was shaved with a pet clipper (SKU #: 09160-210 – Wahl; IL, USA) and 10% povidone-iodine solution was applied to sterilize the incision site. The skulls were perforated at the appropriate location with a dental drill (3 mm). A single dose (2 µL) of 6-OHDA or saline was injected into SN at the following stereotaxic coordinates: antero-posterior (AP): -5.0 mm from bregma; medio-lateral (ML): - 2.1 mm from the midline; dorso-ventral (DV): - 6.0 mm from skull, according to the Rat Brain Atlas in Stereotaxic Coordinates Paxinos (Paxinos and Watson, 2005), using a 10-µL Hamilton® syringe 701SN, needle size 23s ga (Sigma-Aldrich®; MO, USA). Syringe was inserted into the brain at a rate of 2 mm/min and the injection occurred at a rate of 0.5 µL/min. After the injection, the syringe was left in the place for 2 min and then removed at a rate of 2 mm/min. The incision was thoroughly cleaned with povidone-iodine solution and closed using three sutures. Lactated Ringer's solution (1 mL) was injected subcutaneously to replenish electrolytes. Nebacetin® (5 mg/g neomycin sulfate and 250 UI/g of bacitracin zinc, Medley; RS, BRA) was applied topically on the incision to prevent infections. The animals were placed in a controlled temperature recovery cage (37 °C) until recovery of consciousness.

2.6. Immunofluorescence

On the 15th day after 6-OHDA injection, animals were perfused via the vascular system with descending aorta clamped. In this procedure, sterile saline was administered for 10 min followed by more 10 min of 4% paraformaldehyde (PFA) solution in PBS pH 7.4. The brains were carefully extracted and maintained into 4% PFA for 24 h at 4 °C, then were transferred to 15% sucrose solution for 24 h at 4 °C followed by immersion in 30% sucrose for additional 24 h at 4 °C. After lightly dried, brains were frozen at −20 °C. Using a cryostat (Jung Histoslide 2000R; Leica; Heidelberg, Germany) at −20 °C, the SN region was sectioned in slices of 15 µm thickness on the coronal plane, which were collected in PBS containing 0.2% Triton X-100 (PBST). To block non-specific binding, the sections were incubated with 3% albumin for 1 h at room temperature (21 ± 3 °C). Then, the tissue slices were incubated with primary antibodies for 48 h at 4 °C. The details of the antibody source and dilutions are as follows: anti-NeuN (1:400; MAB377) was from Merck Millipore (MA, USA); anti-TH (1:400; 2792S) was from Cell Signaling Technology® (MA, USA); all of them diluted in PBST containing 3% bovine serum albumin. The tissue sections were washed four times in PBST and then incubated with secondary antibodies for 2 h at room temperature. The details of the antibody source and dilutions are as follows: anti-rabbit Alexa 488 and anti-mouse Alexa 555 from Cell Signaling Technology® (MA, USA); all of them diluted 1:500 in PBST. The sections were washed four times in PBST. Then, the tissue slices were incubated for 5 min with DAPI for nucleic acid staining (1:500; D9542 - Sigma-Aldrich®; MO, USA). The sections were washed several times in PBST transferred to gelatinized slides, mounted with FluorSave™ (345789 - Merck Millipore; MA, USA) and covered with coverslips. The images were obtained using a Microscopy EVOS® FL Auto Imaging System (AMAFD1000 - Termo Fisher Scientifc; MA, USA). The quantification of TH and NeuN content was obtained using the software ImageJ measuring the pixels of images with 40X of magnification from slices with 15 µm thickness of each animal.

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL-1β (RAB0272-1 KT) and TNF-α (RAB0479-1 KT) were quantified with commercial kits from Sigma-Aldrich® (MO, USA) following the manufacturer's protocol. For each assay, 100 µL of either CSF or serum were used. IL-1β and TNF-α were measured in the serum. In the CSF, only IL-1β was quantified due to limited sample volume.

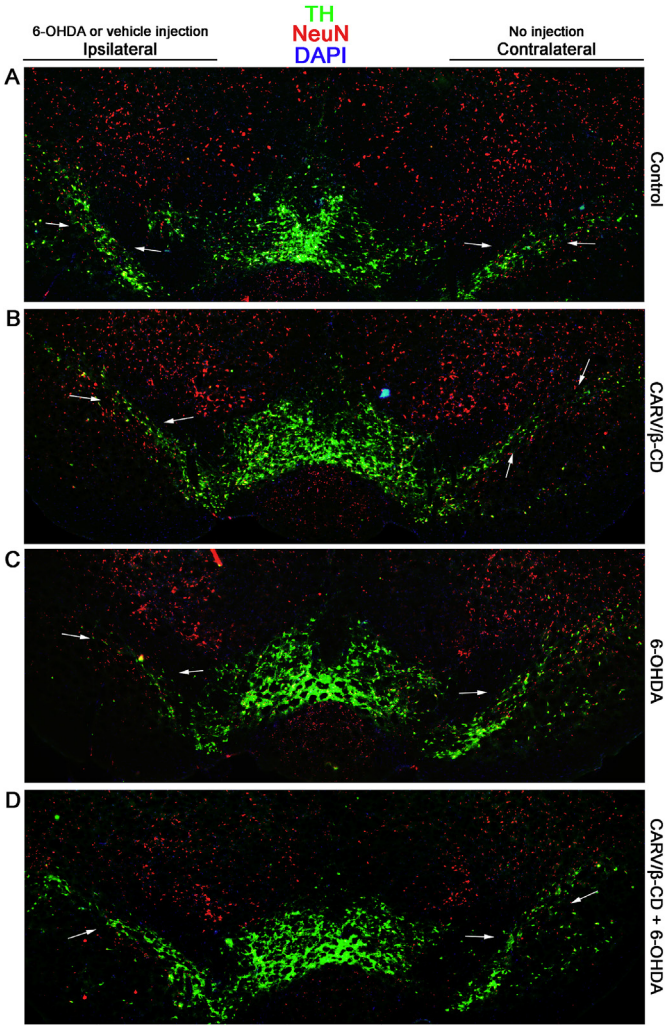

Fig. 1. Effect of CARV/β-CD on the ipsilateral loss of dopaminergic neurons induced by 6-OHDA. Representative immunofluorescence images of SN co-immunostained for TH (green), NeuN (red) and DAPI (blue). Both ipsilateral and contralateral sides are shown. Guide bars represent 1000 µm. **(A)** Control group, **(B)** CARV/β-CD group, **(C)** 6-OHDA group and **(D)** CARV/β-CD + 6-OHDA group. Images are representative of three independent experiments.

2.8. Complete blood count (CBC)

Complete blood count was performed in the blood of each animal using an automated analyzer (ABX Pentra XL 80, Horiba). The following variables were assessed: red blood cell (RBC), hemoglobin (HBG), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width - coefficient of variation (RDWcv), red cell distribution width - standard deviation (RDWsd), platelet count (PLT), mean platelet volume (MPV), plateletcrit (PCT; %), platelet distribution width (PDW), white blood cells (WBC), monocytes (MON), lymphocytes (LYM), segmented neutrophils (NEU), eosinophils (EOS), basophils (BAS), atypical lymphocytes (ALY), large immature cells (LIC).

2.9. AST and ALT

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were measured to determinate hepatotoxicity

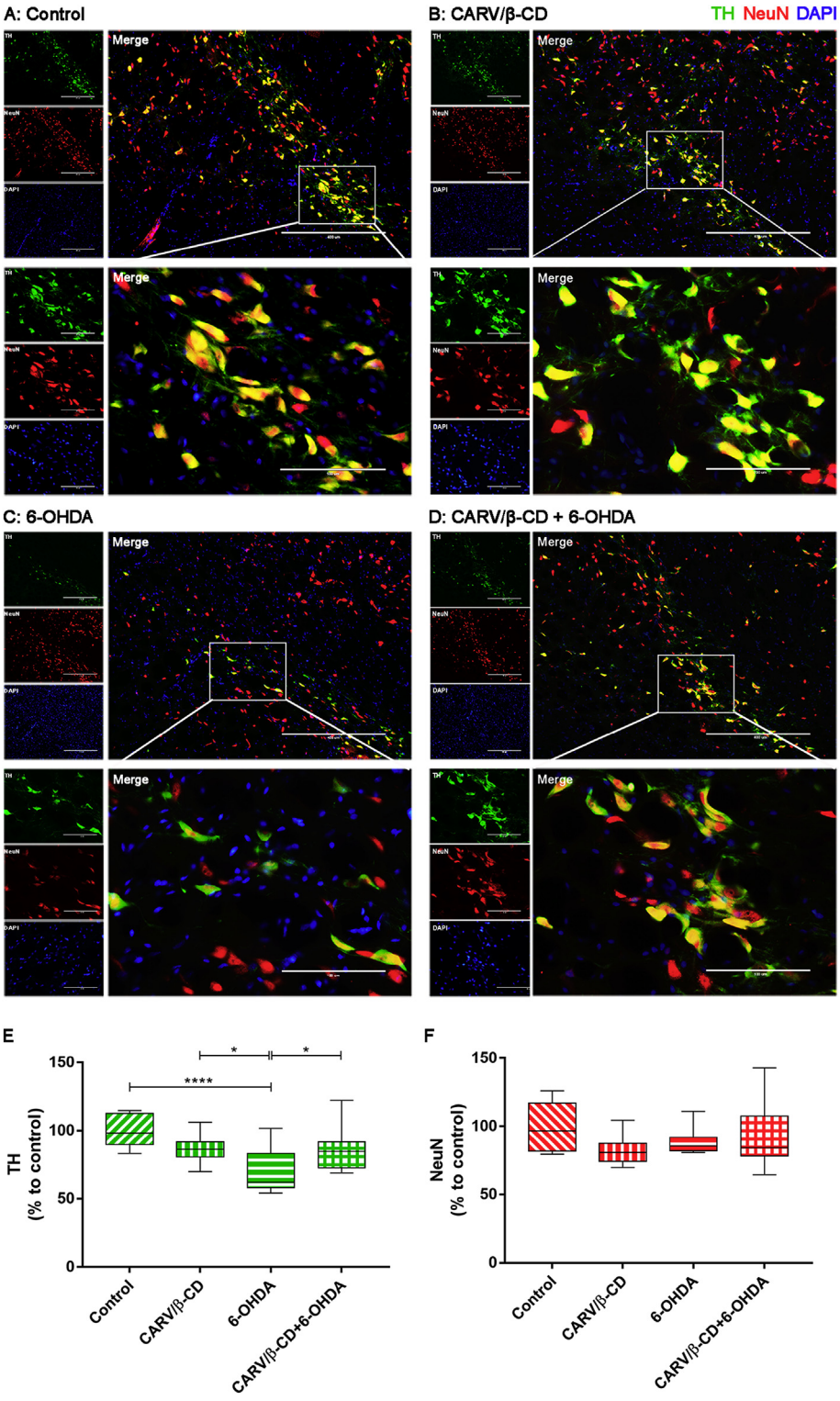

Fig. 2. Effect of CARV/β-CD pretreatment on 6-OHDA-induced dopaminergic loss in SN. Representative immunofluorescence images of SN co-immunostained for TH (green), NeuN (red) and DAPI (blue). The ipsilateral sides are shown. Guide bars represent 400 μm and 100 μm in detail inserts. **(A)** Control group, **(B)** CARV/β-CD group, **(C)** 6-OHDA group and **(D)** CARV/β-CD + 6-OHDA group. Images are representative of three independent experiments. The quantification of **(E)** TH and **(F)** NeuN content was obtained using the software ImageJ measuring the pixels of immunofluorescence images with 40X of magnification. Values represent mean ± SD. The results were expressed as percentage of control. One-way analysis of variance and Tukey's Multiple Comparison *post-hoc* test was applied to all data. The p values are represented as followed: $^{****}p < 0.0001$, $^{*}p < 0.05$.

(Huang et al., 2006), using Labtest kits (Minas Gerais, Brazil) according to manufacturer instructions.

2.10. Mitochondrial superoxide production in cultured cells

SH-SY5Y human neuroblastoma-derived cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F12 Nutrient Mixture, supplemented with 1% of Antibiotic-Antimycotic solution (Gibco, Thermo Fisher, USA) and 10% of Fetal Bovine Serum at 37 °C and 5% CO_2-humidified atmosphere. For assays, cells were plated in 24-well culture dishes and grown for 24 h at 60% confluence. To assess mitochondrial superoxide production in intact, living cells, MitoSOX® Red (Invitrogen, UK), a fluorogenic dye that permeates the membrane and rapidly targets the mitochondria, where it is oxidized by superoxide molecules, was used. MitoSOX Red vial content was dissolved in DMSO for a final concentration of 5 mM. Cells were pretreated with free CARV at 50 μM for 1 h and then incubated with 6-OHDA 30 μM for 24 h. Determination of *in vitro* concentrations of CARV and 6-OHDA was

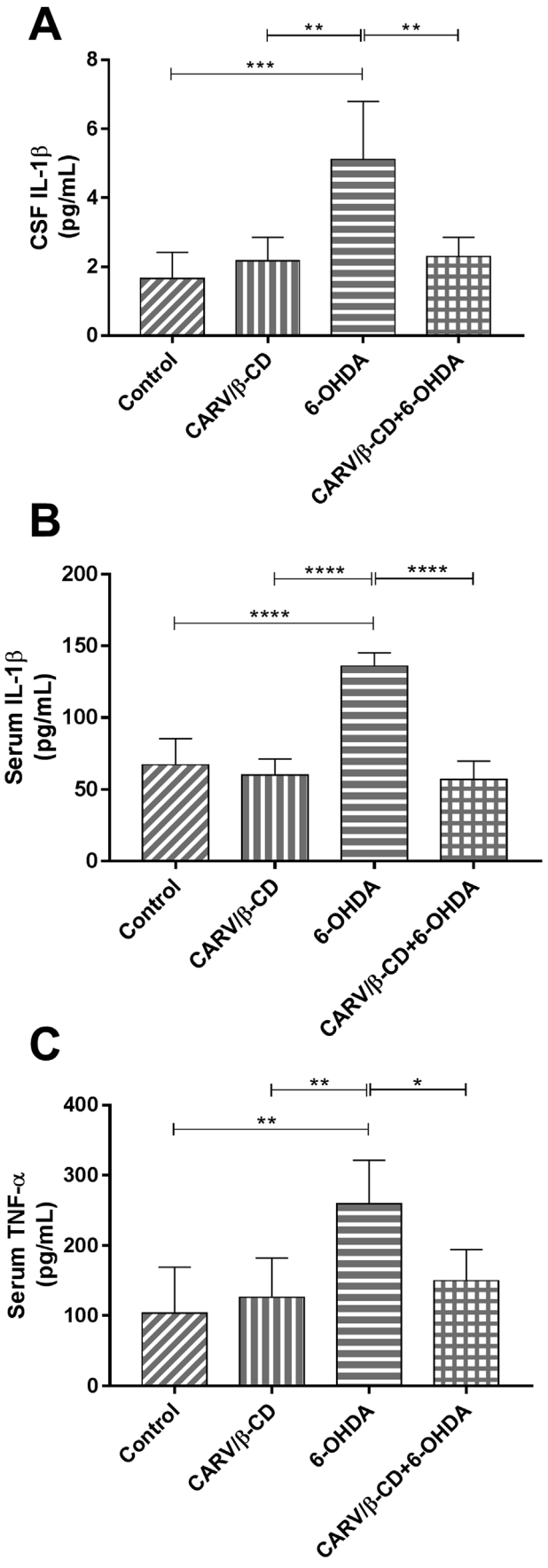

Fig. 3. Effect of CARV/β-CD pretreatment on 6-OHDA-induced release of IL-1β and TNF-α. CSF and serum were analyzed for cytokines by ELISA 15 days after 6-OHDA administration. **(A)** Cerebrospinal fluid was analyzed for IL-1β. **(B)** Serum samples were analyzed for IL-1β. **(C)** Serum samples were analyzed for TNF-α. IL-1β and TNF-α levels are expressed in pg/mL. Values represent mean ± SD. One-way analysis of variance and Tukey's Multiple Comparison *post-hoc* test was applied to all data. The p values are represented as follows: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

performed according a viability assay-based concentration curve (Supplemental Fig. 1). Following treatment, cells were incubated with a solution of MitoSOX Red 2 μM diluted in cell culture medium for 30 min at 37 °C and 5% CO_2. Fluorescence was monitored under an EVOS FL Auto Imaging System (AMAFD1000 – Thermo Fisher Scientific, USA).

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 7.00 (GraphPad Software Inc., San Diego, USA). Data were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison *post-hoc* test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. CARV/β-CD prevented the loss of TH + neurons induced by 6-OHDA

Co-immunostaining of tyrosine hydroxylase (TH - DA neuron marker) with neuronal nuclear antigen (NeuN - neuronal marker) was performed to analyze the effect of CARV/β-CD pretreatment in DA denervation induced by 6-OHDA. In animals treated with CARV/β-CD, the content of TH-positive (TH^+) cells in the SN of both sides of the brain was not different from control group (Fig. 1A and B). In animals subjected to 6-OHDA unilateral injection, TH content decreased in ipsilateral SN, whereas contralateral SN was not affected, as expected (Fig. 1C). Animals pretreated with CARV/β-CD and subjected to 6-OHDA injection did not present differences in TH immunostaining between ipsilateral and contralateral sides (Fig. 1D).

As shown in detail in Fig. 2, the number of TH^+ neurons of ipsilateral side have decreased only in the group that received 6-OHDA. Therefore, the prior treatment with CARV/β-CD prevented the loss of TH^+ neurons induced by 6-OHDA (Fig. 2D and E). The content of NeuN was not different between all groups (Fig. 2F).

3.2. CARV/β-CD prevented the increase of proinflammatory cytokines in CSF and serum of wistar rats treated with 6-OHDA

To analyze the effect of CARV/β-CD pretreatment on 6-OHDA-induced production of proinflammatory cytokines, we evaluated CSF levels of IL-1β (Fig. 3A) and serum levels of IL-1β (Fig. 3B) and TNF-α (Fig. 3C). In animals that received only CARV/β-CD, no significant changes were observed in CSF and serum. On the other hand, 6-OHDA injection led to a significant increase in the levels of all cytokines, but this effect was prevented by CARV/β-CD treatment (Fig. 3A–C).

3.3. CARV prevents the increase in mitochondrial superoxide production induced by 6-OHDA in cultured SH-SY5Y cells

Previous works demonstrated that CARV exerts antioxidant activities in different biological models (Suntres et al., 2015). Since the classical mechanism of 6-OHDA toxicity is linked to disruption of mitochondrial homeostasis associated to increased superoxide production (Drechsel and Patel, 2008), the effect of CARV on 6-OHDA-induced mitochondrial superoxide production was evaluated *in vitro*. MitoSOX Red fluorescence assay was performed to assess mitochondrial

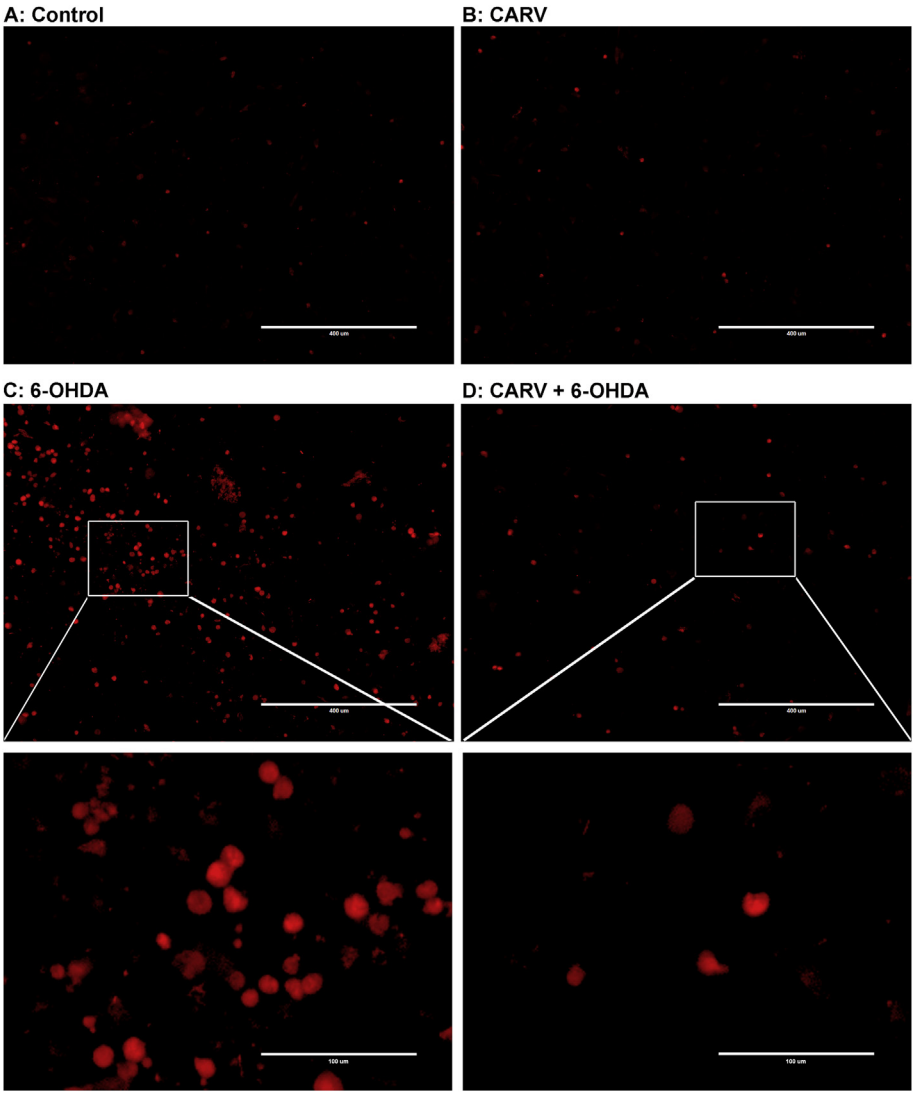

Fig. 4. ***In vitro*** **effect of CARV on 6-OHDA-induced mitochondrial superoxide production**. Cultured SH-SY5Y catecholaminergic-derived cells were pre-treated with CARV at 50 μM for 1 h and then incubated with 6-OHDA 30 μM for 24 h. After treatments, cells were incubated with a solution of MitoSOX Red 2 μM diluted in cell culture medium for 30 min at 37 °C and 5% CO_2. Fluorescence was monitored under an EVOS FL Auto Imaging System (AMAFD1000 – Thermo Fisher Scientific, USA). **(A)** Control (untreated) cells; **(B)** cells treated with CARV 50 μM; **(C)** cells treated with 6-OHDA 30 μM; **(D)** cells treated with CARV 50 μM and 6-OHDA 30 μM. Guide bars represent 400 μm in main figures and 100 μm in detail inserts. Representative images of three independent experiments.

superoxide production in SH-SY5Y catecholaminergic-derived cell line treated with free (uncomplexed) CARV. Superoxide production was poorly detected in control and CARV treated cells (Fig. 4A and B), but incubation with 6-OHDA 30 μM for 24 h significantly enhanced MitoSOX Red fluorescence (Fig. 4C). Pre-incubation with 50 μM CARV for 1 h had a preventive effect against superoxide production induced by 6-OHDA (Fig. 4D). Altogether, these results indicate that CARV prevents mitochondrial superoxide production induced by 6-OHDA.

3.4. CARV/β-CD oral administration did not induce systemic toxicity

We performed a complete blood count (CBC) in order to detect possible alterations on blood cells profile due to CARVβ-CD oral administration. Neither CARV/β-CD nor 6-OHDA administration induced alterations on blood cells numbers compared to control. However, animals that received both CARV/β-CD and 6-OHDA administration presented fewer circulating monocyte cells than animals that received only 6-OHDA (Table 1). We also investigated the CARV/β-CD effect on serum aminotransferases activities to assess a possible hepatotoxic effect. ALT and AST activities were not different between all groups (Table 1).

4. Discussion

A major hallmark of PD is DA neuronal death in the SN, which results in severe depletion of dopamine levels in the striatum (Dauer and Przedborski, 2003). Intracranial injection of 6-OHDA is used as a model of DA denervation, inducing loss of TH^+ neurons projecting from SN to the striatum (Tieu, 2011). Here, the administration of 6-OHDA reduced the number of TH^+ cells in SN and did not affect NeuN staining, confirming that 6-OHDA toxicity was selective to DA neurons. Our main finding is that oral administration of CARV/β-CD prevented the loss of DA neurons induced by an intranigral injection of 6-OHDA.

One of the major contributors to the progressive loss of DA neurons in PD is neuroinflammation (Wang et al., 2015). Previous works reported that sustained expression of inflammatory mediators in SN induces DA neuronal loss (Block and Hong, 2007; Poewe et al., 2017). The 6-OHDA lesion to DA neurons leads to an inflammatory response, in which microglial activation results in production and release of proinflammatory mediators, such as IL-1β and TNF-α (Gasparotto et al., 2017). Previous studies demonstrate that 6-OHDA alters blood–brain barrier (BBB) permeability (Carvey et al., 2005; Olmedo-Diaz et al., 2017), which might explain the increase of proinflammatory cytokines in both CSF and serum. Toxicity of 6-OHDA intranigral injection is acute and the consequent neuronal damage is severe. It is postulated that 6-OHDA uptake and consequent oxidation by DA cells gives rise to toxic reactive species production, causing cell death (Tieu, 2011). The rapid and extensive cell death triggers the recruitment and activation of glial cells, due to the presence of proinflammatory mediators released by apoptotic and necrotic neurons. In fact, neuroinflammation can be

Table 1
Effect of oral administration of CARV/β-CD in blood cell count and serum aminotransferases activities. Red Blood Cell (RBC; 10^6/mm^3), Hemoglobin (HBG; g/dL), Hematocrit (HCT; %), Mean Corpuscular Volume (MCV; μm^3), Mean Corpuscular Hemoglobin (MCH; pg), Mean Corpuscular Hemoglobin Concentration (MCHC; g/dL), Red cell Distribution Width - coefficient of variation (RDWcv; %), Red cell Distribution Width - standard deviation (RDWsd; μm^3), Platelet Count (PLT; 10^3/mm^3), Mean Platelet Volume (MPV; μm^3), Plateletcrit (PCT; %), Platelet Distribution Width (PDW; %), White Blood Cells (WBC; 10^3/mm^3), Monocytes (MON; %), Lymphocytes (LYM; %), Segmented neutrophils (NEU; %), Eosinophils (EOS; %), Basophils (BAS; %), Atypical Lymphocytes (ALY; %), Large immature cells (LIC; %), Alanine aminotransferase (ALT; U/L) and Aspartate aminotransferase (AST; U/L). Values represent mean ± SD. One-way analysis of variance and Tukey's Multiple Comparison *post-hoc* test were applied to all data ($^{\#}p < 0.05$ compared 6-OHDA group).

	Control	CARV/β-CD	6-OHDA	CARV/β-CD + 6-OHDA
Complete Blood Count				
RBC	8.51 ± 0.28	8.50 ± 0.20	8.23 ± 0.50	8.43 ± 0.34
HGB	15.03 ± 0.49	14.56 ± 0.64	14.53 ± 0.88	14.53 ± 0.37
HCT	45.75 ± 2.12	44.36 ± 1.54	44.3 ± 2.45	44.35 ± 1.10
MCV	0.00 ± 0.00	0.00 ± 0.00	27.00 ± 31.19	0.00 ± 0.00
MCH	0.00 ± 0.00	0.00 ± 0.00	8.825 ± 10.19	0.00 ± 0.00
MCHC	0.00 ± 0.00	0.00 ± 0.00	16.38 ± 18.91	0.00 ± 0.00
RDWcv	0.00 ± 0.00	0.00 ± 0.00	6.35 ± 7.33	0.00 ± 0.00
RDWsd	0.00 ± 0.00	0.00 ± 0.00	12.25 ± 14.15	0.00 ± 0.00
PLT	732.25 ± 59.64	763.80 ± 50.13	745.75 ± 24.9	709.25 ± 115.2
MPV	6.63 ± 0.30	6.26 ± 0.32	6.55 ± 0.10	6.53 ± 0.26
PCT	0.48 ± 0.03	0.48 ± 0.03	0.49 ± 0.01	0.46 ± 0.07
PDW	4.83 ± 5.60	0.00 ± 0.00	4.45 ± 5.14	0.00 ± 0.00
WBC	6.13 ± 3.30	6.82 ± 2.88	5.4 ± 0.93	5.55 ± 1.90
MON	1.8 ± 0.35	1.94 ± 0.50	2.65 ± 0.95	1.23 ± 0.52 $^{\#}$
LYM	87.58 ± 3.23	85.00 + 3.09	85.98 ± 3.94	82.43 ± 2.68
NEU	10.58 ± 3.06	12.58 ± 3.72	11.23 ± 3.02	16.25 ± 3.04
EOS	0.05 ± 0.06	0.04 ± 0.05	0.075 ± 0.05	0.075 ± 0.05
BAS	0.00 ± 0.00	0.04 ± 0.5	0.08 ± 0.05	0.03 ± 0.05
ALY	0.25 ± 0.17	0.22 + 0.11	0.33 ± 0.10	0.175 ± 0.05
LIC	0.15 ± 0.24	0.1 ± 0.07	0.13 ± 0.05	0.03 ± 0.05
Aminotransferases Activities				
ALT	79.2 ± 11.34	71.00 ± 10.68	79.60 ± 6.73	75.80 ± 11.28
AST	177.00 ± 65.10	132.33 ± 31.74	161.80 ± 45.18	165.21 ± 61.21

detected before the progression of DA cell death, as it is a rapid response to minimize or prevent cell death (Walsh et al., 2011). However, other studies indicate that activation of glial cells and the release of both pro- and anti-inflammatory cytokines may actually enhance 6-OHDA toxicity at the site of injection (Stott and Barker, 2014).

Works focusing on the BBB integrity in the 6-OHDA model have demonstrated that impairment in BBB selective permeability may account for intensification of 6-OHDA toxicity due to increased iron uptake by the SN, which in turn enhances oxidative stress and sustains neuroinflammation (Olmedo-Diaz et al., 2017). On the other hand, the increase in serum pro-inflammatory cytokines observed here and in previous works may result from the efflux of these compounds from the brain site of lesion, or it may be originated by other systemic response to DA denervation. In a previous study, hepatic mitochondria dysfunction was observed in animals receiving intrastriatal 6-OHDA, and this was postulated to occur in response to increased thyroid hormone levels in the circulation, which subsequently caused liver inflammation and enhanced the circulating levels of proinflammatory mediators (Vairetti et al., 2012). In both *in vitro* and *in vivo* studies, CARV has been demonstrated to be a compound with strong antioxidant properties, as well as anti-inflammatory actions (Cui et al., 2015; Guimaraes et al., 2010; Lima Mda et al., 2013; Xiao et al., 2019). In this context, the capacity of CARV to inhibit proinflammatory cytokines in both CSF and serum may be ascribed mainly to its antioxidant and anti-inflammatory properties, which would prevent both DA cell death and BBB disruption.

CARV anti-inflammatory properties had been associated to inhibition of IL-1β and TNF-α release (Gholijani et al., 2016; Li et al., 2016; Lima Mda et al., 2013). Here, we showed that oral administration of CARV/β-CD prevented the increase of proinflammatory cytokines in both CSF and serum of Wistar rats. Previous studies demonstrated the inhibition of NF-kB signaling pathway by CARV (Cui et al., 2015; Li et al., 2016; Xiao et al., 2019). NF-κB plays a central role in the inflammatory process, regulating the expression of proinflammatory mediators, including IL-1β and TNF-α (Tobon-Velasco et al., 2014); besides, as a redox-sensitive transcription factor, NF-kB is modulated by oxidative stress and antioxidants (Bowie and O'Neill, 2000). CARV/β-CD oral administration may inhibit IL-1β and TNF-α release through modulation of the NF-kB signaling pathway, among other mechanisms. The reduction of brain proinflammatory state, consequently, decreases neurotoxicity (Belarbi et al., 2017; Bordt and Polster, 2014).

It has been reported that the main cause of 6-OHDA toxicity is the oxidative damage due to reactive species production derived from 6-OHDA autoxidation (Soto-Otero et al., 2000), microglial activation (Rodriguez-Pallares et al., 2007) and disruption of mitochondrial homeostasis (Glinka et al., 1996). Previous studies have reported protective effects of CARV i.p. administration in hemiparkinsonian model of striatal damage with 6-OHDA (Baluchnejadmojarad et al., 2014; Dati et al., 2017). Dati et al. (2017) demonstrated that CARV attenuates the upregulation of caspase-3 expression induced by 6-OHDA, which indicates a protective effect through inhibition of pro-apoptotic pathways. Other study has shown CARV antioxidant properties as responsible for its protective effect in a 6-OHDA intrastriatal model (Baluchnejadmojarad et al., 2014). Here we demonstrated *in vitro* that CARV prevented the increase of mitochondrial superoxide production induced by 6-OHDA, indicating a positive action of CARV in mitochondrial homeostasis modulation. Overall, our results demonstrated that both antioxidant and anti-inflammatory properties of CARV may be responsible for the protection against 6-OHDA toxicity.

Despite of CARV potential actions, its exploration by pharmaceutical industry is limited by disadvantages in preparation and storage (Guimaraes et al., 2015). Previous studies have reported that complexation of CARV with β-CD improves solubility, stability and oral bioavailability, providing a delivery shuttle to cross biological membranes (Suntres et al., 2015) and improving drug activity (Guimaraes et al., 2015; Santos et al., 2015). Here, an important finding is that DA toxicity in the nigrostriatal axis was significantly inhibited by oral administration of CARV/β-CD, which constitutes a noninvasive and practical route of drug delivery (Jitendra et al., 2011). Oral administration of CARV/β-CD did not induce alterations on blood cell count

when compared with control group. However, CARV/β-CD pretreated animals presented reduced levels of circulating monocytes when received further treatment with 6-OHDA. The response induced by CARV against the damage in the SN may involve the recruitment of monocytes by the brain, thus reducing the circulating levels of this cell type. Our results also indicate that oral administration of CARV/β-CD is not toxic to liver, since no alterations were found in serum AST and ALT activities. Therefore, the complexation of CARV with β-CD improves oral bioavailability without inducing systemic toxicity, according the parameters analyzed here.

5. Conclusion

The oral administration of CARV/β-CD complex shows neuroprotective actions in the rat model of 6-OHDA-induced DA denervation. Oral treatment with CARV/β-CD for 15 days (25 μg/kg/day) prevented the loss of DA neurons induced by 6-OHDA intranigral injection. This effect might be related with CARV anti-inflammatory properties, since CARV/β-CD decreased the release of inflammatory mediators in both CSF and serum. Antioxidant actions of CARV are probably important as well, since CARV reduced mitochondrial superoxide production induced by 6-OHDA in cultured cells. Our results indicate that CARV/β-CD oral administration did not induce systemic toxicity, since no alterations on blood cell count and serum aminotransferases activities were found. Therefore, the present results indicate a potential pharmacological application of CARV/β-CD complex in neuroprotective therapies for PD and associated disorders.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.021.

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References

Baluchnejadmojarad, t., Hassanshahi, J., Roghani, M., mansouri, M., Raoufi, S., 2014. Protective Effect of Carvacrol in 6-hydroxydopamine Hemi-Parkinsonian Rat Model. Shahed University.

Belarbi, K., Cuvelier, E., Destee, A., Gressier, B., Chartier-Harlin, M.C., 2017. NADPH oxidases in Parkinson's disease: a systematic review. Mol. Neurodegener. 12, 84.

Block, M.L., Hong, J.S., 2007. Chronic microglial activation and progressive dopaminergic neurotoxicity. Biochem. Soc. Trans. 35, 1127–1132.

Bordt, E.A., Polster, B.M., 2014. NADPH oxidase- and mitochondria-derived reactive oxygen species in proinflammatory microglial activation: a bipartisan affair? Free Radic. Biol. Med. 76, 34–46.

Bowie, A., O'Neill, L.A., 2000. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem. Pharmacol. 59, 13–23.

Carvey, P.M., Zhao, C.H., Hendey, B., Lum, H., Trachtenberg, J., Desai, B.S., Snyder, J., Zhu, Y.G., Ling, Z.D., 2005. 6-Hydroxydopamine-induced alterations in blood-brain barrier permeability. Eur. J. Neurosci. 22, 1158–1168.

Costantini, E., D'Angelo, C., Reale, M., 2018. The role of immunosenescence in neurodegenerative diseases. Mediat. Inflamm. 2018, 6039171.

Cui, Z., Xie, Z., Wang, B., Zhong, Z., Chen, X., Sun, Y., Sun, Q., Yang, G., Bian, L., 2015. Carvacrol protects neuroblastoma SH-SY5Y cells against Fe2+-induced apoptosis by suppressing activation of MAPK/JNK-NF-κB signaling pathway. Acta Pharmacol. Sin. 36, 1426–1436.

Dati, L.M., Ulrich, H., Real, C.C., Feng, Z.P., Sun, H.S., Britto, L.R., 2017. Carvacrol promotes neuroprotection in the mouse hemiparkinsonian model. Neuroscience 356, 176–181.

Dauer, W., Przedborski, S., 2003. Parkinson's disease: mechanisms and models. Neuron 39, 889–909.

Deng, W., Lu, H., Teng, J., 2013. Carvacrol attenuates diabetes-associated cognitive deficits in rats. J. Mol. Neurosci. 51, 813–819.

Drechsel, D.A., Patel, M., 2008. Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic. Biol. Med. 44, 1873–1886.

Gasparotto, J., Ribeiro, C.T., Bortolin, R.C., Somensi, N., Rabelo, T.K., Kunzler, A., Souza, N.C., Pasquali, M.A.B., Moreira, J.C.F., Gelain, D.P., 2017. Targeted inhibition of RAGE in substantia nigra of rats blocks 6-OHDA-induced dopaminergic denervation. Sci. Rep. 7, 8795.

Gholijani, N., Gharagozloo, M., Farjadian, S., Amirghofran, Z., 2016. Modulatory effects of thymol and carvacrol on inflammatory transcription factors in lipopolysaccharide-treated macrophages. J. Immunotoxicol. 13, 157–164.

Glinka, Y., Tipton, K.F., Youdim, M.B., 1996. Nature of inhibition of mitochondrial respiratory complex I by 6-Hydroxydopamine. J. Neurochem. 66, 2004–2010.

Guimaraes, A.G., Oliveira, G.F., Melo, M.S., Cavalcanti, S.C., Antoniolli, A.R., Bonjardim, L.R., Silva, F.A., Santos, J.P., Rocha, R.F., Moreira, J.C., Araujo, A.A., Gelain, D.P., Quintans-Junior, L.J., 2010. Bioassay-guided evaluation of antioxidant and antinociceptive activities of carvacrol. Basic Clin. Pharmacol. Toxicol. 107, 949–957.

Guimaraes, A.G., Oliveira, M.A., Alves Rdos, S., Menezes Pdos, P., Serafini, M.R., Araujo, A.A., Bezerra, D.P., Quintans Junior, L.J., 2015. Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with beta-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. Chem. Biol. Interact. 227, 69–76.

Guimaraes, A.G., Xavier, M.A., de Santana, M.T., Camargo, E.A., Santos, C.A., Brito, F.A., Barreto, E.O., Cavalcanti, S.C., Antoniolli, A.R., Oliveira, R.C., Quintans-Junior, L.J., 2012. Carvacrol attenuates mechanical hypernociception and inflammatory response. Naunyn-Schmiedeberg's Arch. Pharmacol. 385, 253–263.

Huang, X.J., Choi, Y.K., Im, H.S., Yarimaga, O., Yoon, E., Kim, H.S., 2006. Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques. Sensors 6, 756–782.

Jitendra, Sharma, P.K., Bansal, S., Banik, A., 2011. Noninvasive routes of proteins and peptides drug delivery. Indian J. Pharm. Sci. 73, 367–375.

McKenzie, Jordan A., Caitlin, L.J.S., Pointer, B., Lowry, Jessica R., bajwa, Ekta, Lee, Carolyn W., klegeris, 2018. Neuroinflammation as a Common Mechanism Associated with the Modifiable Risk Factors for Alzheimer's and Parkinson's Diseases. Curr Aging Sci 10, 158–176.

Kurkov, S.V., Loftsson, T., 2013. Cyclodextrins. Int. J. Pharm. 453, 167–180.

Li, Z., Hua, C., Pan, X., Fu, X., Wu, W., 2016. Carvacrol exerts neuroprotective effects via suppression of the inflammatory response in middle cerebral artery occlusion rats. Inflammation 39, 1566–1572.

Lima Mda, S., Quintans-Junior, L.J., de Santana, W.A., Martins Kaneto, C., Pereira Soares, M.B., Villarreal, C.F., 2013. Anti-inflammatory effects of carvacrol: evidence for a key role of interleukin-10. Eur. J. Pharmacol. 699, 112–117.

Lima, M.M., Martins, E.F., Delattre, A.M., Proenca, M.B., Mori, M.A., Carabelli, B., Ferraz, A.C., 2012. Motor and non-motor features of Parkinson's disease - a review of clinical and experimental studies. CNS Neurol. Disord. - Drug Targets 11, 439–449.

Lins, L., Souza, M.F., Bispo, J.M.M., Gois, A.M., Melo, T.C.S., Andrade, R.A.S., Quintans-Junior, L.J., Ribeiro, A.M., Silva, R.H., Santos, J.R., Marchioro, M., 2018. Carvacrol prevents impairments in motor and neurochemical parameters in a model of progressive parkinsonism induced by reserpine. Brain Res. Bull. 139, 9–15.

Marques, H.M.C., 2010. A review on cyclodextrin encapsulation of essential oils and volatiles. Flavour Fragrance J. 25, 313–326.

Mechan, A.O., Fowler, A., Seifert, N., Rieger, H., Wohrle, T., Etheve, S., Wyss, A., Schuler, G., Colletto, B., Kilpert, C., Aston, J., Elliott, J.M., Goralczyk, R., Mohajeri, M.H., 2011. Monoamine reuptake inhibition and mood-enhancing potential of a specified oregano extract. Br. J. Nutr. 105, 1150–1163.

Melo, F.H., Moura, B.A., de Sousa, D.P., de Vasconcelos, S.M., Macedo, D.S., Fonteles, M.M., Viana, G.S., de Sousa, F.C., 2011. Antidepressant-like effect of carvacrol (5-Isopropyl-2-methylphenol) in mice: involvement of dopaminergic system. Fundam. Clin. Pharmacol. 25, 362–367.

Melo, F.H., Venancio, E.T., de Sousa, D.P., de Franca Fonteles, M.M., de Vasconcelos, S.M., Viana, G.S., de Sousa, F.C., 2010. Anxiolytic-like effect of Carvacrol (5-isopropyl-2-methylphenol) in mice: involvement with GABAergic transmission. Fundam. Clin. Pharmacol. 24, 437–443.

National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, A, 2011. The National Academies Collection: Reports Funded by National Institutes of Health, Guide for the Care and Use of Laboratory Animals. National Academies Press (US)National Academy of Sciences., Washington (DC).

Olmedo-Diaz, S., Estevez-Silva, H., Oradd, G., Af Bjerken, S., Marcellino, D., Virel, A., 2017. An altered blood-brain barrier contributes to brain iron accumulation and neuroinflammation in the 6-OHDA rat model of Parkinson's disease. Neuroscience 362, 141–151.

Oz, M., Lozon, Y., Sultan, A., Yang, K.H., Galadari, S., 2015. Effects of monoterpenes on ion channels of excitable cells. Pharmacol. Ther. 152, 83–97.

Paxinos, G., Watson, C., 2005. The Rat Brain in Stereotaxic Coordinates, 5 ed. Elsevier Academic Press Amsterdam, Boston.

Poewe, W., Seppi, K., Tanner, C.M., Halliday, G.M., Brundin, P., Volkmann, J., Schrag, A.-E., Lang, A.E., 2017. Parkinson disease. Nature Reviews Disease Primers 3, 17013.

Qin, X.Y., Zhang, S.P., Cao, C., Loh, Y.P., Cheng, Y., 2016. Aberrations in peripheral inflammatory cytokine levels in Parkinson disease: a systematic review and meta-analysis. JAMA Neurol 73, 1316–1324.

Rodriguez-Pallares, J., Parga, J.A., Munoz, A., Rey, P., Guerra, M.J., Labandeira-Garcia,

J.L., 2007. Mechanism of 6-hydroxydopamine neurotoxicity: the role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced degeneration of dopaminergic neurons. J. Neurochem. 103, 145–156.
Santos, E.H., Kamimura, J.A., Hill, L.E., Gomes, C.L., 2015. Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 60, 583–592.
Silva, J.C., Almeida, J., Quintans, J.S.S., Gopalsamy, R.G., Shanmugam, S., Serafini, M.R., Oliveira, M.R.C., Silva, B.A.F., Martins, A., Castro, F.F., Menezes, I.R.A., Coutinho, H.D.M., Oliveira, R.C.M., Thangaraj, P., Araujo, A.A.S., Quintans-Junior, L.J., 2016. Enhancement of orofacial antinociceptive effect of carvacrol, a monoterpene present in oregano and thyme oils, by beta-cyclodextrin inclusion complex in mice. Biomed. Pharmacother. 84, 454–461.
Soto-Otero, R., Mendez-Alvarez, E., Hermida-Ameijeiras, A., Munoz-Patino, A.M., Labandeira-Garcia, J.L., 2000. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J. Neurochem. 74, 1605–1612.
Stott, S.R., Barker, R.A., 2014. Time course of dopamine neuron loss and glial response in the 6-OHDA striatal mouse model of Parkinson's disease. Eur. J. Neurosci. 39, 1042–1056.
Suntres, Z.E., Coccimiglio, J., Alipour, M., 2015. The bioactivity and toxicological actions of carvacrol. Crit. Rev. Food Sci. Nutr. 55, 304–318.
Tieu, K., 2011. A guide to neurotoxic animal models of Parkinson's disease. Cold Spring Harb Perspect Med 1, a009316.
Tobon-Velasco, J.C., Cuevas, E., Torres-Ramos, M.A., 2014. Receptor for AGEs (RAGE) as mediator of NF-kB pathway activation in neuroinflammation and oxidative stress. CNS Neurol. Disord. - Drug Targets 13, 1615–1626.
Trabace, L., Zotti, M., Morgese, M.G., Tucci, P., Colaianna, M., Schiavone, S., Avato, P., Cuomo, V., 2011. Estrous cycle affects the neurochemical and neurobehavioral profile of carvacrol-treated female rats. Toxicol. Appl. Pharmacol. 255, 169–175.
Vairetti, M., Ferrigno, A., Rizzo, V., Ambrosi, G., Bianchi, A., Richelmi, P., Blandini, F., Armentero, M.T., 2012. Impaired hepatic function and central dopaminergic denervation in a rodent model of Parkinson's disease: a self-perpetuating crosstalk? Biochim. Biophys. Acta 1822, 176–184.
Walsh, S., Finn, D.P., Dowd, E., 2011. Time-course of nigrostriatal neurodegeneration and neuroinflammation in the 6-hydroxydopamine-induced axonal and terminal lesion models of Parkinson's disease in the rat. Neuroscience 175, 251–261.
Wang, P., Luo, Q., Qiao, H., Ding, H., Cao, Y., Yu, J., Liu, R., Zhang, Q., Zhu, H., Qu, L., 2017. The neuroprotective effects of carvacrol on ethanol-induced hippocampal neurons impairment via the antioxidative and antiapoptotic pathways. Oxid Med Cell Longev 2017, 4079425.
Wang, Q., Liu, Y., Zhou, J., 2015. Neuroinflammation in Parkinson's disease and its potential as therapeutic target. Transl. Neurodegener. 4.
Xiao, Y., Li, B., Liu, J., Ma, X., 2019. Carvacrol ameliorates inflammatory response in interleukin 1β-stimulated human chondrocytes. Mol. Med. Rep. 17, 3987–3992.
Yu, H., Zhang, Z.L., Chen, J., Pei, A., Hua, F., Qian, X., He, J., Liu, C.F., Xu, X., 2012. Carvacrol, a food-additive, provides neuroprotection on focal cerebral ischemia/reperfusion injury in mice. PLoS One 7 e33584.
Zotti, M., Colaianna, M., Morgese, M., Tucci, P., Schiavone, S., Avato, P., Trabace, L., 2013. Carvacrol: from ancient flavoring to neuromodulatory agent. Molecules 18, 6161–6172.