UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE BIOLOGIA MOLECULAR E BIOTECNOLOGIA

TRABALHO DE CONCLUSÃO EM BIOTECNOLOGIA

Efeitos da administração oral do complexo carvacrol /beta-ciclodextrina na denervação dopaminérgica induzida por 6-hidroxidopamina em ratos Wistar.

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Porto Alegre, Rio Grande do Sul

2018

Sumário

Resumo 3
Abstract 4
Introdução Geral 5
Resultados 7
Discussão Geral 28
Conclusão 31
Referências 32

Resumo

O carvacrol (CARV) apresenta efeitos farmacológicos de interesse, como atividades anti-inflamatórias e antioxidantes. No entanto, a sua aplicação é limitada por desvantagens nos processos de preparação e armazenamento. A complexação de monoterpenos por β-ciclodextrina (β-CD) proporciona benefícios na estabilidade, solubilidade e biodisponibilidade oral, sendo capaz de melhorar a atividade farmacológica do CARV. Neste trabalho, foi analisado o efeito protetor do tratamento oral com complexo CARV/β-CD (25 μg/kg) contra a denervação dopaminérgica induzida por injeção intranigral unilateral de 6-hidroxidopamina (6-OHDA - 10 μg por rato), a fim de avaliar uma possível aplicação no tratamento da doença de Parkinson. O pré-tratamento com complexo CARV/β-CD impediu a perda de neurônios dopaminérgicos e os consequentes déficits locomotores induzidos por 6-OHDA. Além disso, os animais que receberam CARV/β-CD apresentaram diminuição na ativação da microglia na substância negra, bem como nos níveis de L-1β no liquido cefalorraquidiano e soro. Portanto, nossos resultados sugerem que a administração oral de CARV/β-CD exerce um efeito protetor contra a denervação dopaminérgica e a neuroinflamação induzida por 6-OHDA.

Abstract

Carvacrol (CARV) presents pharmacological effects of interest, such as anti-inflammatory and antioxidant activities. However, their application is limited by disadvantages in preparation and storage process. The complexation of monoterpenes with β-cyclodextrin (β-CD) provides benefits in stability, solubility and oral bioavailability, being capable of improving the CARV pharmacological activity. Here, the protective effect of oral treatment with CARV/β-CD complex (25 µg/kg) against dopaminergic denervation induced by a unilateral intranigral injection of 6-hydroxydopamine (6-OHDA - 10 µg per rat) was analyzed, in order to evaluate a putative application for Parkinson's disease therapy development. The pretreatment with CARV/β-CD complex prevented the loss of dopaminergic neurons and consequent locomotor deficits induced by 6-OHDA. Besides, animals receiving CARV/β-CD had decreased microglia activation and IL-1β returned to control levels in CSF and serum. Therefore, our results suggest that oral administration of CARV/β-CD exerts a protective effect against dopaminergic denervation and neuroinflammation induced by 6-OHDA.

Introdução Geral

Carvacrol (CARV) é um monoterpeno fenólico encontrado em muitas plantas aromáticas, incluindo o orégano e o tomilho (1). O CARV apresenta propriedades com potencial interesse farmacológico, como atividades anti-inflamatórias, antioxidantes, analgésicas e antimicrobianas (1-3). Porém, características como instabilidade, volatilidade e baixa solubilidade em água são desvantagens nos processos de preparação e armazenamento limitando a aplicação do CARV na indústria farmacêutica (2-4).

Ciclodextrinas (CDs) são oligossacarídeos cíclicos amplamente utilizados em produtos e inovações tecnológicas, sendo um dos agentes complexantes mais comumente utilizados na indústria farmacêutica. CDs são compostos dietéticos solúveis não tóxicos que melhoram a solubilidade, estabilidade, biodisponibilidade oral e atividade farmacológica de moléculas bioativas (4-5). Trabalhos prévios demonstram que a complexação de CARV com β-CD melhora sua eficiência terapêutica (2,3). Portanto, o encapsulamento de CARV por β-CD (CARV/β-CD) representa uma alternativa com valor biotecnológico para o desenvolvimento de novos medicamentos.

A Doença de Parkinson (PD) é uma desordem neurodegenerativa progressiva caracterizada por déficits locomotores, resultantes de dano progressivo na via dopaminérgica nigrostriatal (6, 7). A neuroinflamação é um dos principais contribuintes para a perda de neurônios dopaminérgicos (8). Na patogênese da PD é relatada uma ativação crônica da microglia (9), a qual resulta em um estado pró-inflamatório induzindo neurotoxicidade (10).

A injeção intranigral de 6-hidroxidopamina (6-OHDA) tem sido amplamente utilizada como modelo animal de PD, uma vez que mimetiza os efeitos causados pela perda de neurônios dopaminérgicos que se projetam da substância negra (SN) para o estriado (11). No presente trabalho, investigamos os possíveis efeitos neuroprotetores da administração oral de CARV/β-CD contra a denervação dopaminérgica induzida por injeção intranigral de 6-OHDA. A administração oral de CARV/β-CD reduziu a perda de neurônios dopaminérgicos, déficits locomotores e parâmetros de neuroinflamação induzidos por 6-OHDA. Portanto, a ingestão oral de CARV/β-CD exibe atividade neuroprotetora no sistema dopaminérgico, indicando uma potencial aplicação farmacológica na PD.

Resultados

Os resultados deste trabalho são apresentados no formato de artigo científico, intitulado: **Oral administration of carvacrol/β-cyclodextrin complex protects against dopaminergic toxicity and motor deficit induced by 6-hydroxydopamine in Wistar rats.** Este foi redigido nos formatos do periódico Neurochemistry International.

Oral administration of carvacrol/β-cyclodextrin complex protects against dopaminergic toxicity and motor deficit induced by 6-hydroxydopamine in Wistar rats.

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Abstract

Carvacrol (CARV) presents pharmacological effects of interest, such as anti-inflammatory and antioxidant activities. However, their application is limited by disadvantages in preparation and storage process. The complexation of monoterpenes with β-cyclodextrin (β-CD) provides benefits in stability, solubility and oral bioavailability, being capable of improving the CARV pharmacological activity. Here, the protective effect of oral treatment with CARV/β-CD complex (25 µg/kg) against dopaminergic denervation induced by a unilateral intranigral injection of 6-hydroxydopamine (6-OHDA - 10 µg per rat) was analyzed, in order to evaluate a putative application for Parkinson's disease therapy development. The pretreatment with CARV/β-CD complex prevented the loss of dopaminergic neurons and consequent locomotor deficits induced by 6-OHDA. Besides, animals receiving CARV/β-CD had decreased microglia activation and IL-1β returned to control levels in CSF and serum. Therefore, our results suggest that oral administration of CARV/β-CD exerts a protective effect against dopaminergic denervation and neuroinflammation induced by 6-OHDA.

Keywords: Carvacrol, 6-hydroxydopamine, dopaminergic denervation, neuroinflammation.

1. Introduction

Carvacrol (CARV) is a phenolic monoterpene found in many aromatic plants, including and oregano and thyme (1). CARV presents properties of potential pharmacological interest, such as anti-inflammatory, antioxidant, analgesic and antimicrobial activities (1-3). However, disadvantages in preparation and storage process, due to CARV instability, volatility and low water solubility, can limit the application on pharmaceutical industry (2-4).

Cyclodextrins (CDs) are cyclic oligosaccharides widely used in many products and technologies, being one of the complexing agents most commonly used by pharmaceutical industry. CDs are non-toxic soluble bioavailable dietary compounds, which improves solubility, stability, oral bioavailability and pharmacological activity of bioactive molecules (4, 5). Previous works demonstrated the efficacy of the complexation with β-CD on the improvement of CARV therapeutic effects (2, 3). Therefore, the encapsulation of CARV in β-CD (CARV/β-CD) represents an alternative with biotechnological value to development of new drugs.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by locomotor deficits, which results from progressive damage of the nigrostriatal dopaminergic pathway (6, 7). One of the major contributors to the loss of dopaminergic neurons is neuroinflammation (8). In the pathogenesis of PD a chronic microglia activation is reported (9), which results in a proinflammatory state inducing neurotoxicity (10).

Intranigral injection of 6-hydoxydopamine (6-OHDA) has been extensively used to mimic the effects caused by the loss of dopaminergic neurons from the substantia nigra (SN) projecting into the striatum observed in PD (11). In the present work, we investigated potential neuroprotective effects of CARV/β-CD oral administration against dopaminergic denervation induced by 6-OHDA injected in the SN. The oral treatment with CARV/β-CD prevented the loss of dopaminergic neurons, locomotor deficits and neuroinflammation parameters induced by 6-OHDA. Therefore, oral intake of CARV/β-CD shows a neuroprotective activity in the dopaminergic system, which indicates a potential pharmacological application in PD.

2. Methods

2.1 Ethics Statement

All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health (12) and Behavior recommendations 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 twelve-hour 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 administration.

2.3 CARV/β-CD and 6-OHDA preparation

The CARV/β-CD complex was prepared as previously described (2). Each rat received 25 µg/kg of CARV/β-CD by oral administration (p.o.) (2). 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 heat and light exposure. Each rat received 10 µg of 6-OHDA via intranigral injection (6).

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) (p.o.) for 15 days. On the 15th day, an intranigral injection of saline was administered.

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

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

Behavior tests were performed fourteen days after the induction of the SN lesion. In the following day (15 days after lesion 6-OHDA injection) all the animals were anesthetized with an intraperitoneal injection (i.p.) of ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood was collected from cardiac puncture and cerebrospinal fluid (CSF) was collected from *cisterna magna* with

a syringe (5 animals per group) for analysis of cytokines. The remaining 3 animals per group were perfused via the vascular system for immunofluorescence microscopy assessment of brain parts.

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 securing via 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 the left 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 (13), 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 Behavior tests

2.6.1 Rotarod

The rotatod test was used to evaluate the motor system. The animals were acclimated to the apparatus with three prior training sessions. The protocol was performed with constant 21 rpm. The time on rotarod during experimental sessions was measured and the cut-off time was 240 s (6). The mean of 3 attempts were used for the statistical analyses.

2.6.2 Apomorphine-induced rotation test

Apomorphine-induced rotation test was used to study the dopaminergic receptor sensitivity of the lesioned SN (11). Apomorphine (Sigma-Aldrich®; MO, USA) was administrated subcutaneously, which was prepared 0.1 mg/kg in ascorbic acid 0.2 mg/mL dissolved in sterile saline. After the injection, animals were acclimated for 5 min in cylinder rotameter (400 mm diameter) before the recording of rotations began. Full body ipsilateral and contralateral side rotations were counted. The data were expressed as contralateral-ipsilateral average turns rotations per min (RPM).

2.7 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 ºC. Using a cryostat (Jung Histoslide 2000R; Leica; Heidelberg, Germany) at -20 ºC, 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 nonspecific binding, the sections were incubated with 3% albumin for 1h at room temperature (21 ± 3 °C). Then, the tissue slices were incubated with primary antibodies for 48 h at 4 ºC. The details of the antibody source and dilutions are as follows: anti-GFAP (1:500; G6171) was from Sigma-Aldrich® (MO, USA); anti-Iba-1 (1:500; 019-19741) was from Wako Chemicals USA, Inc. (VA, USA); 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 or 555 and anti-mouse Alexa 488 or 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).

2.8 Enzyme-linked immunosorbent assay (ELISA)

IL-1β (RAB0272-1KT) was quantified with commercial kits from Sigma-Aldrich® (MO, USA) in CSF and serum, following the manufacturer's protocol.

2.9 Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, USA). Data were evaluated by one-way ANOVA analysis and followed by Tukey's Multiple Comparison *post-hoc* test. Differences were considered significant when $p<0.05$.

3. Results

To analyze the effect of CARV/β-CD pretreatment in the dopaminergic cell loss induced by 6-OHDA we conducted co-immunostaining of tyrosine hydroxylase (TH - dopaminergic neuron marker) with neuronal nuclear antigen (NeuN - neuronal marker). The content of NeuN was not different between all groups (Fig. 1). As expected, TH content decreased in ipsilateral SN of animals that received 6-OHDA, whereas contralateral SN was not affected (Fig. S1). Animals treated with CARV/β-CD did not present differences in TH content between ipsilateral and contralateral side (Fig S1). As shown on Fig. 1, oral administration of CARV/β-CD prevented the loss of dopaminergic neurons induced by 6-OHDA.

Locomotor deficit is a characteristic of dopaminergic denervation that can be assessed in behavioral tests (6). General motor performance was assessed using the rotarod test. Animals treated with 6-OHDA presented a decrease in the time on rotarod, whereas animals pretreated with CARV/β-CD did not present significant differences to control group (Table 1). The apomorphine-induced rotation test was performed to evaluate the dopaminergic response in

the nigrostriatal neurons. Animals receiving 6-OHDA had continuous contralateral rotations, whereas all other groups did not show any significant spontaneous rotations, including the group of animals pretreated with CARV/β-CD that received 6-OHDA (Table 1).

To analyze the effect of CARV/β-CD pretreatment over neuroinflammation induced by 6-OHDA we conducted co-immunostaining of Iba-1 (microglial marker) and glial fibrillary acidic protein (GFAP - astrocyte marker). Animals treated with 6-OHDA presented an increased number of microglia and astrocytes (Fig. 2C). The prior treatment with CARV/β-CD reduced microglia activation, whereas did not affect astrocytes activation (Fig. 2D). We also measured IL-1β levels in CSF and serum (Fig. 3A and B). The CARV/β-CD pretreatment prevented the 6-OHDA-induced increase of IL-1β in both CSF and serum.

4. Discussion

As a model of PD, 6-OHDA is used to lesion the nigrostriatal dopaminergic system by inducing the loss of TH+ neurons (11). To assess the neurodegeneration induced by 6-OHDA we conducted co-immunostaining of TH and NeuN. The administration of 6-OHDA reduced the number of TH+ cells and did not affect NeuN staining (Fig. 1C), confirming that 6-OHDA toxicity was selective to dopaminergic neurons. The dopaminergic denervation in SN resulted in locomotor deficit, as confirmed by rotarod test and apomorphine-induced rotations (Table 1). Animals which received the CARV/β-CD treatment before 6-OHDA administration did not present significant differences to control group in locomotor deficit (Table 1) and did not have loss of TH+ neurons (Fig.

1D). Overall these results demonstrate that oral administration of CARV/β-CD prevented the dopaminergic denervation and the consequent locomotor deficit induced by an intranigral injection of 6-OHDA. Previous works had reported that the CARV intraperitoneal administration exerted protective effects in hemiparkinsonian model of striatal damage with 6-OHDA (14, 15). The present results indicate that oral administration of encapsulated CARV is effective against dopaminergic toxicity and represents a viable alternative to develop a compound with a practical application for PD therapy.

One of the major contributors to the loss of dopaminergic neurons in PD is neuroinflammation. In this context, microglia is the major cell type responsible for inflammatory responses (8). The administration of 6-OHDA stimulates microglia activation, demonstrated by the immuostaining pattern of Iba-1 (Fig. 2C), whereas CARV/β-CD pretreatment reduced this effect (Fig. 2D). Long-term overactivation of microglia upregulates the expression of proinflammatory cytokines, contributing to dopaminergic neuron loss. Astrocytes also play a vital role in the neuroinflammatory processes of PD (8). Astrocytes activation was increased by 6-OHDA administration (Fig. 2C), but it was not affected by pretreatment with CARV/β-CD (Fig. 2D). The release of proinflammatory mediators can be amplified by synergic activation of astrocytes and microglia, causing an uncontrolled neuroinflammation that contributes to the loss of dopaminergic neurons (8). However, astrocytes play a dual role in central nervous system (CNS) inflammatory diseases. Depending on the degree of inflammation and the duration of stimuli, astrocytes are also capable to exert an inhibitory effect on microglia, limiting CNS inflammation (16). Therefore, these results might indicate that CARV is able to modulate microglia activation

through astrocytes, contributing to inhibition of microglia responses and limiting neuroinflammation.

CARV anti-inflammatory proprieties had been related to inhibition of IL-1β release (17). IL-1β is a proinflammatory cytokine with an important role during inflammatory response, inducing the production of other inflammatory mediators (17). Our results showed that oral administration of CARV/β-CD prevented the 6-OHDA-induced increase of this cytokine in CSF and serum (Fig. 3A and B). These results demonstrate that oral administration of CARV/β-CD is able to decrease the release of inflammatory mediators, reducing the proinflammatory state induced by 6-OHDA administration. The reduction of brain proinflammatory state decreases neurotoxicity, which might explain the CARV/β-CD protective effect against dopaminergic denervation.

5. Conclusion

The oral administration of CARV/β-CD complex shows a neuroprotective activity in the rat model of 6-OHDA-induced PD. The prior treatment with CARV/β-CD prevented the loss of dopaminergic neurons and consequent locomotor deficits. This protection against dopaminergic denervation might be related with CARV anti-inflammatory proprieties. CARV/β-CD pretreatment limited the neuroinflammation through astrocyte modulation of microglia responses, reducing IL-1β to control levels in CSF and serum. The present results indicate the potential pharmacological application of CARV/β-CD complex in PD, being necessary future studies to evaluate possible effects of CARV/β-CD on long-term dopaminergic degeneration.

6. Conflicts of interest

The authors declare no conflicts of interest.

7. Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) #443514/2014-3 and #401260/2014-3, FAPERGS #16/2551-0000499-4 and #17/2551-0000984-3, Propesq-UFRGS and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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9. Tables and figures

	Control	CARV-βCD	6-OHDA	CARV-βCD + 6-OHDA
Rotarod test	117,67 ± 39,39 **a**	74,27 ± 34,54 **ab**	35,67 ± 42,96 **b**	91,34 ± 34,55 **ab**
Apomorphine-induced rotation test	0 ± 0 **a**	0 ± 0 **a**	2,8 ± 1,72 **b**	0 ± 0 **a**

Table 1: CARV/β-CD pretreatment protected rats from 6-OHDA-induced motor deficits. Locomotor abilities of rats injected with 6-OHDA were examined 14 days after the injection. Rotarod test – the time on rotarod during experimental sessions was measured and the cut-off time was 240 s. Apomorphine-induced rotation test - full body ipsilateral and contralateral side rotations were counted and the data were expressed as contralateral-ipsilateral average turns rotations per min. Values represent mean ± SD from eight rats (n=8) per group. One-way analysis of variance and Tukey's Multiple Comparison *post-hoc* test were applied to all data, means followed by the same letters do not present significant differences.

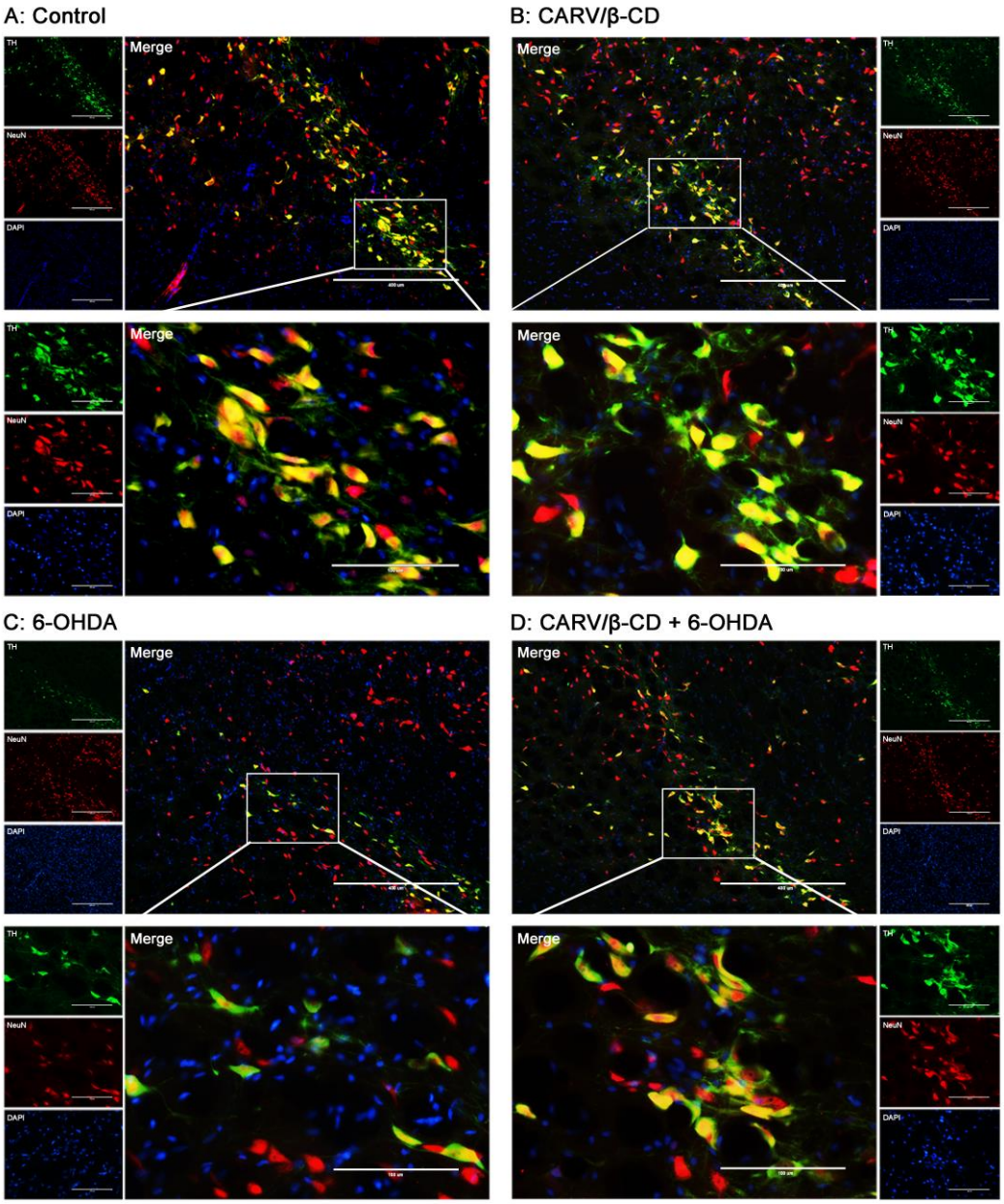

Figure 1: CARV/β-CD pretreatment protected rats from 6-OHDA-induced loss of dopaminergic neurons. Representative immunofluorescence images of SN co-immunostained for TH (green), NeuN (red) and DAPI (blue). The ipsilateral sides are shown. The microscopy images were taken with 400 μm of magnification and the squares represents the location of the approximation of 100 μm. (**A**) Control group (**B**) CARV/β-CD group (**C**) 6-OHDA group (**D**) CARV/β-CD + 6-OHDA group.

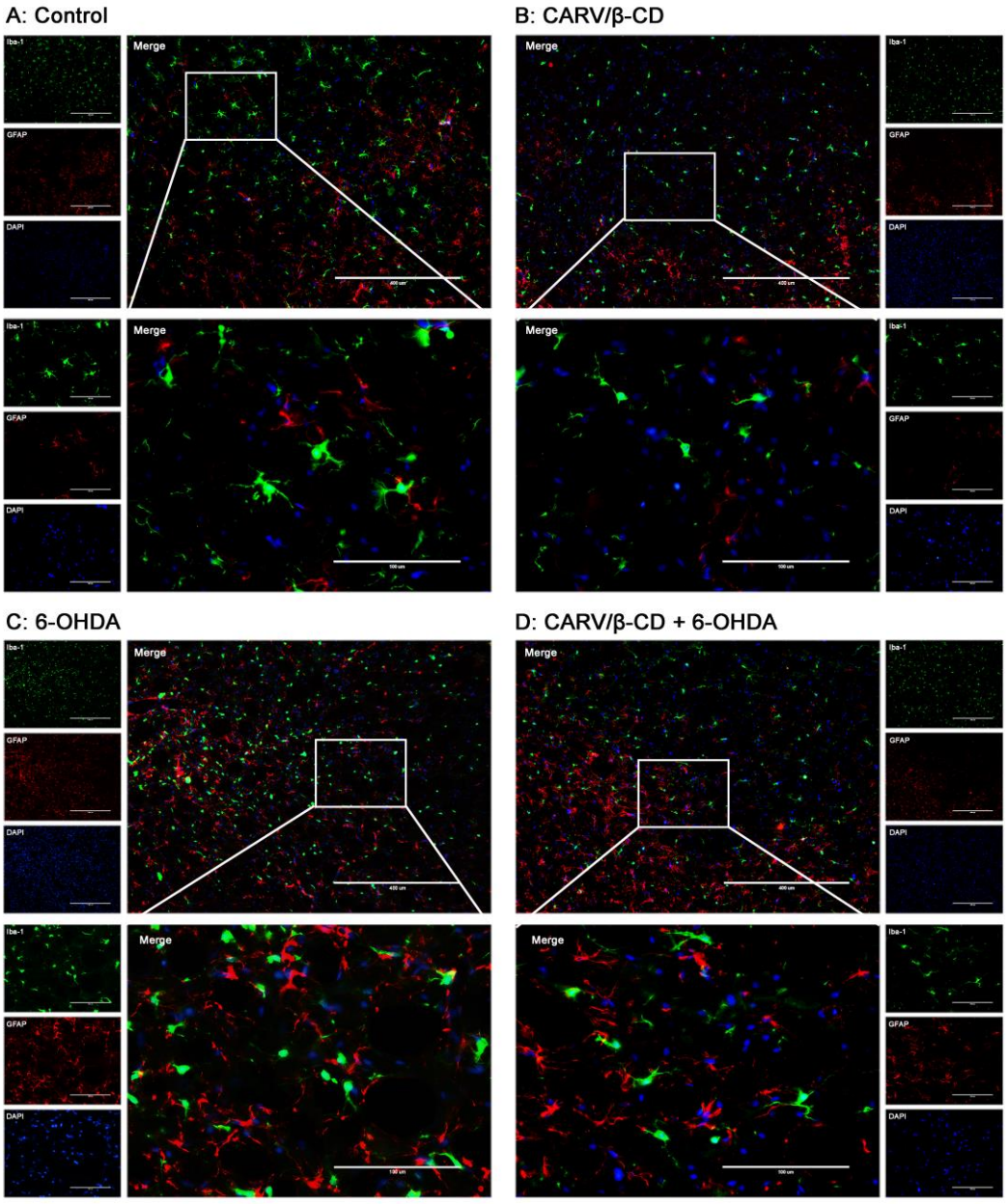

Figure 2: CARV/β-CD pretreatment protected rats from 6-OHDA-induced neuronal inflammation. Representative immunofluorescence images of SN co-immunostained for IBA-1(green), GFAP (red) and DAPI (blue). The ipsilateral sides are shown. The microscopy images were taken with 400 μm of magnification and the squares represents the location of the approximation of 100 μm. (**A**) Control group (**B**) CARV/β-CD group (**C**) 6-OHDA group (**D**) CARV/β-CD + 6-OHDA group.

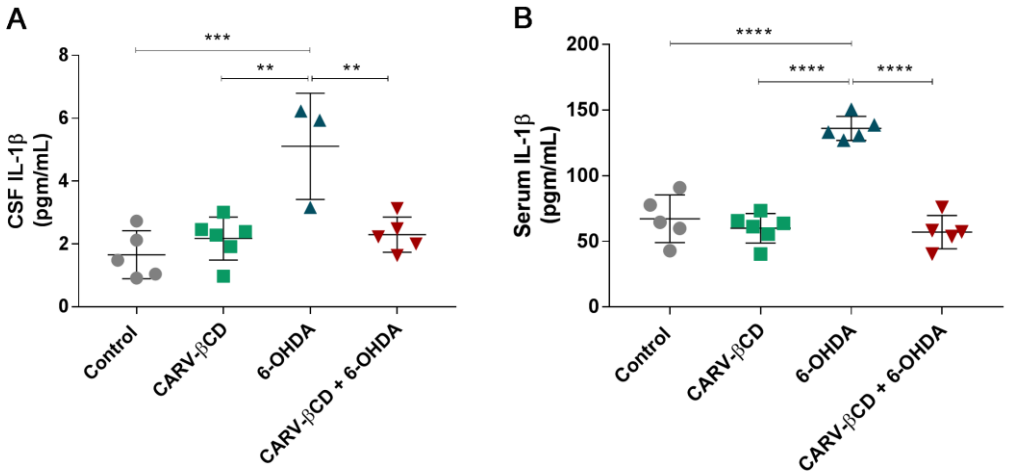

Figure 3: CARV/β-CD pretreatment protected rats from 6-OHDA-induced release of IL-1β. CSF and serum were analyzed by ELISA assay 15 days after 6-OHDA administration. (**A**) Cerebrospinal fluid was analyzed for IL-1β. (**B**) Serum samples were analyzed for IL-1β. IL-1β levels are expressed in pg/mL.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

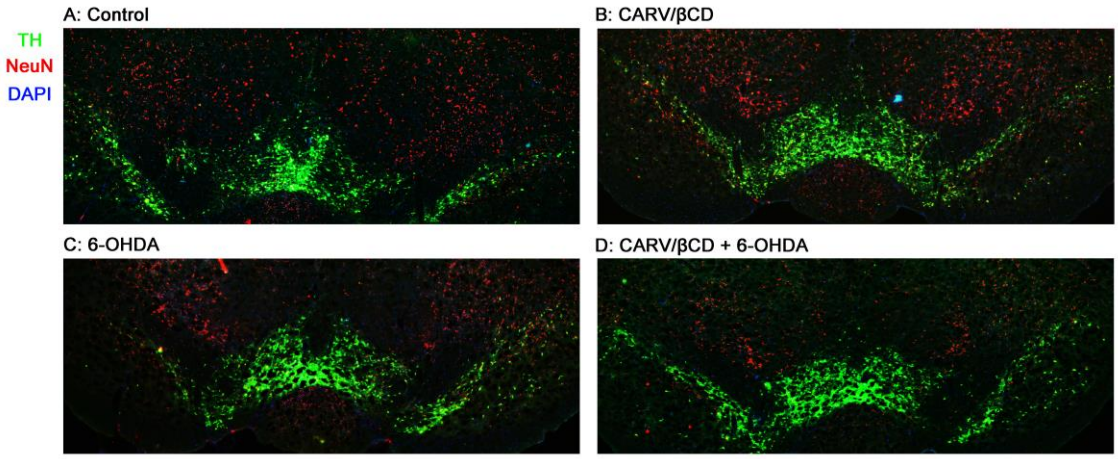

Figure S1: 6-OHDA administration only affect ipisilataral side and CARV/β-CD pretreatment protected rats from 6-OHDA-induced loss of dopaminergic neurons. Representative immunofluorescence images of SN co-immunostained for TH (green), NeuN (red) and DAPI (blue). Both ipsilateral and contralateal sides are shown. The microscopy images were taken with 1000 μm of magnification. (**A**) Control group (**B**) CARV/β-CD group (**C**) 6-OHDA group (**D**) CARV/β-CD + 6-OHDA group.

Discussão Geral

Como modelo de PD, 6-OHDA é utilizada para lesionar o sistema dopaminérgico nigrostriatal induzindo a perda de neurônios TH + (11). Para avaliar a neurodegeneração induzida por 6-OHDA, realizamos co-imunocoloração de TH e NeuN. A administração de 6-OHDA reduziu o número de células TH + e não afetou o conteúdo de NeuN (Fig. 1C), confirmando que a toxicidade 6-OHDA foi seletiva para os neurônios dopaminérgicos. A denervação dopaminérgica na SN resultou em déficit locomotor, conforme confirmado pelo teste rotarod e pelo teste rotacional induzido por apomorfina (Tabela 1). Os animais que receberam o tratamento com CARV/β-CD antes da administração de 6-OHDA não apresentaram diferenças locomotoras significativas em relação ao grupo controle (Tabela 1) e também não apresentaram perda de neurônios TH + (Fig. 1D). Em geral, estes resultados demonstram que a administração oral de CARV/β-CD impede a denervação dopaminérgica e o consequente déficit locomotor induzido por injeção intranigal de 6-OHDA. Trabalhos anteriores relataram efeitos protetores da administração i.p. de CARV sorbre dano induzido por injeção intraestriatal de 6-OHDA (12, 13).

Um dos principais contribuintes para a perda de neurônios dopaminérgicos na PD é a neuroinflamação. Neste contexto, a microglia é o principal tipo celular responsável pelas respostas inflamatórias (8). A administração de 6-OHDA estimula a ativação da microglia, demonstrada pelo conteúdo de Iba-1 (Fig. 2C), enquanto que o pré-tratamento com CARV/β-CD reduz esse efeito (Fig. 2D). A ativação prolongada da microglia aumenta a

expressão de citocinas pró-inflamatórias, contribuindo para a perda de neurônios dopaminérgicos. Os astrócitos também desempenham um papel vital nos processos neuroinflamatórios da PD (8). A ativação de astrócitos foi aumentada pela administração de 6-OHDA (Fig. 2C), mas não foi afetada pelo pré-tratamento com CARV/β-CD (Fig. 2D). A liberação de mediadores pró-inflamatórios pode ser ampliada pela ativação sinérgica de astrócitos e microglia, causando uma neuroinflamação descontrolada que contribui para a perda de neurônios dopaminérgicos (8). No entanto, os astrócitos desempenham um papel duplo em doenças inflamatórias do sistema nervoso central (CNS). Dependendo do grau e da duração do estímulo inflamatório, os astrócitos também são capazes de exercer um efeito inibitório sobre a microglia, limitando a inflamação do CNS (14). Portanto, esses resultados podem indicar que o CARV é capaz de modular a ativação da microglia através de astrócitos, contribuindo para a inibição das respostas microgliais e limitando a neuroinflamação.

As propriedades anti-inflamatórias do CARV foram relacionadas à inibição da liberação de IL-1β (15). IL-1β é uma citocina pró-inflamatória com um papel importante durante a resposta inflamatória, induzindo a produção de outros mediadores inflamatórios (15). Nossos resultados mostraram que a administração oral de CARV/β-CD previniu o aumento, induzido por 6-OHDA, desta citocina no liquido cefalorraquidiano e no soro (Fig. 3A e B). Nossos resultados demonstram que a administração oral de CARV/β-CD é capaz de diminuir a liberação de mediadores inflamatórios, reduzindo o estado pró-inflamatório induzido pela administração de 6-OHDA. A redução do estado

proinflamatório no CNS diminui a neurotoxicidade, o que pode explicar o efeito protetor do CARV/β-CD contra a denervação dopaminérgica.

Conclusão

A administração oral do complexo CARV/β-CD apresenta efeito neuroprotetor contra o dano induzido por 6-OHDA, como modelo animal de PD. O tratamento prévio com CARV/β-CD previne a perda de neurônios dopaminérgicos e consequentes déficits locomotores. Esta proteção contra a denervação dopaminérgica pode estar relacionada com as propriedades anti-inflamatórias do CARV. O pré-tratamento com CARV/β-CD limitou a neuroinflamação através da modulação de respostas microglias por astrócitos, reduzindo IL-1β níveis de controle no liquido cefalorraquidiano e soro. Os presentes resultados indicam uma potencial aplicação farmacológica do complexo CARV/β-CD na PD, sendo necessários estudos futuros para avaliar os possíveis efeitos do pós-tratamento com CARV/β-CD.

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