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The effects of retinol oral supplementation in 6-hydroxydopamine dopaminergic denervation model in Wistar rats

Alice Kunzler[a], Camila Tiefensee Ribeiro[a], Juciano Gasparotto[a], Lyvia Lintzmaier Petiz[b], Helen Thais da Rosa Silva[a], Jeferson Delgado da Silva Jr.[a], Rafael Bortolin[c], Priscila Oliveira de Souza[a], Fabiano Barreto[d], Pedro Espitia-Perez[e], Carlos Eduardo Schnorr[c], Nauana Somensi[a], José Cláudio Fonseca Moreira[a], Daniel Pens Gelain[a,*]

[a] *Departamento de Bioquímica, Centro de Estudos em Estresse Oxidativo, Universidade Federal do Rio Grande do Sul—UFRGS, Brazil*
[b] *Instituto do Cérebro, Laboratório de Neurodinâmica, Universidade Federal do Rio Grande do Norte- UFRN, Brazil*
[c] *Departamento de Ingeniería Civil y Ambiental, Universidad de la Costa, Calle, Barranquilla, Colombia*
[d] *Laboratório Nacional Agropecuário - LANAGRO/RS, Estrada da Ponta Grossa, 3036, Porto Alegre, RS, Brazil*
[e] *Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Calle 38 Carrera 1W, Barrio Juan XXIII, Montería, Córdoba, Colombia*

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ABSTRACT

Vitamin A (retinol) is involved in signaling pathways regulating gene expression and was postulated to be a major antioxidant and anti-inflammatory compound of the diet. Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by loss of nigral dopaminergic neurons, involving oxidative stress and pro-inflammatory activation. The aim of the present study was to evaluate the neuroprotective effects of retinol oral supplementation against 6-hydroxydopamine (6-OHDA, 12 µg per rat) nigrostriatal dopaminergic denervation in Wistar rats. Animals supplemented with retinol (retinyl palmitate, 3000 IU/kg/day) during 28 days exhibited increased retinol content in liver, although circulating retinol levels (serum) were unaltered. Retinol supplementation did not protect against the loss of dopaminergic neurons (assessed through tyrosine hydroxylase immunofluorescence and Western blot). Retinol supplementation prevented the effect of 6-OHDA on Iba-1 levels but had no effect on 6-OHDA-induced GFAP increase. Moreover, GFAP levels were increased by retinol supplementation alone. Rats pre-treated with retinol did not present oxidative damage or thiol redox modifications in liver, and the circulating levels of TNF-α, IL-1β, IL-6 and IL-10 were unaltered by retinol supplementation, demonstrating that the protocol used here did not cause systemic toxicity to animals. Our results indicate that oral retinol supplementation is not able to protect against 6-OHDA-induced dopaminergic denervation, and it may actually stimulate astrocyte reactivity without altering parameters of systemic toxicity.

1. Introduction

Parkinson's disease (PD) is one of the most frequent neurodegenerative pathologies worldwide and it is characterized by the marked loss of dopaminergic neurons projecting from the *substantia nigra* (SN) into the striatum (Bhat et al., 2018). The characteristic pathological features of this disease are the progressive accumulation of Lewy bodies and Lewy neurites in brain stem, spinal cord and cortical regions (Beitz, 2014). Among the considered causes of PD are gene mutations, exposure to toxic substances and aging. At the molecular level, Lewy body formation is a consequence of malignant aggregation of misfolded α-synuclein, which in turn is associated with apoptosis, oxidative stress and mitochondrial dysfunction at several levels, leading to neurodegeneration (Singh et al., 2017).

Vitamin A (retinol) is a micronutrient that is crucial for maintaining vision, promoting growth and development, and protecting epithelium and mucus integrity (Huang et al., 2018). In the central nervous system, retinol, mainly through conversion into retinoic acid and consequent modulation of nuclear retinoic acid receptors (RARs), exerts an essential role in patterning and neuronal differentiation during embryonic development, as well as motor-neuron differentiation and regeneration (Maden, 2007). In the adult nervous system, hippocampal long-term potentiation and depression are dependent on retinol and retinoic acid (McCaffery et al., 2006). Increasing evidence has been implicating

* Corresponding author. Rua Ramiro Barcelos, 2600 – anexo, CEP 90035-003, Porto Alegre, RS, Brazil.
E-mail address: daniel.gelain@ufrgs.br (D.P. Gelain).

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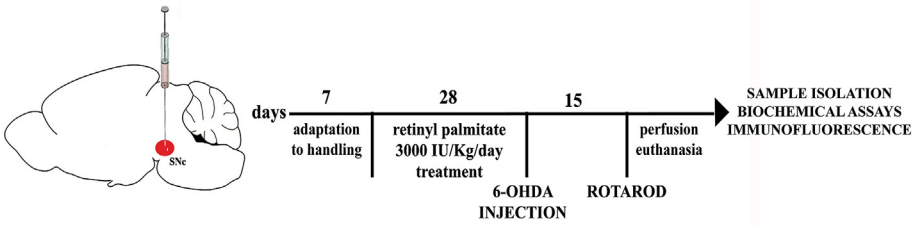

Fig. 1. Schematic overview of experimental design. Wistar rats were acclimatized to manipulation for 7 days. Retinyl palmitate was administrated for 28 days followed by 6-OHDA injection. After 15 days, animals were submitted to behavior analyses, perfusion and euthanasia. Motor incoordination was assessed by rotarod assay.

retinol and retinoic acid in the regulation of synaptic plasticity through cellular mechanisms that involve increasing postsynaptic glutamate receptor levels (Aoto et al., 2008), decreased postsynaptic calcium entry (Wang et al., 2011) and non-genomic actions of RARs on cytoplasmic proteins (Shearer et al., 2012). Besides, the influence of retinoic acid in hypothalamic neurogenesis suggested a link between retinol, stress and depression, as well as other neuropsychiatric conditions (Shearer et al., 2012).

For many years, vitamins were postulated to be major antioxidants (Lobo et al., 2010). Retinol and carotenoids have shown anti-oxidative, cytoprotective and anti-aggregative effects on *in vitro* and *in vivo* models. Some studies suggest that vitamin A deficiency contributes to Alzheimer's disease pathogenesis and cognitive decline in elderly subjects (Zeng et al., 2017). Consumption of foods containing carotenoids and β-carotene significantly reduced the risk of PD (Miyake et al., 2011). However, there is a lack of robust clinical trials exploring the use of retinol or carotenoids in the treatment and prevention of neurodegenerative diseases (Chang et al., 2018).

Unilateral administration of 6-hydroxydopamine (6-OHDA) has been largely used to induce dopaminergic denervation in animal models (Thiele et al., 2012). This model mimics the advanced stages of dopaminergic neurodegeneration in PD, as it affects the neurons that are lost in the course of the disease and it causes similar motor deficits in the animals. In the present work, we used this model to evaluate the potential preventive effects of dietary retinol supplementation against PD. Wistar rats received oral supplementation with retinyl palmitate (i.e., retinol esterified to palmitate) for 28 days, followed by an intranigral 6-OHDA injection. To determine potential neuroprotective effects of retinol supplementation, evaluation of motor performance, content of dopaminergic neurons and neuroinflammation were performed. Systemic parameters of inflammation, hepatotoxicity, oxidative stress and mutagenicity were also assessed, as retinol supplementation has been previously observed to induce toxic effects associated to hypervitaminosis.

The present results indicate that oral supplementation with retinol was not protective against dopaminergic denervation induced by 6-OHDA unilateral injection. Although retinol supplementation prevented the increase in serum TNF-α and IL-1β induced by 6-OHDA, astrocyte and microglia phenotypes were changed in animals treated only with retinol. Besides, although retinol supplementation inhibited the increase in Iba-1 levels caused by 6-OHDA injection, the effect of 6-OHDA on GFAP immunoreactivity was not affected by previous retinol supplementation. These results, altogether, suggest that oral supplementation with retinol does not protect against dopaminergic toxicity in the SN and may disturb astrocytes and microglia functional homeostasis. These results raise concerns about safety of retinol oral supplementation, as systemic parameters of oxidative stress, mutagenicity and inflammation did not indicate toxicity of the dose used in the present protocol.

2. Material and methods

2.1. Animals

The Ethics Committee on Animal Research of the Universidade Federal do Rio Grande do Sul (UFRGS) reviewed and approved the study protocol, which is filed under the project number #21563. Five male Wistar rats (between 280 and 300 g, 60-days-old) were maintained in each cage with *ad libitum* access to food chow and water. The cages were kept in an animal house facility with a 12-h light–dark cycle (7:00 to 19:00 h) at 23 ± 1 °C. The experimental procedures were performed according the directions of the US National Institutes of Health (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011).

2.2. Treatments

The dose of retinyl palmitate (3000 IU/kg/day, equivalent to 900 μg/day for an adult person) was chosen based on the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine of the National Academies (formerly National Academy of Sciences) (Anon, 2001; Trumbo et al., 2001) and recommended by the FDA for adults (2016). Sixty Wistar rats were randomly divided into four groups, as follows: 1) Sham group, treated with vehicle (mineral oil) for 28 days, subjected only to a sham stereotaxic surgical procedure (without 6-OHDA injection); 2) Retinyl palmitate group, which received oral retinyl palmitate (3000 IU/kg/day, dissolved in mineral oil) for 28 days and were also subjected to sham procedure; 3) 6-OHDA, which were treated with mineral oil for 28 days and then subjected to surgery with 6-OHDA injection; 4) Retinyl palmitate + 6-OHDA, which received oral retinyl palmitate for 28 days and then were subjected to surgery with 6-OHDA injection. Before the treatments were initiated, animals were subjected to manipulation (handling and gavage habituation) for 7 days by the same subjects who carried out further manipulations. The animals received the treatment once a day for 28 days with a gavage (Fig. 1). The treatments were carried out at night in order to ensure maximum retinyl palmitate absorption, since retinol is better absorbed during or after a meal, and rats are more active during the night. The animals were treated with vehicle (mineral oil; n = 30 animals) or 3000 IU/kg of retinyl palmitate (n = 30 animals). Oral delivery was performed with a gavage consisting of a metallic gastric tube, in a maximum volume of 0.6 mL.

2.3. Intranigral injection of 6-OHDA

Animals were subjected to a unilateral stereotaxic injection of 6-OHDA in the SN to induce the selective death of dopaminergic neurons projecting from SN into the striatum. In brief, the animals received ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) for anesthesia and were placed on a stereotaxic apparatus (Insight-EFF 338, SP, Brazil). The skulls were surgically perforated and a syringe needle connected to a microinjection pump was inserted into the brain, using the Paxinos and Watson rat brain anatomical coordinates relative to bregma (Paxinos and Watson, 2007): anteroposterior (AP), −8.0 mm; mediolateral (ML), ± 2.1 mm; dorsoventral (DV), −8.00 mm. Two microliters of 6-OHDA solution (12 μg, 0.01% ascorbic acid- Sigma-Aldrich; St. Louis, MO) were injected at a flow rate of 0.5 μL/min. The needle was kept inside the brain for additional 5 min before removal. Animals were sutured and kept in a recovery room for additional 24 h.

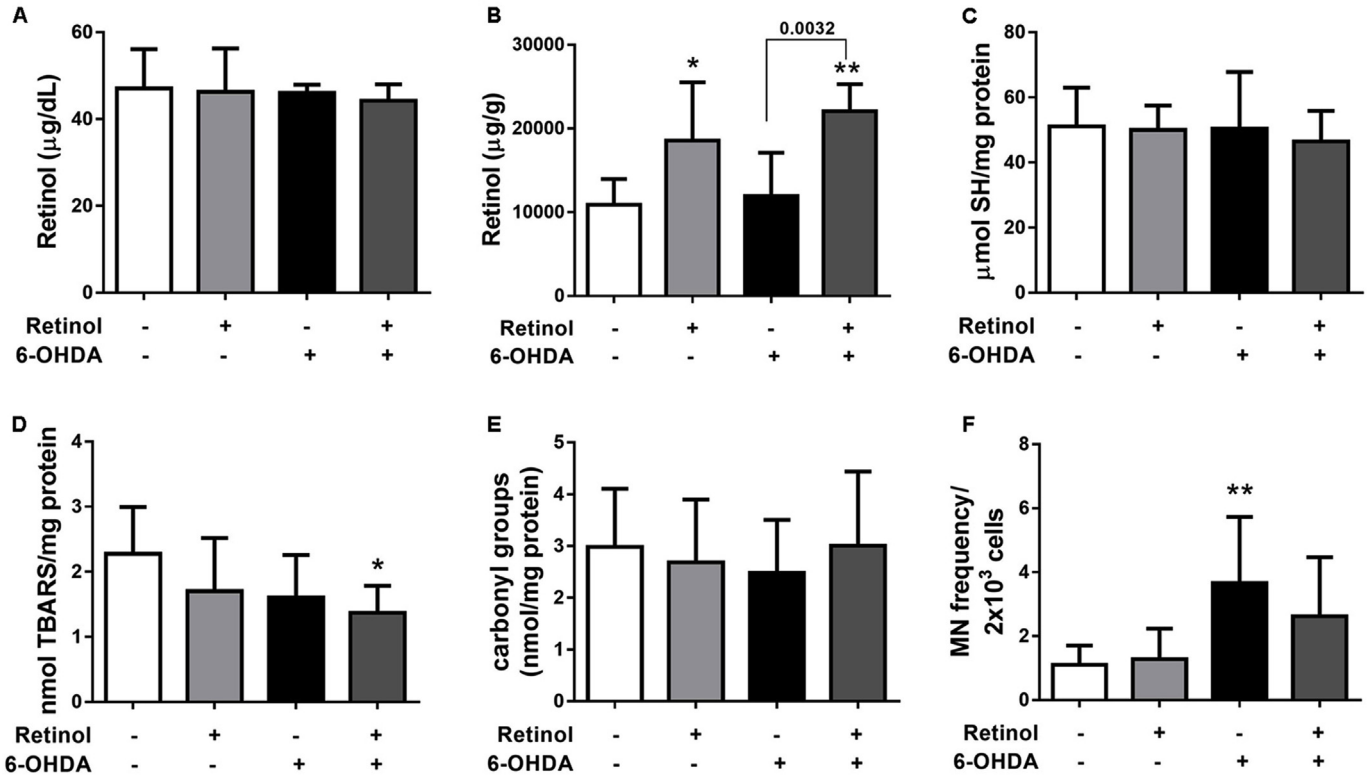

Fig. 2. Systemic effects of retinol supplementation. Total retinoid content in **(a)** serum and **(b)** liver; **(c)** liver sulfhydryl (SH, reduced) groups, **(d)** lipoperoxidation (TBARS levels) and **(e)** protein carbonyl levels (n = 8). **(d)** Micronucleus frequency was analyzed in polychromatic erythrocytes from bone marrow. Values represent mean ± SD. One-way analysis of variance and Bonferroni's Multiple Comparison *post-hoc* tests were applied to all data. Asterisks denote significance level ($p < 0.05$) in relation to control (sham) group. Values for p between specific groups are denoted when difference is significant.

2.4. Behavioral study (motor balance)

Motor balance was evaluated using a rat rotarod apparatus as previously described (Gasparotto et al., 2017). In brief, fifteen days after 6-OHDA injection, animals were placed on the rotarod (set at 18 rpm) and the time span to remain on the rod was recorded automatically. Previously to test sessions, animals were trained and habituated in the apparatus for 2 h. Motor balance was assessed as the length of time the rat remained on the rod before fall in three chances. Data is presented as elapsed time on the rotarod in three test trials.

2.5. Extraction of retinyl palmitate, retinyl acetate and quantification by HPLC in serum and liver samples

Serum (100 µL) was extracted using two-cycle vortex with 150 µL acetonitrile and centrifuged (13,000 × rpm, 10 min). A 200 µL aliquot was transferred for injection to a HPLC system. For liver, pre-weighted samples were homogenized in ethyl acetate (medium speed, cycles of 10 s), vortexed for 10 min (180 × rpm) and centrifuged (4000 × rpm, 10 min). Homogenate samples were evaporated to dryness using a Tecnal TE-0194 evaporator system with nitrogen as carrier gas. Finally, the residue was re-dissolved in methanol and vortexed. A 200 µL aliquot was transferred for injection to a HPLC system.

2.6. LC-MS/MS

Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer SCIEX 5500 QTRAP (Foster City, CA, USA) was used for analysis. Separation was performed using a Zorbax Eclipse XDB-C18 column (C18 3.5 µm, 100 mm × 2.1 mm) coupled to a C18 guard column (Phenomenex, CA, USA). Injection volume was 2 µL and the mobile phase in the gradient elution consisted of ultrapure water with 2 mM ammonium formate (A) and 2 mM ammonium formate acetonitrile, both containing 0.1% formic acid (B). The flow rate was 500 µL min^{-1} and a three-minute equilibrating time was applied. The gradient started with 95% of A buffer decreasing to 5% at 1 min, then kept for 8 min and returned to initial conditions at 9.5 min. A diverter valve was employed to reduce the entry of matrix components in the spectrometer.

Mass spectrometer resolution in multiple reactions monitoring (MRM) was unitary and the dwell time applied was 50 ms. Fragment ions in MRM mode of each precursor protonated molecular ions were produced by collision-activated dissociation (CAD) in a collision cell. Nitrogen was applied as nebulizer gas, curtain gas, heater gas and collision gas. Collision gas (CAD) was set at high. Nebulizer gas (GS1) was set at 30 psi. Heater gas pressure and temperature were set at 55 psi and 700 °C, respectively. Electrospray capillary voltage was set at 5.5 kV. The collision energies were set at maximum for each transition. Quantification was performed by relating peak area of analytes obtained via determination, versus peaks from retinyl palmitate and retinyl acetate standards and expressed as µg/g wet weight for liver samples and µg/mL for plasma samples. To avoid photodecomposition of analytes, all extraction procedures, handling of homogenates and quantifications were performed in light-controlled areas.

2.7. Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-1β, IL-6 and IL-10 were assessed by indirect assay. Blood serum samples (100 µL) were added to ELISA plates and incubated for 24 h. After that, samples were withdrawn and the plates washed three times with Tween-Tris buffered saline (TTBS, 100 mM Tris – HCl, pH 7.5, containing 0.9% NaCl, and 0.1% Tween-20). Subsequently, 200 µL of primary antibody against TNF-α, IL-1β, IL-10 or IL-6 (1:1000) were added and incubated for 24 h at 4 °C. The plates were washed three

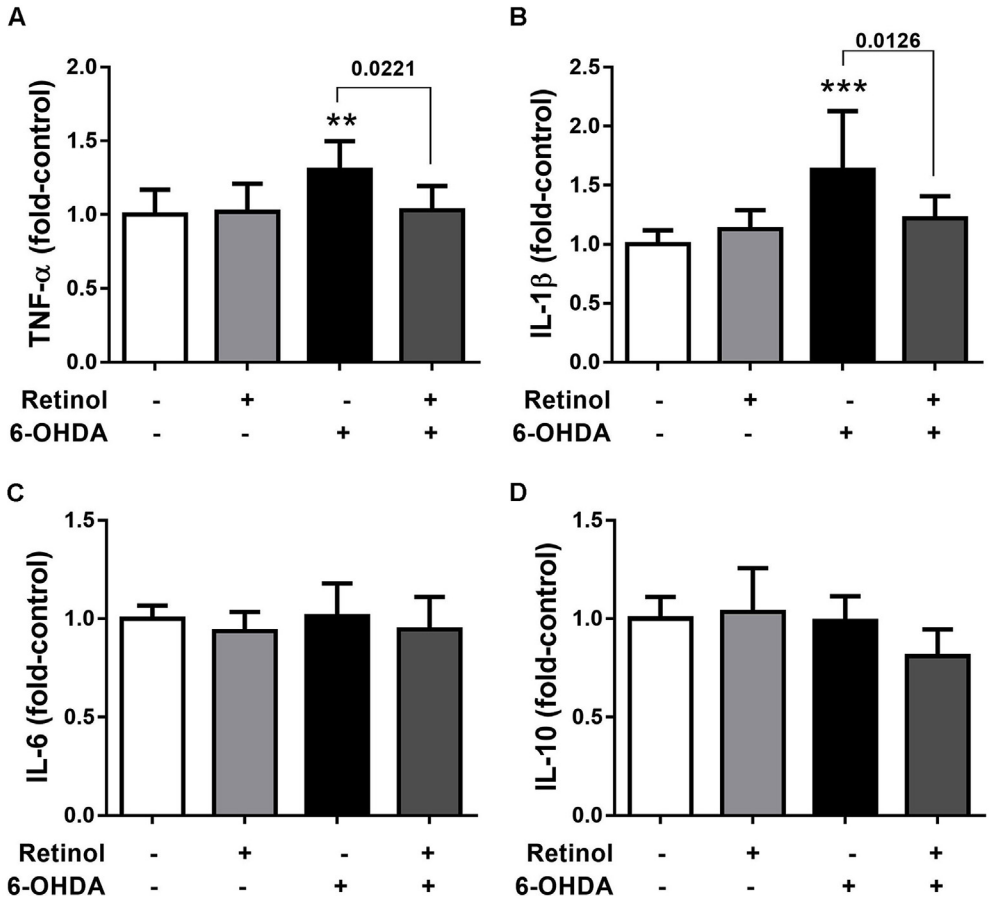

Fig. 3. Serum levels of TNF-α, IL-1β, IL-6 and IL-10. Serum was analyzed by ELISA 15 days after 6-OHDA injection. Serum samples (n = 8) were analyzed for **(a)** TNF-α, **(b)** IL-1β, **(c)** IL-6 and **(d)** IL-10. Values represent mean ± SD. One-way analysis of variance and Bonferroni Multiple Comparison post-hoc tests were applied to all data. Asterisks denote significance level ($p < 0.05$) in relation to control (sham) group. Values for p between specific groups are denoted when difference is significant.

times with TTBS and incubated with rabbit or mouse IgG peroxidase-linked secondary antibody (1:1000) for 1 h. After washing the plate three times with TTBS, 150 µL of substrate solution (TMB spectrophotometric ELISA detection kit) were added to each well and incubated for 15 min. The reaction was terminated with 50 µL/well of 12 M sulfuric acid stopping reagent and the absorbance at 450 nm was read in a microplate reader.

2.8. Immunofluorescence microscopy

To prepare tissues for immunofluorescence microscopy, six rats per group in each experiment were subjected to a perfusion with saline solution via the vascular system with the descendent aorta clamped. Sterile saline (0.9%) was perfused for 10 min, then paraformaldehyde (PFA) solution 4% in PBS (7.4) was perfused for additional 10 min, with a flow rate of 20 mL/min each. After the perfusion, the animals were euthanized and the brains were removed and kept in PFA 4% for 24 h at 4 °C. The brains were then transferred to a sucrose 15% solution for 24 h at 4 °C and then to a sucrose 30% solution for additional 24 h. After that, the brains were dried and frozen in −20 °C.

The slices from SN were obtained using a cryostat at −20 °C (Jung Histoslide 2000R; Leica; Heidelberg, Germany). The SN was sectioned in slices of 20 µm on the coronal plane. Between 20 and 30 slices containing the SN were collected per rat in PBS with Triton X-100 0.1% (PBS-0.1%). The free-floating sections were incubated for 2 h with albumin 5% to block nonspecific binding. Incubations with Iba-1 (1:500; 019–19741 -Wako Chemicals USA, Inc.-VA, USA) GFAP (1:500; #3670 Cell Signaling Technology®) and TH (1:500, #2792, Cell Signaling Technology®), diluted in PBS containing 2% bovine serum albumin, were performed for 48 h at 4 °C. DAPI was used for nucleic acid staining (1:500; D9542, Sigma-Aldrich® MO, USA). The tissue sections were then washed four times with PBS-0.1% and then incubated with respective secondary anti-rabbit or anti-mouse Alexa 488 or 555 from Cell Signaling Technology® (1:500 in PBS and BSA 2%) for 1 h at room temperature. The slices were then thoroughly washed with PBS-0.1%, transferred to gelatinized slides to be assembled with FluorSave™ (345789 – Merck Millipore; MA, USA) and then covered with coverslips. The image acquisition was performed in an EVOS® FL Auto Imaging System (AMAFD1000 – Thermo Fisher Scientific; MA, USA) fluorescent microscope. Quantification analyses were performed with Image J Software.

For quantification of TH-positive cells, zones of the SN were selected and the background fluorescence was subtracted and no thresholding of images was performed. Individual cells with TH-associated fluorescence surrounding DAPI staining were identified and marked by a blind operator and then counted. For GFAP and Iba-1 quantification, total fluorescence per field associated to each protein was quantified. Background fluorescence was subtracted; thresholding was not performed. For background subtraction, a background area of each image was selected and the mean value of pixel intensity of this area was subtracted in the whole field.

2.9. Western blot

Samples of ipsilateral SN (~100 mg) were homogenized in RIPA buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 100 µM PMSF and 1 mM sodium orthovanadate. Sample protein concentration was assayed (Bradford, 1976) and the samples were diluted in Laemmli-sample buffer (62.5 mM Tris–HCl, 1% (w/v) SDS, 10% (v/v) glycerol, pH 6.8) and equal amounts of protein (40 µg/well) were subjected to 10% SDS–PAGE and then transferred to nitrocellulose membranes with a Trans-blot SD semi-dry transfer cell (BioRad). Membranes were blocked with 5% BSA and incubated overnight at 4 °C

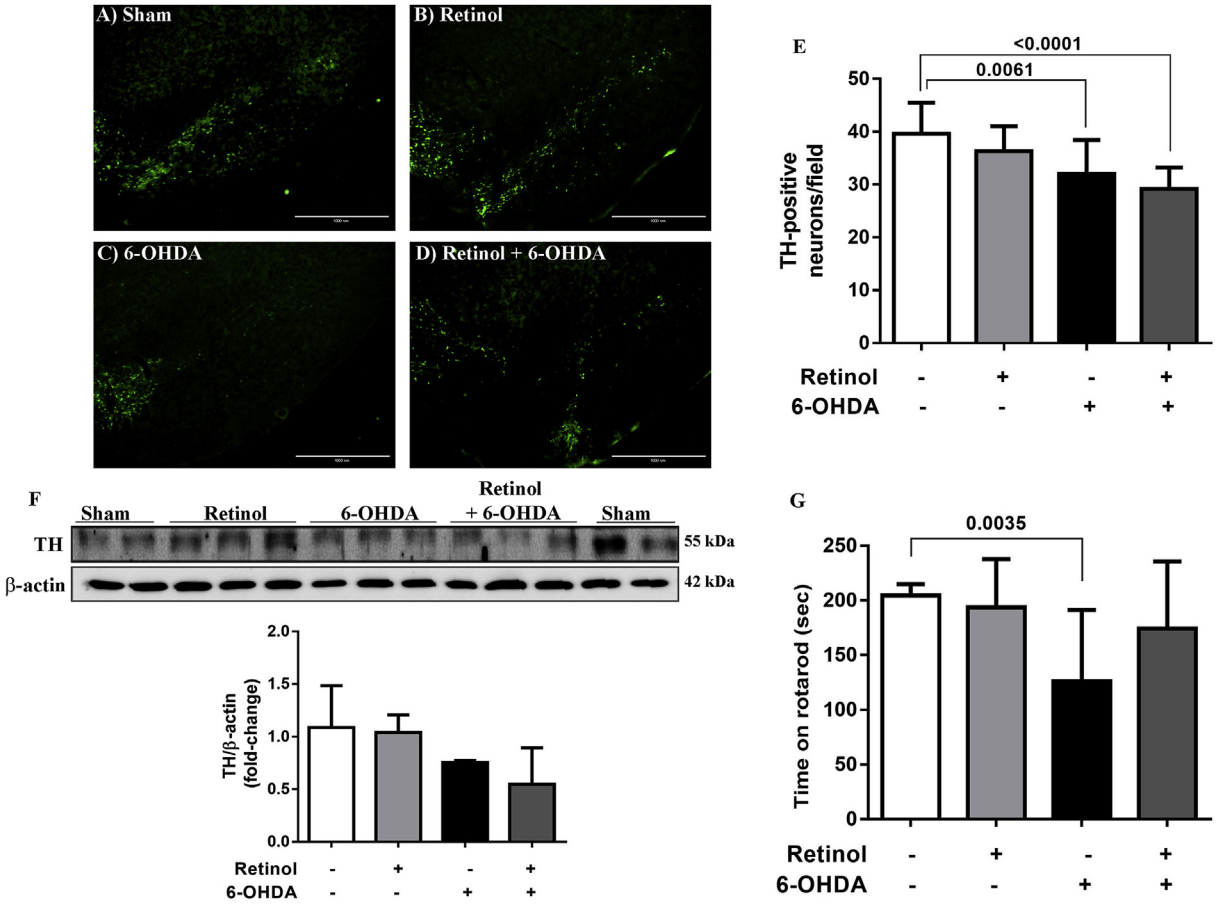

Fig. 4. Effect of retinol supplementation in dopaminergic denervation and rotarod performance. Representative immunofluorescence images of SN (ipsilateral side) immunostained for tyrosine hydroxylase (TH). **(a)** control (sham), **(b)** retinol, **(c)** 6-OHDA and **(d)** retinol + 6-OHDA groups are compared; scale bars represent 1000 μm. **(e)** Quantification of fluorescence intensity of TH staining. Values represent mean ± SD from twelve rats per group (n = 12). **(f)** Representative Western blot for TH and quantification values are depicted. **(g)** Motor incoordination was assessed by rotarod assay. Values represent mean ± SD from twelve rats per group (n = 12). One-way analysis of variance and Bonferroni Multiple Comparison post-hoc tests were applied to all data. Asterisks denote significance level ($p < 0.05$) in relation to control (sham) group. Values for p between specific groups are denoted when difference is significant.

with primary anti-tyrosine hydroxylase (Cell Signaling Technology 2792S) or anti-β-actin (MERCK A1978) at 1:1000 each and washed 4× with TTBS. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary antibody (1:2000) was incubated for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer). Quantification analyses were performed with Image J Software.

2.10. Micronucleus test in polychromatic erythrocytes

To assess retinol systemic mutagenicity, quantification of micronucleus in polychromatic erythrocytes was performed, as retinol supplementation has been previously observed to enhance this effect *in vitro* and *in vivo* (Espitia-Perez et al., 2018; Klamt et al., 2003). The bone marrow was extracted from the two femurs of each animal and the smears were prepared directly on slides, two per animal, with a drop of fetal calf serum. The slides were stained with 10% Giemsa for 5 min, air-dried and coded for blinded analysis. The incidence of micronuclei was observed in 2000 polychromatic erythrocytes for each animal (i.e. 1000 from each of the two slides prepared from the duplicate), using bright-field optical microscopy at a magnification of 200–1000×.

2.11. Oxidative damage and thiol redox status

Carbonyl groups were measured as an index of protein oxidation (Levine et al., 1990). Briefly, the tissue samples were mixed with 10% trichloroacetic acid, the supernatant was removed, and pellets used for reaction with 2,4-dinitrophenylhydrazine. Carbonyl content was determined by the absorbance at 370 nm and results were expressed as nmol carbonyl/mg protein. Lipoperoxidation was assayed through quantification of thiobarbituric acid-reactive substances (TBARS) originated from reaction of TBA with lipoperoxides in an acid-heating medium (Draper and Hadley, 1990). Briefly, samples were deproteinized with 10% trichloroacetic acid and the supernatant was heated with 0.67% TBA for 25 min. TBARS levels were determined by the absorbance at 532 nm and results expressed as nmol TBARS/mg protein. Total reduced thiol (SH) content was determined as an estimation of the overall redox status of the cellular environment assessed by quantification of total reduced thiol (sulfhydryl; SH) groups in samples (Ellman, 1959). Briefly, samples were mixed in a slightly alkaline medium with 10 mM 5,5-dithiobis-2-nitrobenzoic acid prepared in ethanol. SH content was determined after 60 min by the absorbance at 412 nm and results were expressed as μmol SH/mg protein.

2.12. Protein assay and statistical analysis

Protein content was normalized for Western blot samples after determination of cellular total protein content by Bradford method as previously described (Gelain et al., 2011). Data were analyzed by a one-way analysis of variance (ANOVA) followed Bonferroni's *post hoc* test. Data were analyzed in GraphPad® (San Diego, CA; version 5.00).

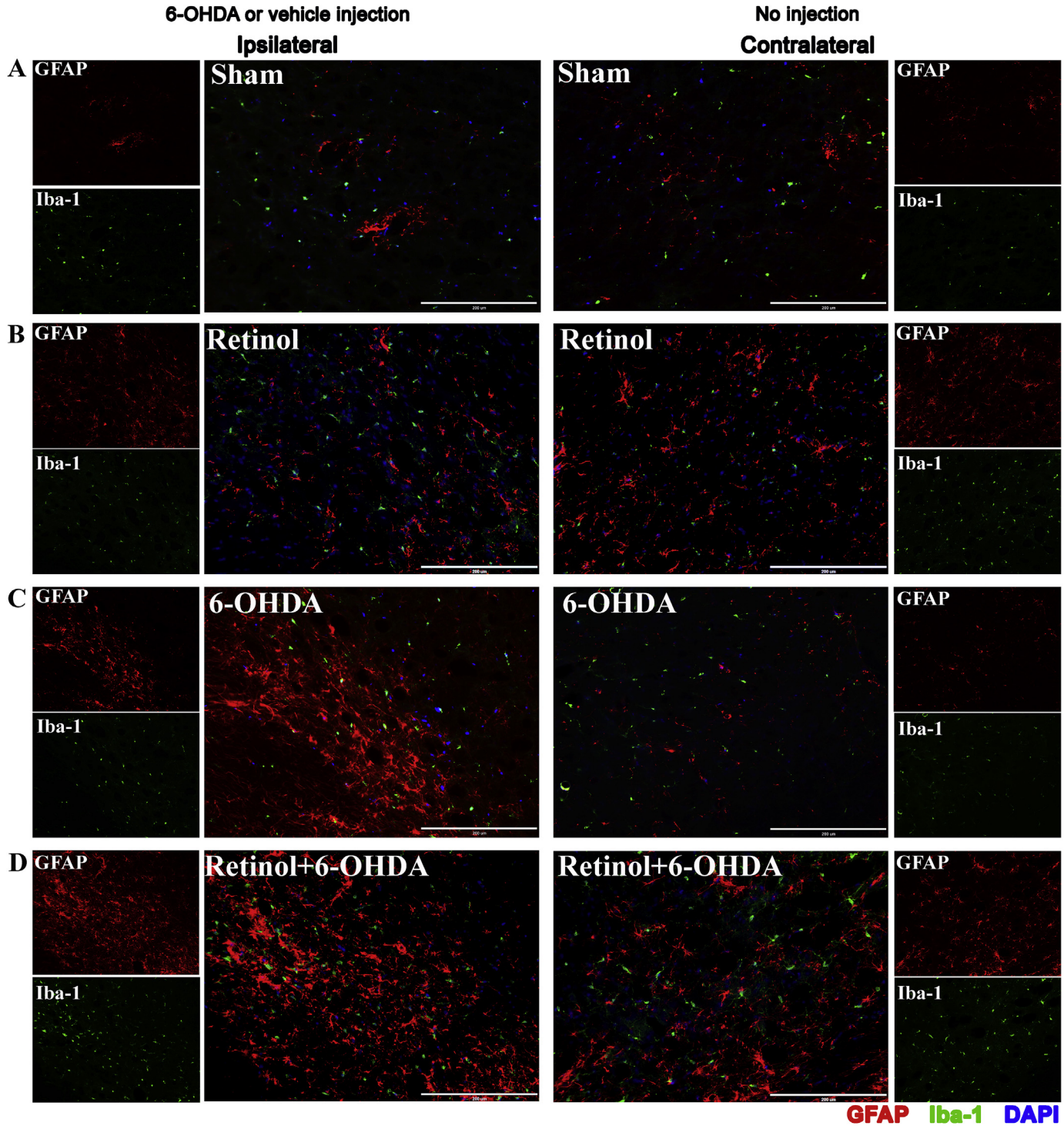

Fig. 5. Effect of retinol supplementation in neuroinflammation parameters. Representative immunofluorescence images of SN (ipsilateral side and contralateral side) immunostained for GFAP (red) and Iba-1 (green). Nuclear content was stained with DAPI (blue). **(a)** control (sham), **(b)** retinol, **(c)** 6-OHDA and **(d)** retinol + 6-OHDA groups are compared; scale bars represent 200 μm.

Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Effects of retinol supplementation over systemic parameters of toxicity and inflammation

It was previously demonstrated that oral supplementation with high doses of retinol (administered as retinyl palmitate) may induce toxic effects through reactive species production in several organs, including the SN (de Bittencourt Pasquali et al., 2012; de Oliveira et al., 2008; Schnorr et al., 2015; Schnorr et al., 2011), as well as to enhance mutagenicity parameters *in vivo* and *in vitro* (Espitia-Perez et al., 2018; Klamt et al., 2003). Thus, we evaluated the effect of supplementation with retinyl palmitate on endogenous retinol levels and also over systemic parameters of oxidative stress, inflammation, mutagenicity and toxicity. HPLC analysis of retinol content demonstrated that retinyl palmitate supplementation did not alter serum levels of retinol

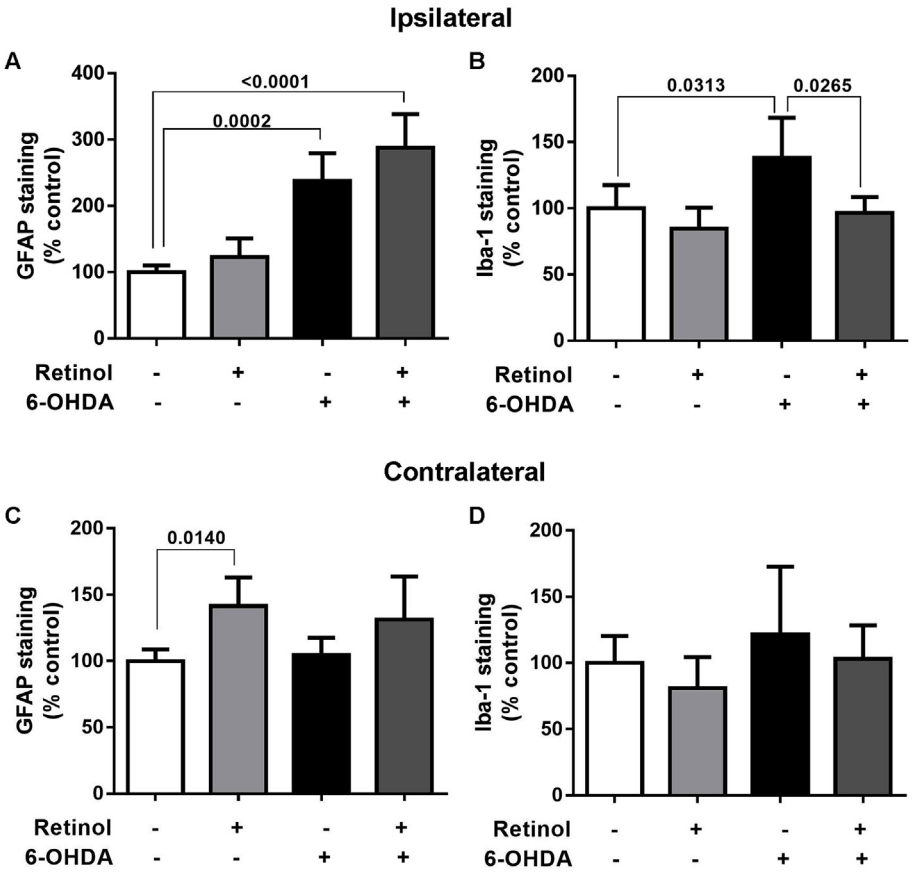

Fig. 6. Quantification of GFAP and Iba-1 staining intensity in ipsilateral SN. Fluorescence intensity of ipsilateral **(a)** GFAP and **(b)** Iba-1, as well as contralateral **(c)** GFAP and **(d)** Iba-1 are depicted. Values represent mean ± SD from twelve rats per group (n = 12). One-way analysis of variance and Bonferroni Multiple Comparison post-hoc tests were applied to all data. Values for p between specific groups are denoted when difference is significant ($p < 0.05$).

(Fig. 2A); however, analysis of retinol levels in liver, which is the main organ of retinol storage, demonstrated that the animals that received retinol supplementation had a significant increase in endogenous retinol content (Fig. 2B). In this organ, the presence of retinol did not cause any changes in the levels of free thiol (sulfhydryl) groups, and did not enhance oxidative damage to lipids and proteins (Fig. 2C–E), which is in agreement with previous data for this dose of retinol (de Oliveira et al., 2009). Also, retinyl palmitate supplementation did not increase the frequency of micronucleus presence in polychromatic erythrocytes from bone marrow, a marker of mutagenicity (Fig. 2F); here, a significant increase was observed in animals treated with 6-OHDA, and this was inhibited in supplemented animals. The circulating levels of TNF-α, IL-1β, IL-6 and IL-10 were also assessed. Retinol supplementation *per se* did not change the levels of these cytokines; besides, the stimulatory effect of 6-OHDA in TNF-α and IL-1β levels was inhibited in rats previously supplemented with retinol (Fig. 3A–D).

3.2. Effect of retinol supplementation in 6-OHDA-induced dopaminergic denervation and motor deficit

Tyrosine hydroxylase (TH) is the gold standard marker of dopaminergic cells in the nigrostriatal axis, thus we analyzed the effect retinol in TH immunofluorescence of the ipsilateral SN. Retinol supplementation did not affect TH staining compared to control (Fig. 4A–B and E); as expected, a significant decrease in TH positive cells in the SN was observed in 6-OHDA-treated animals (Fig. 4C and E). Prior supplementation with retinol did not protect dopaminergic cells from 6-OHDA insult, although a few TH-positive cells are still observed in this group (Fig. 4D and E). Western blot for TH did not reveal a significant effect of retinol pre-treatment against 6-OHDA injection; however, it is important to note that no significant effect of 6-OHDA alone on total TH protein was detected either, although a strong trend towards a decrease in total TH is observed (Fig. 4F). Intriguingly, motor task assessment on rotarod demonstrated that animals supplemented with retinol and subjected to 6-OHDA injection had a similar performance to sham group (Fig. 4G); nonetheless, this effect was not statistically different from the effect observed in the 6-OHDA group, although the performance of the latter was significantly impaired compared to sham group.

3.3. Effect of retinol supplementation in 6-OHDA-induced neuroinflammation

Neuroinflammation exerts a prominent role in the development and progression of neurodegenerative diseases, including PD (Wang et al., 2015a). As the analysis of serum suggested that retinol supplementation could exert an anti-inflammatory effect, we analyzed the staining of glial fibrillary acidic protein (GFAP) and microglia/macrophage-specific calcium-binding protein (Iba-1) in the SN, to assess the activation state of astrocytes and microglia, respectively (Figs. 5 and 6). Immunofluorescence microscopy revealed that GFAP and Iba-1 fluorescence in the ipsilateral side were significantly increased by 6-OHDA injection and that retinol supplementation did not affect the increase in GFAP, but it prevented the effect of 6-OHDA in Iba-1 staining intensity (Fig. 5C and D and 6A-B). Moreover, retinol supplementation, alone, altered the morphological pattern of GFAP and Iba-1 staining in SN in both ispilateral and contralateral sides (Fig. 5B). Statistical analysis of the fluorescence staining quantification in the contralateral side evidences that GFAP staining is increased in the retinol group compared to sham (Fig. 6C); this effect was not observed for Iba-1 staining (Fig. 6D). These results suggested that although microglia reactivity was inhibited in 6-OHDA-treated animals previously supplemented with retinol, retinol treatment *per se* stimulates astrocytes reactivity.

4. Discussion

Parkinson's disease is the most frequent subcortical

neurodegenerative disease, clinically manifested by motor symptoms including rigidity, tremor at rest and slowness of voluntary movements (Sardi et al., 2018). Lipophilic antioxidants such as vitamin A are important nutrients that have been drawing attention in the context of neurodegenerative diseases (Chang et al., 2018). In this study, we demonstrated that retinol supplementation with a retinyl palmitate daily dose of 3000 IU/kg/day did not present a valuable preventive effect upon dopaminergic denervation induced by 6-OHDA. Besides, retinol supplementation *per se* caused morphological alterations in astrocytes and microglia, which may be associated to the activation state of these cell types; indeed, the enhancement of GFAP staining in animals receiving only retinol strongly indicates that stimulation of astrocytes reactivity is induced by retinol supplementation.

The actions of retinol and its metabolites in animal models have been studied for many years. These compounds may prevent cell membranes from free radical damage through readily scavenging peroxyl radicals, and thus prevent lipid, protein, and DNA oxidation (Lobo et al., 2010). However, this is the first time that oral supplementation with retinol is tested as a potential prevention factor against nigrostriatal degeneration in an animal model using 6-OHDA. Vitamins in general play an important role in the prevention of different diseases (Burton and Ingold, 1984). In the context of neurodegenerative pathologies, retinol supplementation was suggested for a potential approach to Alzheimer's disease (AD) prevention and treatment, as its marginal deficiency was associated with increased risk for AD pathogenesis (Grimm et al., 2016; Mohammadzadeh Honarvar et al., 2017; Zeng et al., 2017). Early post-treatment with retinoic acid protected against neurodegeneration in a PD model (Yin et al., 2012) and reversed locomotor reduction activity in a rotenone model study (Ulusoy et al., 2011).

In this study we used the rotarod test to evaluate motor incoordination induced by 6-OHDA. This test is used as a common standard to evaluate motor balance in hemi-parkinsonian rats (Assini and Abercrombie, 2018; Haddadi et al., 2014; Lai et al., 2019). We found intriguing that the rotarod performance of animals receiving retinol supplementation and subjected to 6-OHDA was not different from control (sham) group, although statistical analysis indicated that it was not different from 6-OHDA group as well. Although the decrease in TH levels by 6-OHDA was not affected by retinol supplementation, we should take into account that we have no information about the dopamine levels of the animals in the present study. Dopamine production can be upregulated by the modulation of TH activity in the remaining cells, a common response to dopaminergic neurodegeneration in order to compensate for dopaminergic loss (Dunkley et al., 2004). In cultured chromaffin cells, retinol was reported to induce TH phosphorylation and consequent activation (Gelain et al., 2007).

The main mechanism of 6-OHDA toxicity is believed to consist of autooxidation and generation of reactive species inside the neurons, leading to a pro-oxidant state and mitochondrial dysfunction (Rodriguez-Pallares et al., 2007). In this context, retinol was suggested to have antioxidant properties (Dao et al., 2017; Shi et al., 2018) and the generation of an antioxidant environment was the original hypothesis for the rationale for the present work. However, we were unable to detect significant changes in the parameters of oxidative damage, as opposed to previous observations (Real et al., 2017; Xie et al., 2018). In these and other works, 6-OHDA administration into the striatum was observed to produce significant changes in lipoperoxidation and protein carbonylation levels, among other redox-associated modifications. Here, we injected 6-OHDA directly into the SN and this tissue was collected 15 days after injection. Most studies that evaluated oxidative stress in the 6-OHDA-induced dopaminergic denervation model have been performed with striatal injection, so it is a possibility that the effects over redox parameters may differ between protocols.

Neuroinflammation is a phenomenon that is observed during progression of several psychiatric and neurodegenerative disorders, such as depression, schizophrenia, Alzheimer's disease and is a common factor reported in PD progress (Taylor et al., 2013; Wang et al., 2015a). Also, glial activation and subsequent release of neurotoxic proinflammatory factors are believed to play an important role in the pathogenesis of several neurological disorders (Leonoudakis et al., 2017). Retinol has been reported as an anti-inflammatory compound (Karkeni et al., 2017), reducing the levels of pro-inflammatory cytokines (Petiz et al., 2017a, 2017b) and all-*trans* retinoic acid acted as a preventive factor against lipopolysaccharide (LPS)-induced aged neuroinflammation (Behairi et al., 2016). However, pro-inflammatory actions of retinoids have also been described; all-*trans* retinoic acid treatment in Lupus-Prone *MRL/lpr* mice resulted in astrogliosis (Theus et al., 2017), while retinol supplementation was observed to enhance TNF-α and IL-1β in Wistar rats submitted to exercise (Petiz et al., 2017b). Here, we demonstrate that retinol supplementation was able to block the increase in the levels of TNF-α and IL-1β in serum, which is suggestive of an anti-inflammatory effect; however, immunofluorescence microscopy revealed changes in morphology associated to GFAP and Iba-1 staining in the SN of Wistar rats.

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 (Wang et al., 2015b). The administration of 6-OHDA stimulated Iba-1 staining whereas retinol pretreatment prevented this effect. 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 (Wang et al., 2015b). Morphological alterations in astrocytes were evident in all groups, including the group treated only with retinol, which might suggest that retinol supplementation modulates the activation state of these cells. Analysis of the effect of retinol in GFAP and Iba-1 fluorescence of the contralateral SN, which excludes the effect of 6-OHDA, confirmed that retinol alone had a significant effect in increasing GFAP staining. In the ipsilateral side, the dramatic effect of 6-OHDA over GFAP may eclipse the statistical difference between retinol and sham groups, but the effect of retinol is similar in both sides. The release of proinflammatory mediators can be amplified by synergistic activation of astrocytes and microglia, causing an uncontrolled neuroinflammation that contributes to the loss of dopaminergic neurons (Wang et al., 2015b). However, astrocytes play a dual role in central nervous system inflammatory diseases. Depending on the degree of inflammation and the duration of stimuli, astrocytes are capable to exert an inhibitory effect on microglia, limiting neuroinflammation (Liu et al., 2011). Therefore, it is possible that retinol supplementation is able to modulate microglia activation through astrocytes, contributing to inhibition of microglia responses and limiting neuroinflammation. More studies are necessary to elucidate the role of retinol in astrocytes function.

Previous works demonstrated that retinol supplementation may induce several toxic effects to peripheric organs and brain structures. These effects are generally associated to the intake of high doses and are related to pro-oxidant actions leading to severe oxidative stress (de Bittencourt Pasquali et al., 2012; De Oliveira and Moreira, 2008; de Oliveira et al., 2008). Taking these previous works into account, we opted to test the effect of oral supplementation using a dose described in the intake recommendations for vitamin A and other nutrients provided in the Dietary Reference Intakes developed by the Food and Nutrition Board (FNB) at the Institute of Medicine of the National Academies (Anon, 2001, 2016; Trumbo et al., 2001). We also performed assays to quantify the amount of retinol in liver and serum of the animals. Retinol serum concentration is maintained in homeostasis if organ stores are not significantly compromised (de Pee and Dary, 2002). As the liver is the major organ responsible for storage of retinol, liver retinol content is a more reliable indicator of vitamin A status of a given individual (Tanumihardjo et al., 2016). Serum retinol levels were within the physiological range (reference range 30–72 μg/dL) (Maqbool et al., 2008) in all groups of animals evaluated in this work. As expected, liver retinol quantification demonstrated higher levels in the

animals subjected to retinol supplementation compared to other groups, indicating that the present protocol of oral supplementation was effective and increased the levels of vitamin A in the body. Besides, no alterations were found in liver lipid peroxidation, protein carbonylation and thiol oxidation, confirming that retinol supplementation at 3000 IU/kg/day did not induce oxidative damage, which is in agreement with data from previous work (de Oliveira et al., 2009).

In conclusion, findings from this study reveal that a protocol of oral supplementation for 28 days with retinol using a daily recommended dose of retinyl palmitate (3000 IU/kg/day) did not prevent dopaminergic denervation in the SN of Wistar rats subjected to unilateral injection of 6-OHDA. Evaluation of cellular morphology and staining intensity associated to GFAP and Iba-1 indicates that retinol supplementation is capable to modulate astrocytes and microglia and that these cells respond differently to 6-OHDA insult in supplemented animals. Systemic measures indicated that this protocol of supplementation was not toxic for the animals, which raises concerns about possible silent neurotoxic effects taking place in the brain, as GFAP levels were significantly increased by retinol alone. Precaution in the interpretation of current data is recommended when considering the clinical context of PD and other neurodegenerative conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.002.

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