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JEFERSON DELGADO DA SILVA JUNIOR

Efeitos do retinol (vitamina A) frente à citotoxicidade causada pela 6-hidroxidopamina em células SH-SY5Y

Orientador: Prof. Dr. Daniel Pens Gelain

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Resumo

Retinol (vitamina A) é um componente essencial na dieta, devido a suas ações biológicas variadas nos tecidos, incluindo o sistema nervoso central. O retinol age como anti ou como pró-oxidante, a depender do ambiente, uma propiedade chamada redox-ativa. A doença de Parkinson (DP) é uma patologia predominantemente relacionada à idade na qual neurônios dopaminérgicos na substância negra são progressivamente denervados. A 6-hidroxidopamina (6-OHDA) é uma neurotoxina capaz de induzir parkinsonismo *in vivo*. Neste trabalho, avaliamos se o retinol é capaz de prevenir a injúria causada por 6-OHDA na linhagem celular de neuroblastoma humano SH-SY5Y, um modelo de estudo da DP *in vitro*. As células foram pré-tratadas com retinol 1 e 5 μM 1 h antes de receber 6-OHDA 30 e 50 μM e as análises foram feitas após 24 h. A viabilidade celular foi avaliada pelos ensaios de incorporação de suforrodamina e de atividade de lactato-desidrogenase no meio de cultura; efetuamos ensaio de DCFH para produção de espécies reativas (ER); ELISA e Western blot para o imunoconteúdo de 4-hidroxinonenal (4-HNE) e nitrotirosina (nitroTyr). Além disso, foram realizados ensaios espectrofotométricos para conteúdo de grupos sulfidril (SH) e atividade de catalase (Cat), superóxido-dismutase (SOD), glutationa-peroxidase (GPx) e glutationa-S-transferase (GST). O retinol foi incapaz de prevenir a morte celular provocada pela 6-OHDA, assim como a produção de ER e dano oxidativo. A atividade de Cat e GPx não sofreu alteração em nenhum grupo experimental. A atividade de SOD aumentou no grupo retinol + 6-OHDA em relação ao controle, enquanto a atividade de GST aumentou no grupo somente retinol em relação ao grupo somente 6-OHDA. Concluímos que o retinol é incapaz de conferir neuroproteção neste modelo de estudo da DP, e estudos futuros com outros modelos são necessários para obtermos uma conclusão sobre as propriedades anti ou proneurodegenerativas do retinol em relação à DP.

Neurochemical Research

Retinol Effects Against 6-Hydroxydopamine-Induced Citotoxicity in SH-SY5Y Cells

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Abstract

Retinol (vitamin A) is an essential component in diet due to several biological actions in tissues, including central nervous system. Retinol acts either as anti or as prooxidant, depending on the environment, a property called redox-active action. Parkinson's disease (PD) is a predominantly age-related pathology in which dopaminergic neurons in the substantia nigra are progressively denervated. 6-hydroxydopamine (6-OHDA) is a neurotoxin able to induce parkinsonism *in vivo*. Here we tested if retinol prevents 6-OHDA-injury in SH-SY5Y human neuroblastoma cell line, a model for PD study *in vitro*. Cells were pretreated with retinol 1-5 µM 1 h before receiving 6-OHDA 30-50 µM. Analyses were performed after 24 h. We evaluated cell viability by sulforhodamine incorporation and lactate-dehydrogenase activity in culture medium assays; we performed DCFH assay for reactive species (RS) production; ELISA and Western blot for 4-hydroxynonenal (4-HNE) and nitrotyrosine (nitroTyr) immunocontents; and spectrophotometric assays for sulfhydryl (SH) groups content, as well as catalase (Cat), superoxide-dismutase (SOD), glutathione-peroxidase (GPx), and glutathione-S-transferase (GST) activities. Retinol was unable to prevent 6-OHDA-induced cell death, as well as RS production and oxidative damage. Cat and GPx activities did not change in any experimental group. SOD activity was increased in retinol + 6-OHDA group relative to control. GST activity was increased in retinol alone group relative to 6-OHDA alone group. We conclude that retinol is unable to confer neuroprotection in this PD study model, and future studies with other PD models are needed to have a better conclusion about retinol anti or proneurodegenerative properties in PD.

Introduction

Vitamin A is a group of related essential molecules in our diet, which comprises forms such as retinol and retinoic acid. Retinol is the most abundant form of vitamin A and is characterized by a hydrocarbon chain with conjugated double bonds with an alcohol group. Retinoic acid is the carboxylic acid form of the vitamin A. Vitamin A is a necessary factor in a wide range of biological processes, such as cell differentiation [1], including neuronal differentiation [2-3], development and maintenance of central nervous system [4-5], and immune system correct function [6]. Vitamin A, through its several

forms, has cellular targets such as intracellular receptors, which act directly on the DNA or in a non-genomic way, activating cell signaling cascades leading to various cell processes [7]. Also, vitamin A exhibits antioxidant or prooxidant activities depending on the microenvironment where it is found, a property called redox-active action [7].

Parkinson's disease (PD) is one of the most common neurodegenerative diseases in the world. It is associated with senescence and its frequency increases with age [8]. Its main hallmarks are molecular aggregates of α-synuclein, called Lewy's bodies, and denervation of the nigrostriatal tract [9], which main consists by dopaminergic neurons. The latter is directly responsible for the principal symptoms experienced by patients, such as akinesia, bradykinesia, muscular rigidity, difficulties in speaking and walking, tremors and so on. Therefore, PD is a motor-based pathology [10]. The symptoms start when approximately 50 to 80 % of the dopaminergic neurons are lost [11]. Its etiology is not well-established, however, it is known that the nervous tissue of the patients is found prooxidant, with activated inflammatory status [12]. Also, mitochondrial failure is a process linked to PD [13].

One of the major aims of scientists so far is to investigate substances which could prevent PD establishment in humans. For this, PD models were created to facilitate the investigations. One of those models is the 6-hydroxydopamine (6-OHDA)-induced denervation of the nigrostriatal tract. 6-OHDA is a dopamine analogue [14] and enters cells through dopamine transporters [15]. 6-OHDA exerts its neurotoxic effects by inhibiting mitochondrial complex I, leading to respiratory chain impairment and RS formation in mitochondria [16, 17]. Also, 6-OHDA itself is able to catalyze RS generation in cells [16]. In Wistar rats, intracranial administration of 6-OHDA leads to parkinsonism, and this neurotoxicity model is common in PD studies [14, 18]. *In vitro* models were also created, and SH-SY5Y cell line is a very popular model for PD [19]. This cell line is derived from human neuroblastoma and is able to express tyrosine-hydroxylase, the rate-limiting enzyme for catecholamine synthesis [20]. Neuroprotection studies with this cell line are very common and can fit for the first step to investigate antineurodegenerative potential of vitamin A.

PD is a cureless condition and demands for novel prevention strategies. Retinol is an essential vitamin abundant in diet and exhibits redox-active properties. Oxidative stress is a condition linked to PD, and RS are produced by 6-OHDA. With those statements, we hypothesize that retinol could prevent 6-OHDA-induced injury in SH-

SY5Y human neuroblastoma cell line, supporting further studies with retinol neuroprotection against PD.

Materials and Methods

Chemicals

All-trans retinol, 6-hydroxydopamine hydrobromide (6-OHDA), dimethyl sulfoxide (DMSO), ascorbic acid, sulforhodamine B (SRB), 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA), Tween-20, β-mercaptoethanol, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), epinephrine, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH), t-butil-hydroperoxide (tBHP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture reagents were purchased from Gibco (Invitrogen Corporation, Carlsbad, CA, USA) and were of cell culture grade. Lactate-dehydrogenase (LDH) activity assay kit was obtained from LabTest Diagnóstica (Lagoa Santa, MG, Brazil). Sodium dodecyl sulfate (SDS)-polyacrilamide gel electrophoresis (PAGE) reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA), nitrocellulose membrane (Hybond ECL), enhanced chemiluminescence kit (ECL plus). Primary antibodies anti-4-hydroxynonenal (ab46545) and anti-nitrotyrosine (ab7048) were purchased from Abcam (Cambridge, UK).

Cell Culture and Treatment

Human SH-SY5Y neuroblastoma cell line was purchased from European Collection of Cell Cultures (ECACC). Cells were grown in Dulbecco's Modified Eagle Medium with Ham's F12 Medium (DMEM-F12) (1:1) supplemented with 10% inactivated fetal bovine serum (FBS) plus 1% antibiotic/antifungal mix, in a 37° C, 5% CO_2 humidified cell incubator. Culture medium was replaced every 2-3 days, until 90% confluence was reached, then cells were cultured in 6- (Western Blot and ELISA experiments) or 96-wells plates (SRB, LDH and DCFH-DA assays) or 25 cm^2 flasks (sulfhydryl groups, Cat, SOD, GST and GPx assays) until 70% confluence for the experiments. Retinol and 6-OHDA solutions were prepared immediately before experiments and kept protected from light and heat during all procedures. Retinol was dissolved in DMSO and its concentration was determined by absorbance at 325 nm. 6-OHDA was dissolved in 0,01% ascorbic acid in saline solution to 10 mM. Concentrated

solutions were added to the wells, in order to reach the final concentrations. Vehicle final concentrations did not exceed 0,01%. For the treatment (except for DCFH-DA assay), culture medium was replaced with FBS-deprived DMEM-F12 1 h before retinol administration, and 6-OHDA was administered 1 h after retinol. Retinol concentrations tested were zero, 1 and 5 µM. 6-OHDA concentrations tested were zero, 30 and 50 µM.

Cell viability by SRB and LDH assays

To assess the cell viability after retinol/6-OHDA treatment, sulforhodamine B (SRB) and lactate-dehydrogenase (LDH) activity assays were performed. SRB assay is based on the sulforhodamine incorporation to the proteins, which reflects directly the amount of adhered cells in the well. LDH assay measures the enzyme's activity in the culture medium in which the treatment was performed, then the cell death was evaluated. For the SRB assay, cells were treated and the culture medium was removed after 24 h. The cells were fixed with 10% trichloroacetic acid solution for 1 h, washed with distilled water and left to dry. 100 µL of SRB solution (0,4% SRB in 1% acetic acid) was added to each well by 15 min, then washed with 1% acetic acid until the excess stain was all removed. The plate was left to dry and the incorporated SRB was resolubilized with 10 mM Tris base solution. The absorbance at 515 nm was read. LDH activity assay was performed by the manufacturer's instructions. Briefly, a solution containing pyruvate plus reduced β-nicotinamide adenine dinucleotide was added to the cell-free culture medium where the cells were treated and the absorbance was monitored for at least 3 min at 340 nm and 37° C. Results were expressed as the percentage of absorbance relative to average absorbance of control group (non-treated cells).

Production of intracellular ROS by DCFH assay

For DCFH-DA assay, the culture medium was replaced with 1% FBS DMEM-F12. DCFH-DA was added to the cells and left to incorporate for 1 h. The DCFH-DA-containing medium was discarded, and FBS-deprived DMEM-F12 was added to the cells, along with retinol plus 6-OHDA, or vehicle, to prevent any early intracellular DCFH-retinol interactions. The fluorescence was read at 1, 4 and 24 h of treatment with an emission wavelength at 532 nm and excitation wavelength at 485 nm. SRB assay was then performed to evaluate the cell viability after DCFH-DA assay. Results were divided by SRB units (1 SRB unit was assumed to be the average absorbance of control group) and expressed in arbitrary units.

Tyrosine nitrosylation and 4-hydroxynonenal (4HNE) production by WB and ELISA

To assess the amount of nitrotyrosine (nitroTyr) and 4-HNE generated after treatment, enzyme-linked immunosorbent assay (ELISA) and Western Blot (WB) were performed. Tyrosine nitrosylation is resulted by the generation of reactive nitrogen species (RNS) in the cell, whereas 4-HNE production and consequent linkage to proteins is resulted by lipid peroxidation. Therefore, both assays evaluate the oxidative stress in the cell. Cells were treated in 6-wells plates by 24 h. For ELISA, cells were scraped in PBS and protein content was assessed by the method described by Lowry et al. [21]. Then, the samples were normalized to have the same protein concentration. For the experiment, 10 µg of protein were added to a 96-wells plate and the volume was completed to 100 µL with PBS. The plate was left to adsorb overnight. The well supernatant was discarded, and samples were washed three times with wash buffer (0,05% Tween-20 in PBS 10 mM). The primary antibodies diluted 1:4000 in wash buffer were added to the wells (100 µL) and left overnight. The supernatant was discarded and the plate was washed three times with wash buffer. The horseradish-containing secondary antibodies (anti-rabbit for 4-HNE and anti-mouse for nitroTyr) diluted 1:2000 were added to the wells (100 µL) and left 2 h. The supernatant was removed and 100 µL of ELISA substrate solution was added and left to turn blue for 5 min. After, 2 M sulfuric acid was added to stop the reaction and the absorbance was read at 450 nm. Results were expressed as percentage of absorbance relative to the average absorbance of the control group. For WB, cells were scraped in 2% SDS containing buffer and protein content was assessed by the method described by Lowry et al. [21]. Then, the samples were normalized to have the same protein concentration. Sample buffer (20% glycerol + 1 M Tris base + 4% SDS + 0,1 M dithiotreitol + bromophenol blue 0,2%) plus 10% β-mercaptoethanol were added, and samples were boiled 100° C for 5 min. Samples were migrated in 12 % poliacrylamide SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford) with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Protein content and electrophoresis success were evaluated with Ponceau S staining, and the membranes were washed with TTBS (0,1% Tween-20 plus 0,2 M Tris base in saline) until the stain disappeared. The membranes were blocked with 5% bovine serum albumin for 1 h and washed three times with TTBS. The membranes were exposed overnight to the primary antibody (1:1000) diluted in TTBS. Then, the

membranes were washed with TTBS three times and exposed to the secondary antibody (anti-rabbit 1:2000 for 4HNE and anti-mouse 1:2000 for nitroTyr) for 2 h. The membranes were washed three times with TTBS and exposed to the Supersignal West Pico Chemiluminescent kit (Thermo Scientific). The immunocontent of 4-HNE and nitroTyr was detected by enhanced chemiluminescence with a CCD camera (GE ImageQuant LAS 4000). Quantitative analysis was performed with Image J. software. The membranes were washed with TTBS and exposed to β-actin primary antibody and the protocol was repeated. To evaluate the relative immunocontent of nitroTyr and protein-linked 4HNE, the protein β-actin was selected for varying the least with the treatments. Results were expressed as β-actin-4-HNE/total β-actin and β-actin-nitroTyr/total β-actin immunocontent, in arbitrary units.

Sulfhydryl (SH) groups assay

To assess the levels of SH groups in proteins, cells were seeded in 25 cm^2 flasks, treated, and scraped in PBS. Protein content was measured by a colorimetric method developed by Lowry et al. [21], and samples were normalized to have the same protein concentration. For the experiment, 30 µg of protein were exposed to 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) for 1 h and the absorbance was read at 412 nm. Results were expressed as µmol SH groups/mg protein.

ROS-detoxifying enzymes activity assays

To evaluate the activity of catalase (Cat), superoxide-dismutase (SOD), glutathione-peroxidase (GPx) and glutathione-S-transferase (GST), spectrophotometric assays were performed, according to each enzyme analyzed. SOD detoxifies superoxide anion to hydrogen peroxide, which is decomposed by Cat to H_2O and O_2. GPx decomposes H_2O_2 by using GSH, generating oxidized glutathione (GSSG). GST detoxifies xenobiotics by using GSH. Cat activity assay consisted of adding 1 M hydrogen peroxide to the samples. Absorbance was monitored for 5 min (15-15 s) at 240 nm. Results were expressed as Cat units (U Cat)/mg protein (1 U Cat is the decomposition of 1 µmol H_2O_2 per minute at 25° C in pH 7.0, mediated by Cat). SOD activity was determined by measuring the decomposition of adrenaline to a chromogen mediated by superoxide, which is inhibited by SOD activity. Different concentrations of protein were incubated with glycine buffer and adrenaline and the absorbance was measured for 20 min (15-15 s) at 480 nm and 32° C. Results were expressed by SOD units (U SOD)/mg

protein (1 U SOD is the SOD activity which inhibits 50% the formation of the chromogen). GST activity assay was performed by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). CDNB and GSH were added to 30 µg of protein, and the formation of the CDNB-GS conjugate was monitored by absorbance at 340 nm by 5 min (15-15 s) at 32° C. Results were expressed as GST units (U GST)/mg protein (1 U GST is the generation of 1 µmol CDNB-GS per minute, mediated by GST). GPx activity was determined indirectly by detecting the decomposition of NADPH mediated by glutathione-reductase, which converts GSSG to GSH. GPx detoxifies H_2O_2 by converting GSH to GSSG. Then, the higher the GPx activity is, the faster is NADPH decomposed by the GR reaction. GSH, GR, NADPH and t-butil hydroperoxide (tBHP) were added to 30 µg protein and the absorbance was monitored at 340 nm (15-15 s) for 10 min, at 37° C. Results were expressed as µmol NADPH decomposed per minute per mg protein.

Statistical Analysis

Results were expressed as the mean ± SEM with 3-6 replicates according to the assay. D'Agostino and Pearson test was applied for normality check. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. Analyses were performed using the GraphPad software® (San Diego, CA; version 7.00). Differences were considered to be significant when $p < 0.05$.

Results

Retinol did not prevent cell death and RS production induced by 6-OHDA

To test our hypothesis, SH-SY5Y cells were pretreated with retinol 1 or 5 µM for 1 h before addition of 6-OHDA 30 or 50 µM in FBS-deprived DMEM-F12. It was shown that retinol 5 µM was the highest concentration which preserved cell viability [22], therefore it was the highest concentration chosen in this study. The cell viability was evaluated after 24 h treatment by SRB incorporation and LDH activity assays. SRB incorporation assay is a useful method to measure the cell survival after treatment, since sulforhodamine stains the cell proteins adhered to the well bottom. LDH assay is based on the measurement of the enzyme's activity on the cell-free culture medium where cells were treated. LDH is leaked free to the culture medium when cell membrane is ruptured, so this assay is a useful cell death measurement. In our experiments, SRB and LDH

activity assay results corresponded faithfully to each other. We observed that 6-OHDA 30 or 50 µM alone decreased cell viability in a concentration-dependent manner (Figs. 1A-D). Retinol 5 µM alone increased cell viability by LDH assay (Fig 1D), but not in SRB assay (Fig. 1C). Retinol 1 µM did not change cell viability significantly (Figs. 1A and B). Also, vehicle group did not change cell viability significantly. There was no significant change in SRB stain or cell-free LDH activity with the addition of retinol 1 or 5 µM 1 h before 6-OHDA 30 or 50 µM, relative to 6-OHDA alone groups (Figs. 1A-D).

We evaluated the reactive species (RS) production in SH-SY5Y treated with retinol 5 µM plus 6-OHDA 30 µM by the oxidation of DCFH. This assay was made in a cotreatment regimen, since retinol could interact with DCFH before 6-OHDA addition. Briefly, DCFH-DA was added to the culture, the DCFH-DA-containing medium was discarded following 1 h and fresh medium was added to the culture, along with retinol plus 6-OHDA. DCFH oxidation was analyzed 1, 4 and 24 hours after treatment started. We observed a marked increase in DCFH oxidation in 6-OHDA group in all times analyzed, relative to control (Figs. 2A-C). Retinol + 6-OHDA group was not significantly different from 6-OHDA group (Figs. 2A-C). Surprisingly, retinol alone group was not significantly different from control group (Figs. 2A-C). We also compared each treatment along the different times analyzed (Fig. 2D). There was a little increase in DCFH oxidation in both control and retinol groups, and a large increase in 6-OHDA alone and retinol + 6-OHDA, and these groups were very alike (Fig. 2D).

Retinol did not prevent 6-OHDA-induced changes in oxidative stress parameters

To evaluate if retinol could protect SH-SY5Y cells from 6-OHDA-induced damage through lipid peroxidation and RNS production, we pretreated the cells with retinol 5 µM for 1 h before 6-OHDA 30 µM addition, and after 24 h cells were homogenized and normalized, so we performed an ELISA and Western blot for 4-hydroxy-nonenal (4-HNE) and nitrotyrosine (nitroTyr). 4-HNE is a lipid peroxidation product which can be found free or linked to proteins. Tyrosine can be nitrosylated spontaneously by RNS both in free form and as a peptide residue. Our results showed that total 4-HNE and nitroTyr levels did not change in any group relative to control (Figs. 3A and B). Then, we evaluated 4-HNE and nitroTyr groups specifically on β-actin, relative to total β-actin. This analysis indicates the levels of 4-HNE groups and tyrosine nitrosylation in an abundant, constitutive protein, β-actin, and might reflect faithfully the

damage by which the cell is submitted. We found that β-actin-linked-4-HNE immunocontent increase in both 6-OHDA alone and retinol + 6-OHDA groups, relative to control, and there is no significant difference between these groups (Fig. 3C). Interestingly, retinol alone group showed some tendency to increase β-actin-linked-4-HNE immunocontent ($p = 0.0548$), relative to control group (Fig. 3C). It was observed a large increase in β-actin-nitroTyr immunocontent only in retinol + 6-OHDA group, relative to control (Fig. 3D). 6-OHDA alone group showed some tendency to increase its β-actin-nitroTyr immunocontent relative to control, though it was not statistically significative ($p = 0.0572$; fig. 3D). Additionally, we performed a sulfhydryl (SH) groups spectrophotometric assay, based on the reaction of DTNB with SH groups, with formation of TNB, a yellow-colored product. We could not find any difference in total SH- groups in any of the groups (Fig. 3E).

Retinol modulates the activity of SOD and GST, but not Cat and GPx

SH-SY5Y cells were pretreated with retinol 5μM 1 h before the administration of 6-OHDA 30 μM, a potent neurodegenerative toxin, and the activity of catalase (Cat), superoxide-dismutase (SOD), GST (glutathione-S-transferase) and GPx (glutathione-peroxidase) were analyzed after 24 h treatment. These analyses are based on spectrophotometric readings of H_2O_2 decomposition, adrenaline-originated chromogen formation, CDNB-glutathione formation, and NADPH decomposition, respectively. Surprisingly, SOD activity increased significantly only in retinol + 6-OHDA group, relative to control (Fig. 4A). Interestingly, GST activity was significantly different only between retinol alone and 6-OHDA alone groups (Fig. 4B). Neither Cat, nor GPx activity assays showed significant difference among groups (Figs. 4C and D).

Discussion

Retinol, or vitamin A, is an essential factor necessary for the nervous system development and maintenance [4,5], as well as many other functions. Literature has attributed retinol a redox-active ability, due to its both anti and prooxidant potentials [22, 23]. It is well-known that prooxidant microenvironments favor the outbreak of neurodegenerative diseases [12], such as Parkinson’s and Alzheimer’s diseases. Therefore, antioxidants may protect the cells against RS injury. So far, researchers looked

forward substances that could prevent or delay neurodegenerative diseases, and retinol was pointed as candidate. Due to its abundance in diet and moderate physiological concentrations in cells [24], its role in relation to Parkinson's disease (PD) pathogenesis deserves to be investigated. Here we used a catecholaminergic neuroblastoma cell line, SH-SY5Y, along with a potent neurotoxin, 6-hydroxydopamine (6-OHDA), to make an *in vitro* model for neurodegeneration. We tested if retinol could prevent the cell injury caused by 6-OHDA in the SH-SY5Y cell line.

Succinctly, SH-SY5Y cells were pretreated with retinol by 1 h before being exposed to the neurotoxin 6-OHDA, and analyses were performed after 24 h. SRB incorporation assay and LDH activity on culture medium assay were made to quantify cell survival and cell death, respectively. According to our results, retinol at 1 or 5 μM did not protect SH-SY5Y cells from death induced by 6-OHDA 30 or 50 μM, in both cell viability assays. To verify RS production in cells, DCFH-DA was administered 1 h before cotreatment retinol 5 μM plus 6-OHDA 30 μM. The cotreatment regimen was chosen in order to avoid any undesirable interaction between retinol and DCFH before cells were exposed to 6-OHDA, provided that retinol could act as prooxidant before 6-OHDA administration. Retinol is a redox-active molecule and it was shown that retinol alone promotes a prooxidant environment [22]. Therefore, it could oxidize DCFH before 6-OHDA and lead to mistaken results. Here we showed that retinol, at 5 μM, is unable to impair RS generation by 6-OHDA in SH-SY5Y cells. Taking into account that retinol could not prevent cell death by 6-OHDA, it is not surprising that retinol could not prevent RS generation within the cell. Including, RS generation may have caused cell death indicated by SRB and LDH activity assays, since RS are well known mediators of cell degeneration.

Provided that retinol could not protect SH-SY5Y from cell death and RS production induced by 6-OHDA, we investigated if retinol could protect cells from oxidative damage. Surprisingly, total 4-HNE immunocontent did not change in any experimental group, which is contrary to the finding that malondialdehyde levels, another lipid peroxidation product, increase with 6-OHDA alone treatment [25]. Additionally, there was no change in nitroTyr immunocontent levels in any treatment group. In addition to that, there was no significant difference in SH groups in any treatment. Nonetheless, we found differences in 4-HNE and nitroTyr immunocontents specifically in β-actin. 6-OHDA exerts its neurotoxicity by inhibiting mitochondrial complex I and by generating

itself RS in cytosol [16, 17]. The lack of increased total 4-HNE and nitroTyr immunocontent, as well as total SH groups may be explained by an increased protein turnover in cells. Damaged proteins are targeted for digestion by proteasomes and lysosomes. As RS production increases in 6-OHDA-treated cells, cell components become more oxidized, producing nitroTyr and 4-HNE residues in proteins, as well as lower SH- groups content. As oxidized protein levels increase, the general protein turnover rate increases, in order to reestablish functional protein levels and cell health. Although there is a higher protein turnover in cells, we found significant differences in 4-HNE and nitroTyr immunocontents in β-actin. B-actin is a constitutive, abundant protein, which is present in almost all intracellular environments and therefore is preferably exposed to RS generation sites, such as mitochondria. 6-OHDA inhibits mitochondrial complex I, leading to respiratory chain impairment and subsequent RS generation in mitochondria [16, 17]. Since cytoskeleton is present nearby, it may be preferably oxidized by mitochondria-produced RS. Also, cytoskeleton is present near cell membranes, then lipid peroxidation products such as 4-HNE may link preferably to it. Retinol was unable to protect cells against oxidative damage markers linked to β-actin, which is in agreement with our previous results. Shortly, 6-OHDA leads to RS production, which causes oxidative damage and increases oxidative stress in cells. Protein degradation machineries in cells may be highly active, so we could only find differences only specifically in *β-actin 4-HNE and nitroTyr immunocontent*; cell death is probably induced by this whole injury; and *retinol could not impair any of those processes*.

Given that retinol did not prevent the oxidative damage induced by 6-OHDA, we checked if the cells were at least reacting to this challenge. We evaluated the activity of enzymes linked to detoxification of RS, whose production was increased both in 6-OHDA and retinol + 6-OHDA groups and induced lipid peroxidation in cells. We found an increase in SOD activity in retinol + 6-OHDA group with no change in Cat activity. We did not find any significant change in 6-OHDA alone group, and this finding is not in agreement with literature, in which SOD activity was found to be decreased with 6-OHDA treatment [26]. SOD converts the anion superoxide to hydrogen peroxide, which is decomposed to water and O_2 by Cat. We did not observe changes in GPx activity in any group. We could only find significant differences in GST activity in retinol alone group, relative to 6-OHDA alone group. Both enzymes use GSH to depurate H_2O_2 and xenobiotics, respectively. Retinol pretreatment was able to increase SOD activity in SH-

SY5Y cells exposed to 6-OHDA. Neither Cat, nor GPx activities were increased by retinol pretreatment. Both enzymes depurate SOD-produced H_2O_2. As such, this imbalance between generation and depuration of H_2O_2 may lead to an accumulation of this ROS, leading to cell oxidative damage, although DCFH oxidation was not significantly different between 6-OHDA and retinol + 6-OHDA groups. GST activity was greater in retinol alone group in relation to 6-OHDA alone group. Probably, this enzyme is upregulated to detoxify the excess retinol by which the cells were exposed, despite no difference was found relative to control group.

Some authors have already investigated relationships between vitamin A intake and PD. Kim et al did not find any correlation between retinol serum levels and PD progression in PD patients vs control [27]. In addition, a meta-analysis carried out by Takeda et al showed no association between vitamin A and PD progression, but the authors conclude that data were insufficient to draw definite conclusions [28]. 9-cis-retinoic acid improved cell health in 6-OHDA-damaged primary cultured dopaminergic neurons and 6-OHDA-injected rats [29]. Retinoic acid, in a rotenone-rat model, was able to improve locomotor activity, but not dopamine striatal levels [30]. Vitamin A could exert antineurodegenerative properties by the form of retinoic acid, and not in the retinol form. Although, more studies are needed to confirm this.

Conclusion and Future Directions

In conclusion, all our experiments indicate that retinol was unable to prevent 6-OHDA-induced cell degeneration in SH-SY5Y cells. We could not find differences between retinol pretreatment 6-OHDA group and 6-OHDA alone group in any experiment, except for SOD activity assay, which was higher in pretreatment group. These results suggest that retinol might not be able to protect dopaminergic cells against development of PD. Despite these results, this PD study model presents some intrinsic issues. This neuroblastoma human cell line is derived from catecholaminergic neurons and is used as an *in vitro* model for PD, since dopaminergic cells are the main type affected in this pathology. Since it is a tumor-derived cell line, there are some issues in considering if this model faithfully reflects the neurodegeneration which occurs in PD. SH-SY5Y phenotype varies greatly with culture medium conditions [19, 31] and it may have influenced the retinol’s ability to cause greater effects in our study model. Also, the SH-SY5Y models of neurodegeneration do not exhibit the cellular mechanisms of PD with fidelity [19]. In this thread, retinol might actually not be ineffective or harmful to

cells in relation to PD pathogenesis. More studies are needed to conclude if retinol influences PD development. We suggest the utilization of other parkinsonism-inducing neurotoxins, such as rotenone and MPTP, and other PD study models, such as PC12 cell line, dopaminergic neuron primary culture and *in vivo* models, to help elucidate if retinol has anti or proneurodegenerative action.

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Figures

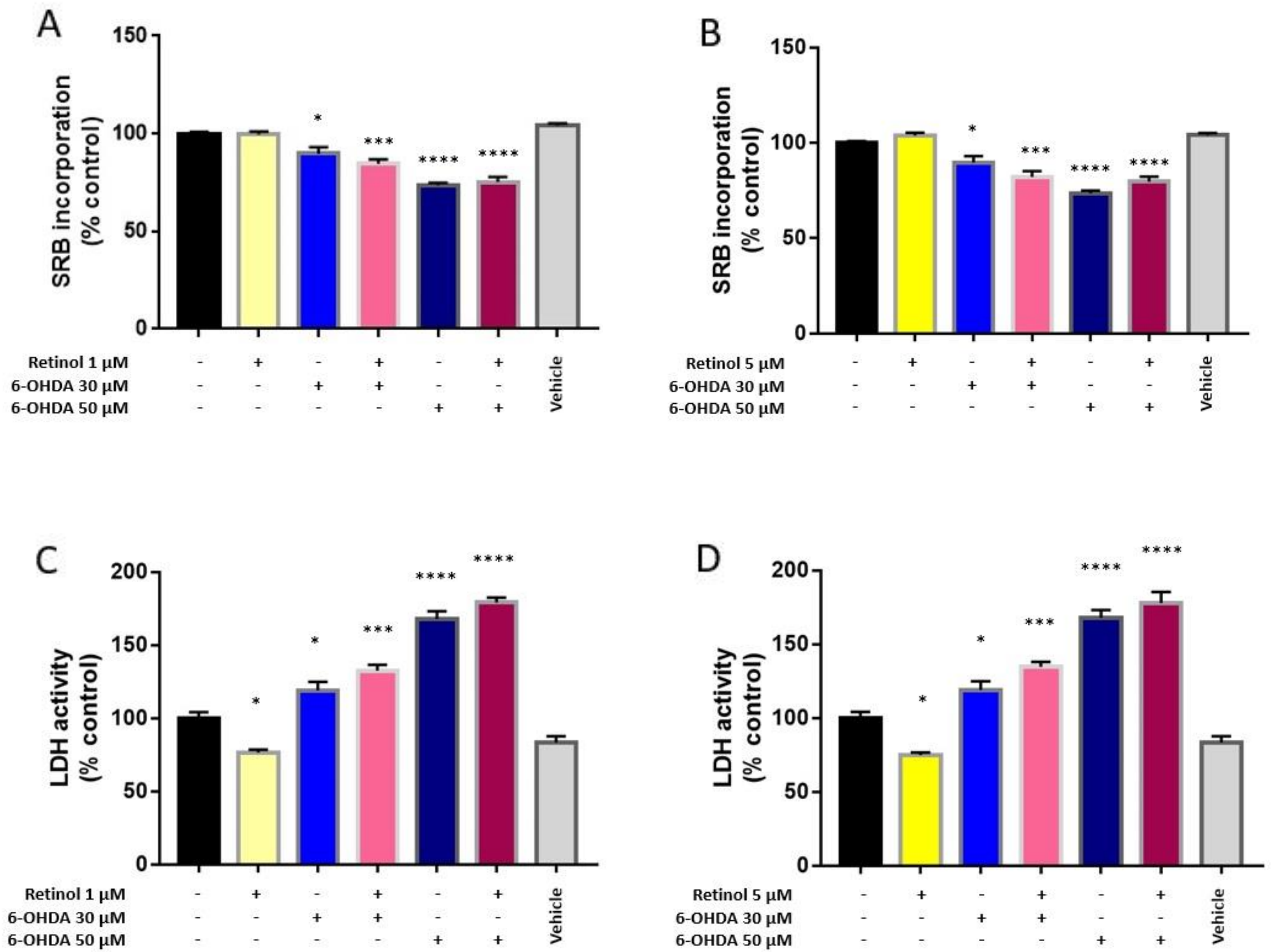


Fig. 1 Effects of retinol pretreatment against 6-OHDA challenge on SH-SY5Y line cell viability. **A** SRB incorporation in 24 h 6-OHDA-treated cells after 1 h retinol 1 µM pretreatment. **B** SRB incorporation in 24 h 6-OHDA-treated cells after 1 h retinol 5 µM pretreatment. **C** LDH activity assay on culture medium in 24 h 6-OHDA-treated cells after 1 h retinol 1 µM pretreatment. **D** LDH activity assay on culture medium in 24 h 6-OHDA-treated cells after 1 h retinol 5 µM pretreatment. Vehicle is DMSO 0.075% + NaCl 0.0045% + ascorbic acid 0.00005% (final concentrations in the well). Graphs depict mean ± SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$ compared to control group.

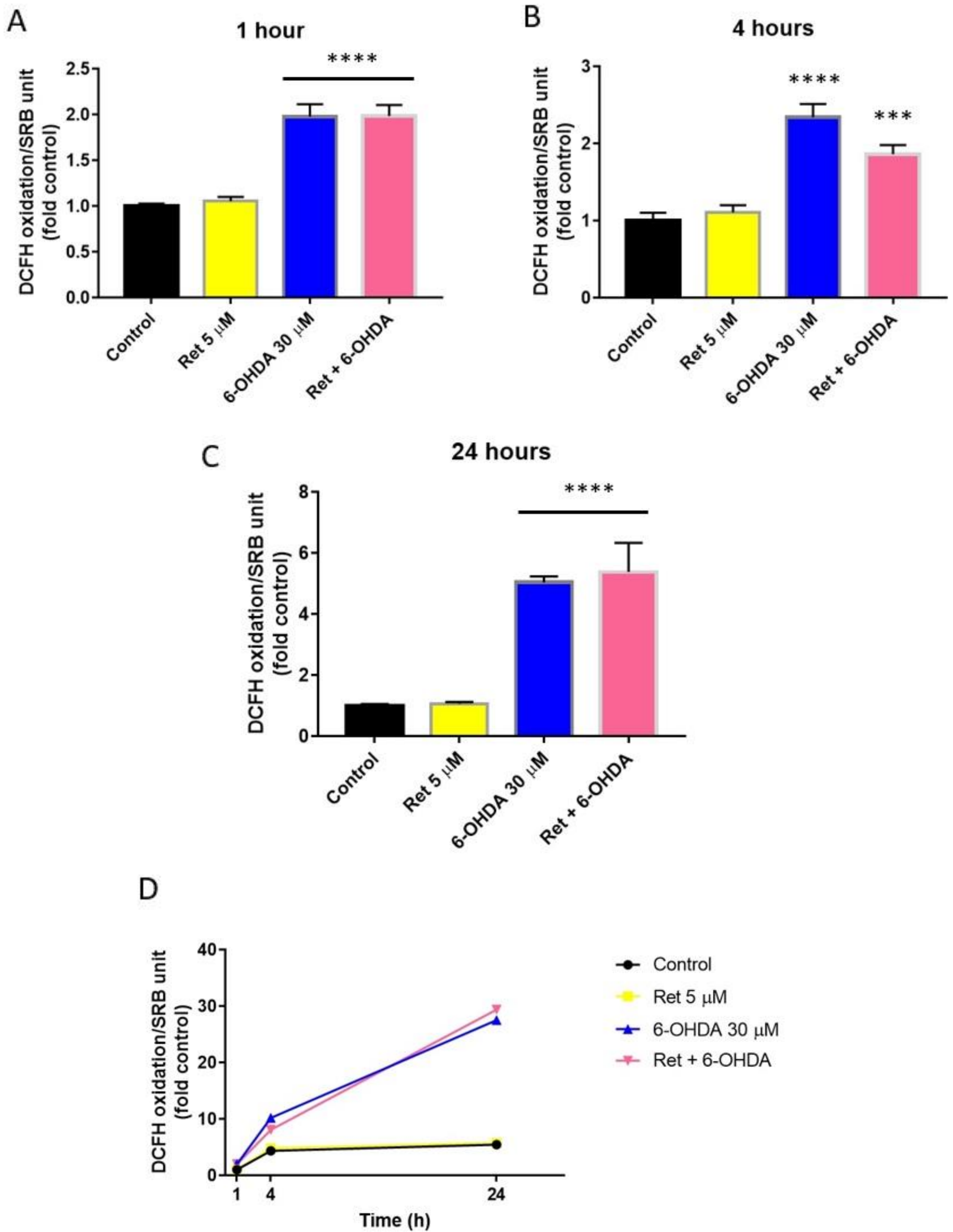
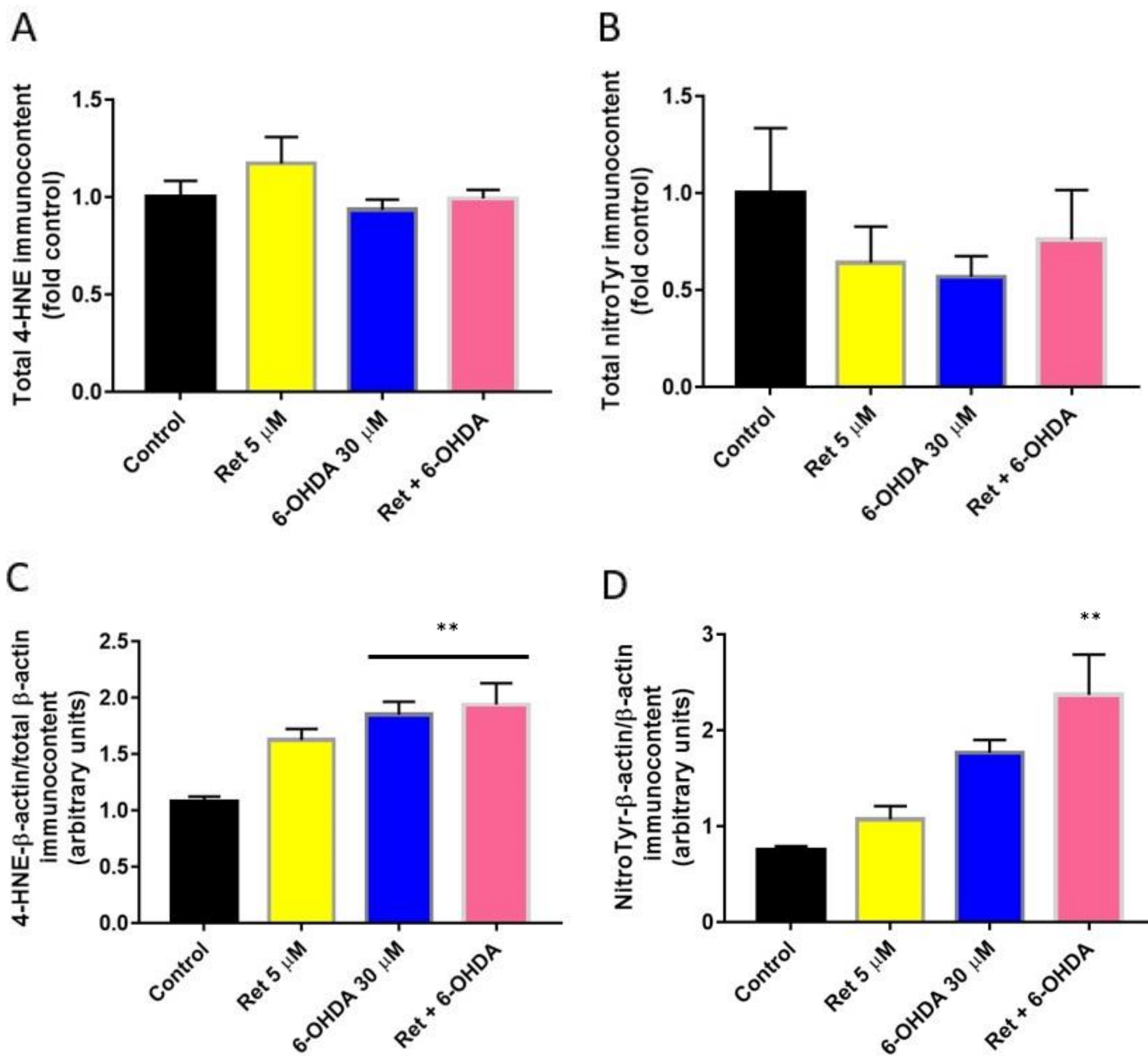


Fig. 2 Effect of retinol plus 6-OHDA cotreatment on RS production. **A** DCFH oxidation at 1 h retinol 5 µM plus 6-OHDA 30 µM cotreatment. **B** DCFH oxidation at 4 h retinol 5

μM plus 6-OHDA 30 μM cotreatment. **C** DCFH oxidation at 24 h retinol 5 μM plus 6-OHDA 30 μM cotreatment. **D** DCFH oxidation at 1, 4 and 24 h retinol 5 μM plus 6-OHDA 30 μM cotreatment. Graphs A, B and C depict mean ± SEM. Graph D depicts mean. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. ***$p <$ 0.001 and ****$p < 0.0001$ compared to control group.



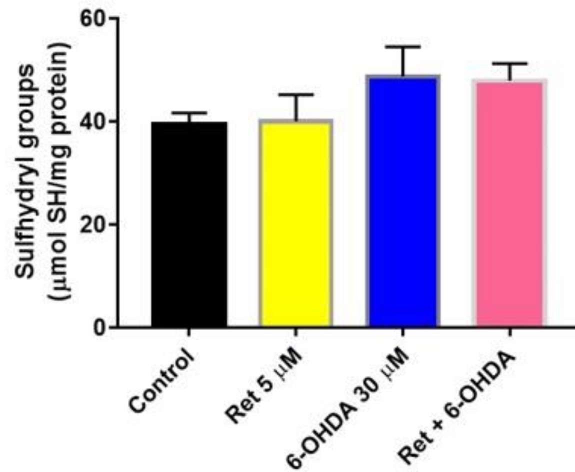


Fig 3 Effect of retinol pretreatment against 6-OHDA challenge on oxidative stress markers. Cells were pretreated with retinol 5 µM 1 h and 6-OHDA 30 µM was added by 24 h. **A** Total 4-hydroxy-nonenal immunocontent. **B** Total nitrosylated tyrosine immunocontent. **C** 4-hydroxy-nonenal residues linked to β-actin immunocontent, divided by total β-actin immunocontent. Line indicates groups in the same statistical group. **D** Nitrotyrosine immunocontent in β-actin, divided by total β-actin immunocontent. **E** Total sulfhydryl groups by TNB formation. Graphs depict mean ± SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$ and **$p < 0.01$ compared to control group.

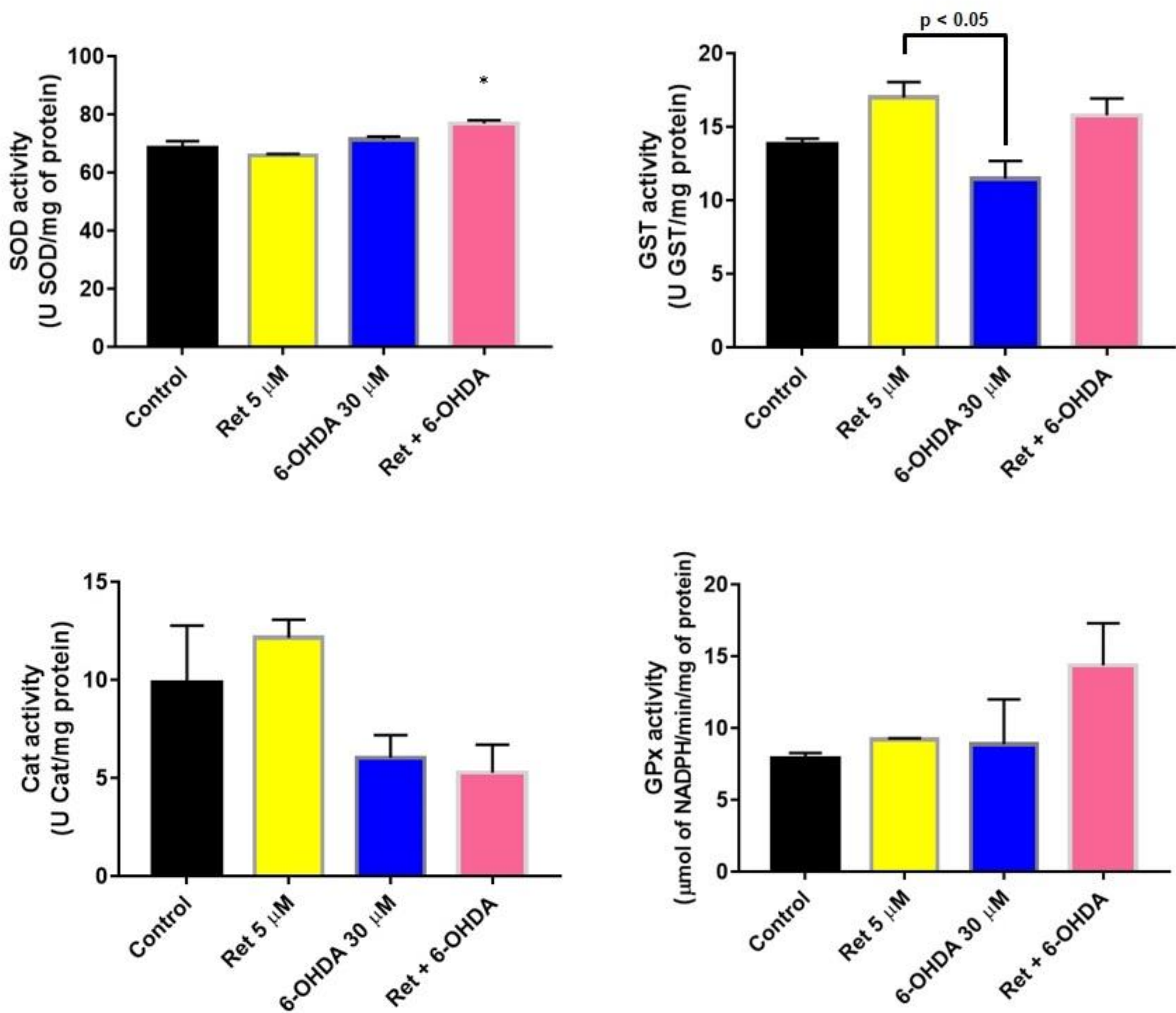


Fig 4 Effect of retinol pretreatment against 6-OHDA challenge on antioxidative enzymatic defenses. Cells were pretreated with retinol 5 μM 1 h and 6-OHDA 30 μM was added. Analyses were made following 24 h. **A** superoxide-dismutase activity measured by oxidation of adrenaline. **B** glutathione-S-transferase activity measured by CDNB-glutathione formation. **C** catalase activity measured by H_2O_2 decomposition. **D** glutathione-peroxidase activity measured indirectly by NADPH decomposition. Graphs depict mean ± SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$ compared to control group.

Anexo – normas de publicação para a revista Neurochemical Research

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