

Universidade Federal do Rio Grande do Sul

Instituto de Biociências

Comissão de Graduação em Ciências Biológicas

Trabalho de Conclusão de Curso em Ciências Biológicas

**Avaliação de Parâmetros de Viabilidade Celular e Marcadores de Neurotoxicidade  
em Linhagem de Neuroblastoma Humano SH-SY5Y Tratadas com Retinol**

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Artigo redigido no formato do periódico científico Molecular and Cellular  
Biochemistry.

Porto Alegre, 2014

## Resumo

A Vitamina A (retinol), exerce importantes funções em processos de diferenciação e crescimento, porém a suplementação com retinol é muitas vezes mal controlada. Muitos trabalhos anteriores demonstraram que o excesso ou a falta de suplementação de retinol ocasiona muitos efeitos deletérios como a alteração em tecidos epiteliais, mas essa suplementação também surge como alternativa para o tratamento da doença de Alzheimer, devido à habilidade de afetar componentes vitais dessa doença. Muitos estudos anteriores, tanto em culturas celulares quanto em modelos animais, mostraram que o retinol pode induzir a produção de radicais livres, o estresse oxidativo e dano em biomoléculas. Aqui nós objetivamos analisar os efeitos causados pelo retinol em parâmetros de viabilidade celular e marcadores de neurotoxicidade em um modelo celular de neuroblastoma humano (SH-SY5Y). As células SH-SY5Y foram tratadas com doses fisiológicas de retinol (1, 2.5 e 5 $\mu$ M) e doses maiores (7, 10 e 20 $\mu$ M). O retinol, nas concentrações 7, 10 e 20 $\mu$ M, aumentou a produção de radicais livres e diminuiu a viabilidade celular. A dose de 7 $\mu$ M aumentou a expressão de P-TAU e a dose de 10 $\mu$ M diminuiu a expressão de RAGE, mas a co-administração do Trolox<sup>®</sup>, um antioxidante análogo da vitamina E, reverteu o efeito causado pelo retinol em P-TAU, diminuiu a expressão de  $\alpha$ -sinucleína e potencializou o efeito em RAGE. Levados em consideração, nossos resultados indicam um efeito pró-oxidante do retinol em células SH-SY5Y, modulando vias de sinalização celular e sugerindo um possível uso dos antioxidantes como o Trolox<sup>®</sup> para terapia e tratamento dessa doença neurodegenerativa.

Palavras-chave: Retinol, Estresse Oxidativo, RAGE, P-TAU,  $\alpha$ -sinucleína

## Evaluation of Parameters of Cell Viability and Markers of Neurotoxicity in Lineage of Human Neuroblastoma SH-SY5Y Treated with Retinol

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### Abstract

Vitamin A (retinol) plays important roles in growth and differentiation processes, but supplementation with retinol is often poorly controlled. Many previous studies have shown that excess or lack of retinol supplementation causes many harmful effects such as changes in epithelial tissues, but this supplementation also appears as an alternative for the treatment of Alzheimer's disease due to the ability to affect vital components of this disease. Many previous studies, both in cell cultures and in animal models, have shown that retinol can induce the production of free radicals, oxidative stress and biomolecules damage. Here we aimed to analyze the effects caused by retinol on parameters of cell viability and markers of neurotoxicity in a cellular model of human neuroblastoma (SH-SY5Y). SH-SY5Y cells were treated with physiologic doses of retinol (1, 2.5 and 5 $\mu$ M), and larger doses (7, 10 and 20 $\mu$ M). The retinol concentrations 7, 10 and 20 $\mu$ M, increased production of free radicals and decreased cell viability. The dose of 7 $\mu$ M increased the expression of P-TAU and 10 $\mu$ M decreased the expression of RAGE, but the co-administration of Trolox<sup>®</sup> antioxidant, an analog hydrophilic to vitamin E, reversed the effect caused by retinol in P-TAU decreased the expression of  $\alpha$ -synuclein and potentiated the effect on RAGE. Taken together, our results indicate a pro-oxidant effect of retinol on SH-SY5Y cells, modulating cell signaling pathways and suggesting a possible use of antioxidants such as Trolox<sup>®</sup> for therapy and treatment of this neurodegenerative disease.

Keywords: Retinol, Oxidative Stress, RAGE, P-TAU,  $\alpha$ -synuclein

## **1. Introduction:**

Retinol (Vitamin A) is essential for the survival of vertebrates that must be obtained from dietary food sources and their oxidation into active metabolites such as retinoic acid nutrient is essential for the life of vertebrates. The regulation of the expression of many protein-coding [1] and the regulation of a large unknown number of regulatory noncoding RNA is other function performed by retinol [2]. Studies on the effects of deficiency or excess of vitamin A [3], and the lack of relevant components of cell signaling pathways showed that retinol is essential for embryonic development [4] as well as it has a high relevance to the grocery cell phenotype in adult organisms [5, 6]. This dependence shows not only limited on embryogenesis but also remains for the entire life of the organism [6]. Studies about retinol deficiency in adult animals caused many defects in epithelial tissue, causing a metaplastic keratinization, changing the morphology [5]. Furthermore, retinol is also important for the development and keeping pulmonary alveolar as well keeping the innate and adaptive immunity [7]. Recent studies have shown also that the components of Vitamin A also influence the adipogenesis and the obesity [7, 9]. The deficiency or excess of vitamin A in humans could represent a significant medical problem in the developed world, however this question require further studies to improve the knowledge regarding this issue [6]. Several studies have demonstrated that the vitamin A has a very important regulatory role, being responsible for the regulation of fertility, maintenance of vision and prevention of neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's diseases [4]. Moreover, vitamin A is synthesized in greater amounts in the central nervous system (CNS) and regulates a number of genes responsible in large part for the development and maintenance of the CNS functions [10, 11].

Patients diagnosed with AD disease have low levels in the serum and plasma of vitamin A and  $\beta$ -carotene [12]. Furthermore, it was reported that among 442 patients aged over 65 years who had a  $\beta$ -carotene supplementation showed better memory performance [12].

Nowadays, the concerns with memory loss and loss of several motor functions of the body have led the society to seek treatments and therapies to combat these neurodegenerative diseases. Since then, scientists had been studying more deeply the AD, because the elderly human population is increasing significantly through the years [12]. AD is a chronic neurodegenerative disease characterized by the accumulation of neuritic plaques and neurofibrillary tangles, being related to the accumulation of amyloid- $\beta$  peptide ( $A\beta$ ) in brain tissues and cytoskeletal changes due to hyperphosphorylation of neuronal proteins, such as Tau protein. Those molecular modifications are considered the main hallmarks in this disease [13]. Currently, several evidences showed the relevance of oxidative stress as a pivotal factor for pathogenesis of AD and its association with the molecular hallmarks that potentiates the redox imbalance and consequently the neuronal damage [14, 15]. It has been found that retinoids have very important roles in the signaling pathways of inflammation, and elevated inflammatory responses are associated with cognitive decline and neuronal loss observed in animal models and patients with DA [16]. However, treatments and therapies by using retinoids can reduce proinflammatory gene expression and can prevent mitochondrial dysfunction and oxidative damage [17]. Previous studies showed that there is a strong relationship between the disruption of signaling retinoid compounds and the occurrence of Alzheimer's disease [18, 19]. Thus, retinoids can be promising alternatives in therapeutics and treatment of Alzheimer's disease due to the ability to affect the vital components of the disease such as preventing the formation of

amyloid plaque, inflammation and expression of related proteins as RAGE, TAU hyperphosphorylation and amyloid beta (A $\beta$ ) accumulation [17].

The study of inflammation related proteins as RAGE, TAU and  $\alpha$ -synuclein is relevant for better understanding the mechanisms of the development of this neurodegenerative disease. RAGE is a member of the immunoglobulin superfamily that can bind to other ligands such as AGEs and  $\beta$ -amyloid proteins [20]. The interaction of these ligands and receptors can activate transduction signals pathways of cellular inflammation, death and disruption of cellular functions [21]. Several studies have demonstrated that RAGE plays an important role in the pathogenesis of AD by increasing  $\beta$ -amyloid peptide [22], and consequently the neurotoxicity, leading to memory impairment of different transgenic mice models [23, 24]. Thus, activation and increased RAGE expression and other proteins related to glycation end products (AGEs) imply in acceleration of the deterioration of memory and various cerebellar synaptic functions leading to cognitive and motor impairment [25]. TAU protein is a member of the *Microtubule associated proteins (MAPs)* which is predominantly found in neurons and highly soluble, has important roles in the regulation of cytoskeletal plasticity during development of embryogenesis and cell signaling in membranes [26]. In pathological conditions this protein is highly phosphorylated assuming this way, and insoluble and aggregated form [27], disrupting the microtubule network and consequently leading to synaptic loss, another prevalent feature in AD [28, 29]. For this reason, there is great scientific interest in find accurate biomarkers to improve the early diagnosis of AD [30]. The  $\alpha$ -synuclein is a small, intricately folded and soluble protein found mainly in neurons, but it is also distributed in many cellular compartments, having a very important role in synaptic function and modulation of neurotransmitters [31]. The increased level of  $\alpha$ -synuclein in cerebrospinal fluid results from a significant expression of this protein in patients with

Alzheimer's disease [32]. Furthermore, the correlation between the decline in cognition and progression of synaptic loss in patients with Alzheimer's disease are related to increased levels of  $\alpha$ -synuclein [33]. This may be due to the fact that, physiologically, the high concentration of presynaptic  $\alpha$ -synuclein and its association with synaptic vesicles suggest a very important role in the modulation of neurotransmitters in the cerebrospinal fluid [31]. Therefore, this protein may be an important biological marker in the diagnosis and monitoring this disease [33].

Thus, for the treatment of neurodegenerative diseases was used a well-described cell culture model of human neuroblastoma SH-SY5Y cells [34], an immortalized cell line that displays an adult neuronal morphology phenotype. Moreover, RAGE, TAU and  $\alpha$ -synuclein proteins are used as molecular markers for studies with this model. Therefore, the objectives of this work was to evaluate the effects caused by retinol on cell viability parameters and markers of neurotoxicity in a model of dopaminergic neuron (cell line SH-SY5Y) used for the study.

## **2. Materials and Methods**

### **2.1 Chemicals**

Glycine, hydrogen peroxide ( $H_2O_2$ ), EDTA, trypsin, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO), diacetate 2',7'-dichlorofluoresceina (DCFH -DA), sulforhodamine (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The materials used in cell culture were purchased from Gibco® Invitrogen (Sao Paulo, SP, Brazil) and the Bank of cells of Rio de Janeiro (BCRJ, Rio de Janeiro, Brazil). Reagents and materials for electrophoresis / immunoblot were purchase from Bio-Rad Laboratories (Hercules, USA), GE Healthcare Operating Headquarter (Sao Paulo, Brazil), and Sigma. The rabbit polyclonal primary

antibody  $\beta$ -actin mouse monoclonal primary antibody Tau (Tau46), primary mouse Phospho-Tau (Ser396) monoclonal antibody and anti-rabbit and anti-mouse immunoglobulin conjugated with linked with a peroxidase were purchased from Cell Signaling (Beverly, USA). Polyclonal primary antibody rabbit RAGE was obtain from Abcam (Cambridge, UK). The primary antibody mouse monoclonal  $\alpha$ -synuclein was purchase from Sigma Chemical Co. (St. Louis, MO, USA). Immunoblot chemiluminescence detection was carry out with the West Pico detection kit from Pierce (Rockford, USA).

## **2.2 Cell Culture and Treatments**

The lineage of human neuroblastoma SH- SY5Y were obtain from Cell Bank of Rio de Janeiro ((BCRJ) Rio de Janeiro, Brazil). Cells were grown and maintained in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) inactivated with 2 mM glutamine, 0,28 mg/ $\mu$ L of gentamicin and 250 mg of amphotericin B in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. The cells were grown until they reached 80-90 % confluence and then they were trypsinized. After 24 hours of trypsinization, approximately half the volume of the old culture medium was replaced with fresh cell culture medium 10 % FBS DMEM:F12. The cell medium was replace every 2 days. All treatments were performed when the cells had approximately 75% confluence with culture medium 1 % FBS, and further incubated for 24 hours. For each test, the retinol concentrations in 1, 2.5, 5, 7, 10, and 20 $\mu$ M, respectively, were diluted in DMEM:F12 1% FBS for cell treatment.



### **2.3 MTT Assay**

On this colorimetric assay, the viability of human neuroblastoma line SH- SY5Y cells was evaluated by MTT assay as previously described [35]. This method is based on the ability of viable cells to reduce MTT (3-(4,5 -dimethyl)-tetrazolium bromide 2,5-diphenyl) and form blue formazan product . The MTT solution (sterile stock solution of 5 mg/ml) was added to the incubation of the wells with a final concentration of 0.2 mg/mL. The cells were left for 45 min at 37 ° C in an atmosphere containing 5% CO<sub>2</sub>. The medium was then removed and the plates with DMSO were stirred for 30 min. The plate was then scanned on a spectrophotometric plate (SpectraMax 190. Molecular Devices) and the quantized data reader. The optical density of each well was measured at 550 nm (test) and 690 nm (reference). The data were expressed as a percentage of the formation of formazan in untreated cells (control).

### **2.4 Sulforhodamine B Assay**

This colorimetric assay estimates cell number through protein staining of total cell with SRB [36]. First, the cells were fixed with a layer of 100 µL of 40% trichloroacetic acid cooled with ice at the top of the culture medium and incubated at 4°C for 1 h. The plates were then washed five times with cold water. The excess water was then decanted and the plates were allowed to air dry. The SRB solution (100µl; 0.4% acetic acid 1%) was added to each well and left in contact with the cells for 30 min. The cells were then washed with 1% acetic acid, rinsed four times until only the cells remained adherent dye. After drying in air, was added to the plates, then 100 µL of 10 mM Tris base reagent with pH 10.5 to each well to dissolve the sulforhodamine dye. The plates were gently shaken for 20 min on a gyratory shaker and the absorbance of each well was

measured and read at 492nm. Cell survival was measured as the percentage uptake in comparison with the absorbance of the control (untreated cells).

## **2.5 DCFH-DA Assay**

The intracellular production of reactive oxygen species was determined by DCFH-DA assay [37]. The technique is based on the ability of DCFH to be oxidized into a highly fluorescent product (DCF) in the presence of reactive oxygen species (ROS). This technique can be used as an index to measure the levels of production of free radicals in cells. Thus, the SH-SY5Y cells were seeded in 96- well plates and treated with retinol concentrations ranging from 1 $\mu$ M to 20 $\mu$ M. Subsequently, 100 $\mu$ L of a solution of DCFH-DA was dissolved in DMEM: F12 culture medium and 1 % FBS was added to each well. The cells were incubated at 37 ° C and 5% CO<sub>2</sub> to allow cellular uptake of DCFH-DA. Thereafter, this medium was discarded and new culture medium of 1 % FBS was added to each well. The 1 mM H<sub>2</sub>O<sub>2</sub> was used as a positive control in DCF fluorescence. On this endpoint technique, DCF fluorescence was read on a fluorescent plate reader at a temperature of 37°C (Spectra Max M2, Molecular Devices, USA) with emission wavelength 535nm and excitation wavelength of 485nm . Results were expressed as percentage of the fluorescence of DCF compared to the untreated control.

## **2.6 Western Blot Assay**

To perform immunoblot experiment, SH-SY5Y cells were washed with PBS (phosphate-buffered saline), lysed and resuspended in 0.25 mL 1x RIPA (radioimmunoprecipitation buffer- 20 mM Tris-HCl pH 7.5, 150mM NaCl, 1 mM Na<sub>2</sub> EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM b- glycerophosphate, 1mM in Na<sub>3</sub>PO<sub>4</sub> and 1 $\mu$ g/mL leupeptin), and centrifuged for 10 minutes (14,000xg, 4°C). The supernatant was removed to

perform protein quantification by Bradford method [38]. Later, it was added to Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% ( w/v ) SDS, 10% (v/v) glycerol) and equal amounts of cell protein (60 µg/well) were fractionated on an SDS gel and then electro- transferred to nitrocellulose membranes . The amount of protein and efficiency of the electrotransfer to nitrocellulose membranes were verified by Ponceau staining and were blocked and subsequently incubated with a buffered saline Tween-Tris (TTBS 100mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. The membranes were incubated overnight at 4°C with each antibody was incubated in TTBS separately, at different dilutions, as suggested by the manufacturers, and then washed with TTBS each membrane. Secondary antibodies anti- rabbit IgG and mouse connected to the peroxidase were dilute at 1:5000 concentration, than incubated with the membranes for 2 hours at room temperature. After this period, the membranes were washed again with TTBS, and the chemiluminescence immunoreactive bands were detected by exposure of the membranes to a film using a kit for this luminescence Chemi Super Signal West Pico (Pierce). The densitometry analysis of the bands was performed by ImageJ® software. Bands were designed to be linear in the range used for densitometry. All results were expressed as a ratio relative to the internal control β-actin or the respective total protein.

## **2.7 Statistical Analysis**

The amount of protein in the samples was quantified by normalizing the data in accordance with the method based on Bradford [38]. Statistical analyzes were performed using GraphPad® 5.0 software and the results were expressed as the mean ± SD (standard deviation) of three independent experiments (n = 3). Data were analyzed by analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were considered significant at \* p <0.05, \*\* P <0.01 and \*\*\* P <0.001.

### 3. Results

#### 3.1 Cellular Viability assay

To evaluate the effects of retinol concentrations in cell viability in the human neuroblastoma SH-SY5Y cell line, we performed MTT and SRB-based viability assays. For this, different concentrations of retinol were tested. Cells were incubated for 24 h with retinol from physiologic doses (1 to 5  $\mu\text{M}$ ) to higher doses (7 to 20  $\mu\text{M}$ ). The higher doses of retinol had a significant effect in reducing cell viability. In both MTT and SRB assays, a significant decrease in cell viability was observed with retinol 7  $\mu\text{M}$ , and an extensive cytotoxic effect was observed with 10 and 20  $\mu\text{M}$ . Vehicle (ethanol 0.2 %) had no effect (Figures 1A and B).

Considering these results, higher concentrations caused a cytotoxic effect with a prominent cell death. Previous studies observed that concentrations of retinol higher than 5 $\mu\text{M}$  caused oxidative stress and consequent loss of viability to different cell types [39- 42]. Thus, we decided to evaluate the involvement of oxidative stress in the effects of retinol in SH-SY5Y cells. For this purpose, we co-incubated cells with retinol and the antioxidant Trolox<sup>®</sup> (100  $\mu\text{M}$ ) and evaluated MTT reduction and SRB incorporation. Trolox<sup>®</sup> co-treatment blocked the cytotoxic effect of the higher concentrations of retinol (10 and 20  $\mu\text{M}$ ), as observed in both MTT and SRB-based assays. These results indicate the involvement of ROS in the loss of cell viability caused by retinol at 10 and 20  $\mu\text{M}$  (Figures 2 A and B).

#### 3.2 Intracellular ROS production

Oxidative stress commonly observed when pro-oxidants, such as free radicals, ROS, and reactive nitrogen species override cellular antioxidant systems and induce cellular damage. ROS lead to disruption of cellular membrane integrity and mitochondrial

dysfunctions. Known environmental toxins like rotenone, have been shown to induce apoptosis through cellular signaling mechanism initiated by ROS production [43]. To evaluate the production of ROS in SH-SY5Y cells incubated with retinol, we performed the real-time DCFH oxidation assay in living cells [37]. SH-SY5Y cells were treated for 0 hour, 1 hour, 4 hours and 24 hours with retinol concentrations ranging from 1 $\mu$ M to 20 $\mu$ M. Cells whose retinol treatment was higher than 5 $\mu$ M showed a significant increase in the rate of production of free radicals, suggesting that cell death is by oxidative stress originates (Figures 3 A-D). Even more, other studies demonstrated that oxidative stress is a key player in neuronal cell death [44-46].

### 3.3 Expression of RAGE, $\alpha$ -synuclein and TAU proteins

Since the brain consumes about 20 % of the body's oxygen supply, and much of it is converted into oxygen reactive species, this offer an explanation for the importance of the study and its key role in neurodegenerative diseases [47]. The literature shows many data correlating oxidative stress and production of free radicals to pathogenicity and progression of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington diseases. This mechanism is greatly contributing to neuronal degeneration, leading to accumulation and increased levels of free radicals in the cerebellar regions [48]. Thus, we decided to investigate the effect of retinol treatment on biochemical parameters of these diseases and neurodegenerative processes in a model of human neuronal cell culture. As well as vitamin D prevented the increase of RAGE in aortic vessels, inhibiting RAGE-mediated oxidative stress, causing either vessel protective effects [49]. Our results showed a downregulation of RAGE protein at a concentration of 10 $\mu$ M, enhanced by co-treatment with the Trolox<sup>®</sup> antioxidant, showing a regulation of pro-inflammatory pathways (Figures 4 A and B). Moreover, the treatment also significantly increased the immunocontent of the tau protein at a concentration of 10 $\mu$ M

retinol, while co-treatment with the Trolox<sup>®</sup> antioxidant significantly reduced its immunocontent (Figures 5 A and B). Studies based on other data showed the association between high levels of expression of TAU protein in cellular models of neuroblastoma as in M17 cells and the critical involvement of oxidative stress whose effects are attenuated by co-administration of antioxidants [50]. Finally, we decided to study the involvement of  $\alpha$ -synuclein protein which has a significant role to be associated with membranous and vesicular structures vesicle recycling and storage of neurotransmitters in synaptic transmission are critical for normal cerebellum [51]. We have found that increasing the immunocontent of  $\alpha$ -synuclein protein, along with retinol treatment, was accompanied by a significant decrease of expression when co-administered with the Trolox<sup>®</sup> antioxidant at concentrations 7 and 10 $\mu$ M (Figures 6 A and B).

#### **4. Discussion**

Other previous studies showed that under physiological cytosolic concentrations of retinol do not cause cellular damage [39, 53]. Incubation with 5 $\mu$ M retinol in Sertoli cells caused no significant morphological change [41]. However, the increase in concentration at levels above 7 $\mu$ M in cultured Sertoli cells showed a significantly increased oxidative damage caused by a pro-oxidant effect of retinol [41]. Furthermore, our results about increased cell death and the consequent decrease in cell viability in 10 $\mu$ M and 20 $\mu$ M are probably related to the activity of the regulatory mechanisms of vitamin A. It has been observed in other studies that several cellular oxidative damage parameters measured as lipid peroxidation, decreased activity of antioxidant enzymes and levels of carbonyl, increased production of free radicals at concentrations between 7 and 14 $\mu$ M [35]. Recent studies suggest that the role of retinol in different cellular systems is varied, being vital to many cellular processes and acting as a pro-oxidant

inducing cellular damage at concentrations higher than those found in the physiological conditions [40]. Because of these, we decided to test the Trolox<sup>®</sup>, an analog hydrophilic to vitamin E, aiming investigate the role of ROS in pro-oxidant scenario induced by retinol treatment. Previous studies have shown a great ability to reverse the deleterious effects of cytotoxic and Trolox<sup>®</sup> in Sertoli cells including the decrease of signaling inflammatory proteins as RAGE [42]. Furthermore, Trolox<sup>®</sup> decreased the damage in lung cells of smokers, decreasing significantly, DNA damage [53]. In addition, the effects of supplementation with Trolox<sup>®</sup> can reverse the accumulation of free radicals and reduce oxidative stress characteristics, having a neuro-protective effect in the brain of Wistar rats [54]. Thus, we decided to check the function of ROS in increased cell death and the effects caused by co-treatment of Trolox<sup>®</sup> concentrations higher than 7 $\mu$ M retinol. It was found in similar studies that the retinol at high concentrations exerts a significant increase in production of free radicals, being blocked by co-treatment with 100 $\mu$ M Trolox<sup>®</sup> in Sertoli cells [40]. Thus, our findings in cell viability regarding to the protective effect caused by Trolox<sup>®</sup>, while co-treatment with retinol, decreased the effects of cell death, corroborating the data found in previous studies. Furthermore, increased production of ROS occurred with increasing retinol concentrations, leading to decrease of cell viability, emphasizing the role of oxidative stress in such scenario. Besides, another study performed by our group used cell lung cancer (A549), found that retinol in highest concentrations (10 $\mu$ M and 20 $\mu$ M) decreased RAGE immunocontent and the immunofluorescence, indicating that this mechanism is dependent on the production of reactive oxygen species [55]. However, co-treatment with Trolox<sup>®</sup> inhibited the effect of retinol on the down-regulation of RAGE [55]. In this study, our results partially confirm this proposition, since there was also down-regulation of retinol in the highest concentration (10 $\mu$ M), but the co-treatment with Trolox<sup>®</sup> potentiated this

effect. Regarding the TAU protein, studies show that this protein has a major influence on the initial stage and progression of AD, consisting of one of the targets of various antioxidants [56]. Furthermore, a combined therapy of antioxidants may improve the synaptic plasticity during the initial stages of AD offering new options for treatment [57]. Considering this, our results showed that retinol in higher concentration (10 $\mu$ M) increased the expression of phosphorylated tau, whereas co-administration with the Trolox<sup>®</sup> down-regulated the expression of this protein. This indicates a possible protective effect of antioxidants for the treatment of this disease that could help many older people and represents a new hope. However it is necessary to have some caution in the administration and use of supplementation in high doses of retinol, because Vitamin A, when administered in high doses, induces a pro-oxidant effect, changing the redox environment of the *substantia nigra* of rats brain [58]. Moreover, high doses of retinol even shown that they can cause adverse skeletal effects, then is recommended caution in the use of high doses of retinol [59]. Finally, previous studies have indicated that melatonin has a direct and indirect effect in reducing the expression of  $\alpha$ -synuclein in postnatal rats [60]. Thus, this confirm our results about the effects of decreased expression of  $\alpha$ -synuclein when co-treatment with Trolox<sup>®</sup> in human neuroblastoma cells.

In conclusion, the results presented here demonstrate that retinol may be a possibility for use in combination with antioxidants for the treatment of AD therapies, however, the isolated use of retinol, in high concentrations, can cause loss of cell viability, favoring increase of ROS. This, in turn, is involved in various signaling proteins related to AD and co-treatment with Trolox<sup>®</sup> was able to attenuate expression of these proteins. Thus, the results obtained here will be useful for future work, suggesting that more studies be



conducted to further evaluate the effects of retinol combined with antioxidants as a new alternative for the treatment of this neurodegenerative disease.

### **Acknowledgments**

The Brazilian research funding agencies FAPERGS, CAPES, CNPq and PROPESQ-UFRGS

**Conflict of Interest** None.

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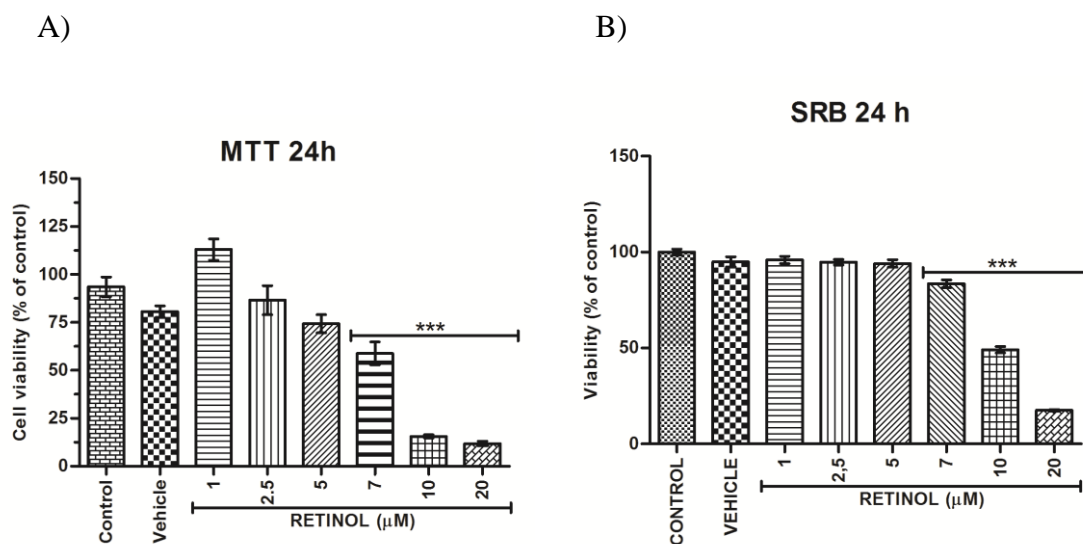
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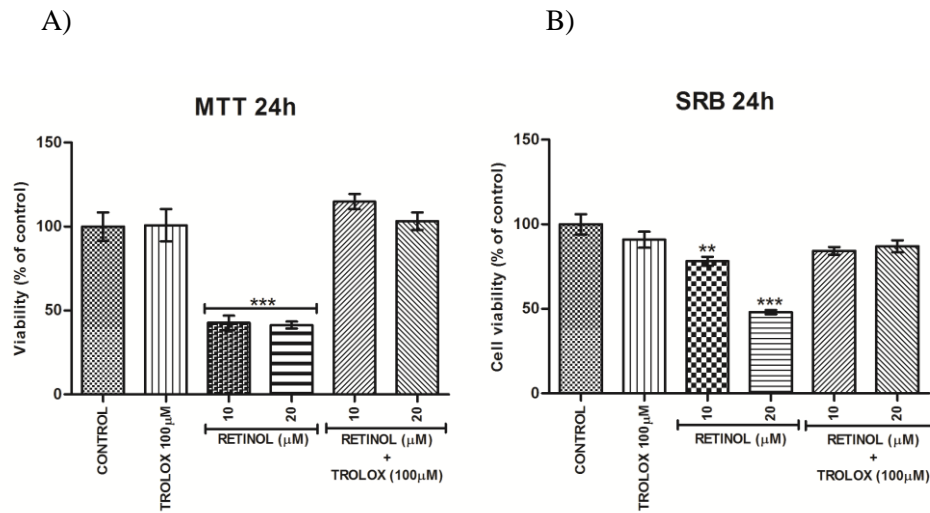
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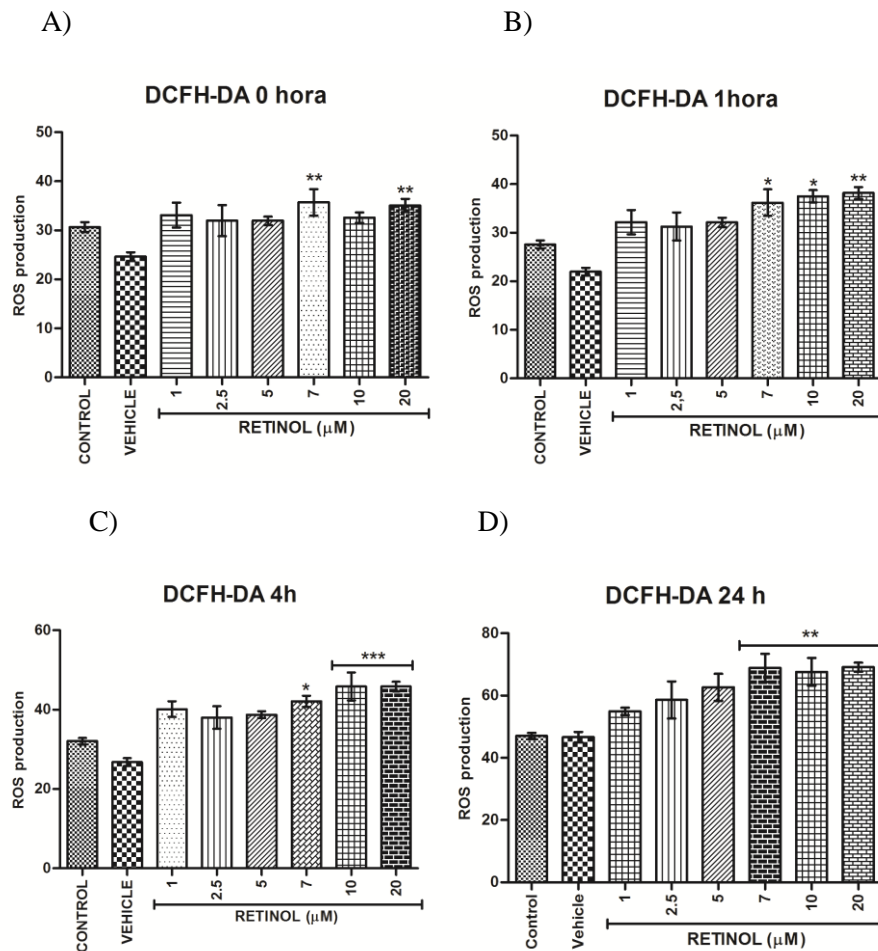
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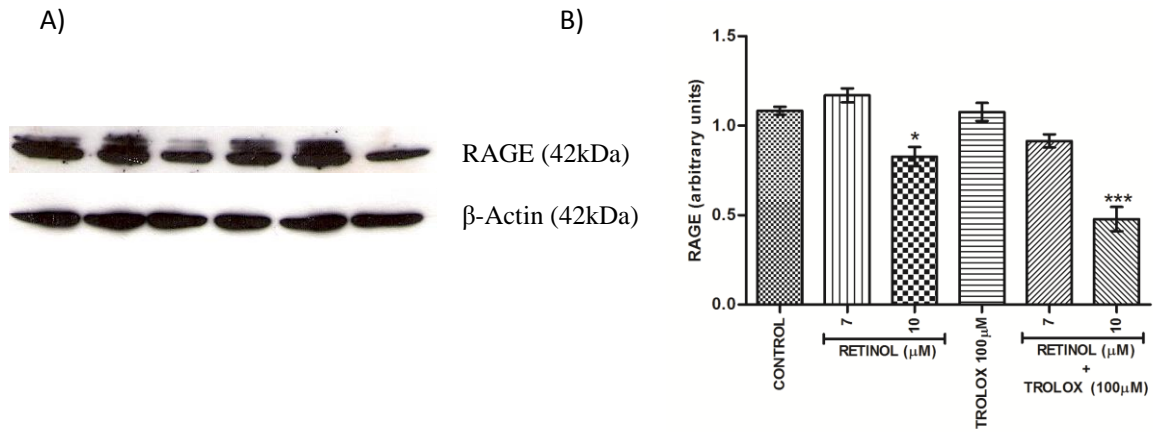
**Figure 1:** A) Reduction of MTT; B): Quantification of Sulforrodamine B; Different Retinol Concentrations (μM). Values expressed as Mean ±SEM (n=6-12 and at least 3 independent experiments). ANOVA followed by Tukey Multiple Comparison Test. \*P<0,05, \*\*P<0,001, \*\*\*P<0,0001; compared to control.



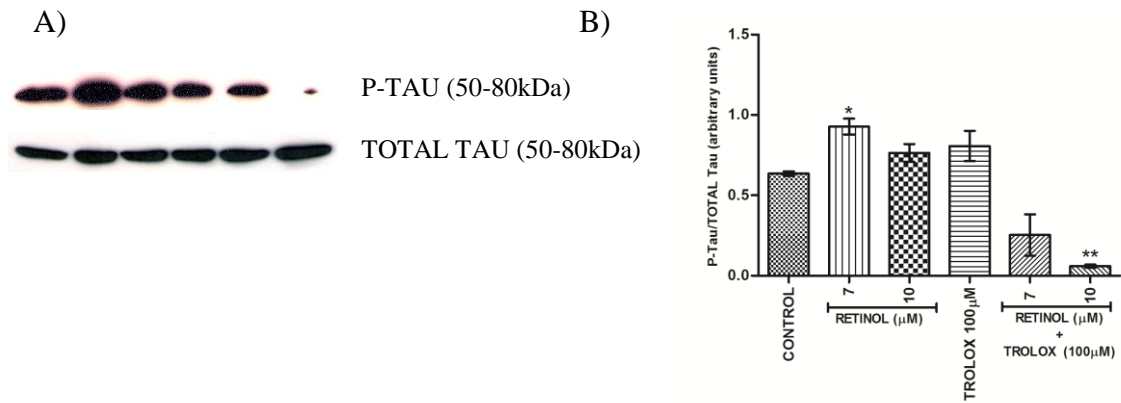
**Figure 2:** A) Reduction of MTT; B) Quantification of Sulforhodamine B; Different Retinol Concentrations ( $\mu$ M) with Co-treatment with Trolox®. Values expressed as Mean  $\pm$  SEM (n=6-12 and at least 3 independent experiments). ANOVA followed by Tukey Multiple Comparison Test. \*P<0,05, \*\*P<0,001, \*\*\*P<0,0001; compared to control.



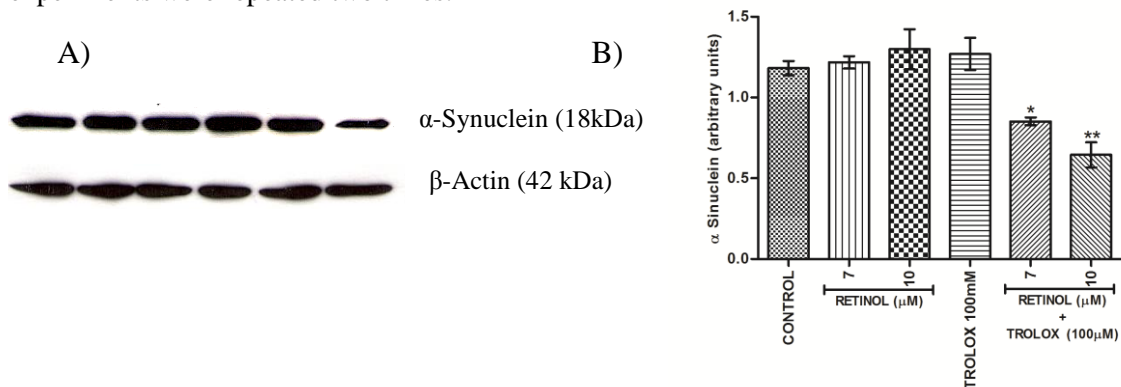
**Figure 3:** Measuring the production of ROS: A) 0 hour; B) 1 hour; C) 4 hours; and D) 24 hours – Retinol ( $\mu$ M). Values expressed as Mean  $\pm$  SEM (n= 6-12, and at least 3 independent experiments). ANOVA followed by Tukey Multiple Comparison Test. \*P<0,05; \*\*P<0,001; \*\*\*P<0,0001; compared to control.



**Figure 4:** A) RAGE Protein Content. B) Total Quantification Values. Representative immunoblots as Mean  $\pm$  SEM are depicted. The same analysis was applied to verify RAGE immunocontent. One-way ANOVA with Tukey's post hoc test was applied. Asterisks different from control (\* $P$ <0.05, \*\*\* $P$ <0.001). The experiments were repeated three times.



**Figure 5:** A) P-TAU and TOTAL TAU Protein Content. B) Total Quantification Values. Representative immunoblots showing detection of phosphorylated TAU (P-TAU) are depicted. The immunocontent of P-TAU is quantified relative to TOTAL TAU. One way ANOVA with Tukey's post hoc test was applied. Asterisks different from control (\* $P$ <0.05, \*\* $P$ <0.01). The experiments were repeated two times.



**Figure 6:** A)  $\alpha$ -Synuclein Protein Content. B) Total Quantification Values. Representative immunoblots as Mean  $\pm$  SEM are depicted. The same analysis was applied to verify  $\alpha$ -Synuclein immunocontent. One-way ANOVA with Tukey's post hoc test was applied. Asterisks different from control (\* $P$ <0.05, \*\* $P$ <0.01). The experiments were repeated three times.