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PREVENTIVE USE OF RESVERATROL INCREASES ITS ANTIOXIDANT CAPACITY IN NEURO-2A CELLS

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ABSTRACT

Cerebral aging is a phenomenon determined almost exclusively by oxidative stress, accumulation of macromolecules damaged by oxidation and changes in the structure and function of neurons, being a risk factor for several diseases. Many data support the important role of oxidative stress in the aging process and neurodegenerative diseases, suggesting the beneficial effect of antioxidants as an adjuvant therapy. Thus, the objective of our work was to verify whether the neuroprotective effect of resveratrol is altered if N2-A cells are inserted earlier or later in an "oxidative stress environment" caused by exposure to H_2O_2 thus generating support for health professionals in the clinical approach. For that, we performed the production of nitric oxide and peroxynitrite, cellular reducing capacity by MTT, the production of Reactive Oxygen Species (ROS) by chemiluminescence assay and the activity of catalase and superoxide dismutase. We observed that resveratrol (5μM) in N2-A cells exerts antioxidant and neuroprotective power. This occurs mostly in the form of prevention, when the cells were pre-stimulated with resveratrol before inducing an oxidative stress environment.

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INTRODUCTION

The effects of ageing on the brain and cognition are widespread and have multiple etiologies. Ageing has its effects on the molecules, cells, vasculature, gross morphology, and cognition. As we age our brains shrink in volume, particularly in the frontal cortex. As our vasculature ages and our blood pressure rises the possibility of stroke and ischemia increases and our white matter develops lesions. Memory decline also occurs with ageing and brain activation becomes more bilateral for memory tasks. This may be an attempt to compensate and recruit additional networks or because specific areas are no longer easily accessed. Genetics, neurotransmitters, hormones, and experience all have a part to play in brain ageing (Peters, 2005). The brain is especially susceptible to the assaults perpetrated by ROS. This is because the brain as an organ is a major metabolizer of oxygen (20% of the body consumption) and yet has relatively feeble protective antioxidant mechanisms. Thus, it is especially vulnerable to oxidative stress (Popa-Wagner *et al*. 2013).

Mitochondrial energy metabolism yields several reactive oxygen species (ROS) including oxygen ions (O_2^-, the primary ROS), free radicals, and peroxides (inorganic and organic). A wealth of evidence suggests that high levels of ROS are intimately linked to the appearance of neuronal death in various neurological disorders. These include chronic diseases (Parkinson's disease or Alzheimer's disease), acute injury of the brain (brain trauma and cerebral ischemia) or psychiatric disorders (autism, attention déficit hyperactivity disorder, depression, and schizophrenia) (Guglielmotto *et al*. 2009). An increase in oxidative and nitrooxidative stress and a decrease in the antioxidant capacity of the brain are key factors involved in the etiology of neuropsychiatric diseases (Valko *et al*. 2007; Michel *et al*. 2012; Popa-Wagner *et al*. 2013). In this context, Resveratrol (3,5,4′-trihydroxy-trans-stylbene) is a polyphenolic compound found in several plant species, including grapes and peanuts. Among its potential biological effects is the ability to promote the activity of several antioxidant enzymes, enzymes that show a reduction in activity throughout aging and in neurodegenerative diseases

(Tao *et al*. 2016).The cellular pathogenesis of these physical disorders related to aging have not yet been fully elucidated, and currently, there are no interventions based on mechanisms aimed at the root cause to improve this inability in elderly, which are available for clinical application (Truong *et al*. 2018). Therefore, it is important to study what would be the mechanisms involved in the performance of potentially important molecules in the control this imbalance between: generation of oxidizing molecules and antioxidant capacity intrinsic; as is the case with resveratrol.Thus, the objective of our work was to verify whether the neuroprotective effect of resveratrol is altered if N2-A cells are inserted earlier or later in an "oxidative stress environment" caused by exposure to H_2O_2 thus generating support for health professionals in the clinical approach.

MATERIALS AND METHODS

Cellular Culture of Neuro 2-A Lineage: The Neuro 2-A cell line (ATCC CCL131) (N2-A) was purchased from the Cell Bank of Rio de Janeiro (Brazil). For culturing, they were placed in sterile 75 cm^2 growth bottles containing Dulbecco's Phosphate Buffered Saline (DMEM) culture medium. It was added 10% (v/v) foetal bovine sérum (FBS). The bottles were incubated in an oven at 37°C humidified with 5% carbon dioxide (CO_2). The medium was replaced every two or three days, according to the confluence of the cell monolayer and subcultures. When the bottles reached 80% confluence, the medium was aspirated, and the cell monolayer washed twice with calcium-free and magnesium-free Phosphate Buffer Solution (PBS). Subsequently, 5 mL of trypsin solution (0.20% trypsin solution and 0.02% EDTA) was used to detach the monolayers. After detachment, the cells were withdrawn from the flask, placed in Falcon tube with 5 mL of DMEM, and centrifuged at 400g for 10 minutes. After centrifugation, the supernatant was removed, and the pellet re-suspended in 1 mL DMEM. The cells were then counted with 0.3% Trypan Blue in the Neubauer chamber (OPITIK LABOR).

For the experiments described below, the N2-A cells were incubated in two different ways: Pre-stimulus and Post-stimulus. This methodology aimed to assess whether resveratrol acts as a prevention and treatment for oxidative stress.

- ***Pre-stimulation*:** N2-A were stimulated with resveratrol for 24 hours and washed with Hanks before being exposed to H_2O_2 for 20 minutes.
- ***Post-stimulation*:** N2-A were exposed to H_2O_2 for 20 minutes, washed with Hanks and stimulated by resveratrol for 24 hours.

Padronization of H_2O_2 and resveratrol concentration: The cells ($5x10^3$) were stimulated with increasing concentrations of H_2O_2: 2.6%v/v; 5.25%v/v; 10,5%v/v; 21%v/v; 31,5%v/v and 35%v/vfor 20 minutes at 37°C. The Trypan Blue assay was used to evaluate cell viability. Results were expressed as a percentage of viability ± SE. Chemiluminescence assay was also performed to assess concentrations in which would show cell viability within acceptable parameters (cell viability >70%). The resveratrol concentration used in the assays were previously chosen from the literature (Lastra *et al*. 2007). Besides, was defined based on the Dose-Response-Curve by resazurin assay in N2-A. For this, $5x10^3$ cells/well were stimulated with increasing concentrations of resveratrol: 0.63 µM; 1.25 µM; 2.5 µM; 5 µM; 10 µM and 20 µM for 24 hours at 37°C. After treatment, 100µL of resazurin (0.125 mg/L) was added to wells and the plate was incubated for 3 hours at 37°C. Absorbance was read at 570 nm in microplate reader (Thermo Plate). Results were expressed as absorbance *vs* concentration ± SE.

Cell viability assay: The resazurin colorimetric assay was performed to verify cell viability against H_2O_2, Resveratrol and H_2O_2 + Resveratrol treatments. For this, $5x10^3$ cells/well were plated in the presence of DMEM and FBS 10% (final volume = 200µL). The plates were incubated in a humidified oven at 37ºC for 24 hours for cell adhesion and cell monolayer formation. After this time, the supernatant was removed and realized the two different types of treatments in N2-A cells (*Pre-stimulus and Post-stimulus*). The plates were again incubated for 24 hours. After treatment, the supernatant was removed and the wells were washed with PBS. 100 µL/well of a solution containing 0.125mg/L of resazurin in DMEM was added and incubated for 3 hours at 37°C. The absorbance was read at 570 nm in microplate reader (Thermo Plate). Results were expressed as a percentage of viability ± SE.

Evaluation of reducing capacity in N2-A: The MTT assay is a model for assessing the cellular reducing capacity quickly and objectively, based on a colorimetric reaction. Mitochondria is an extremely important organelle in this study, as it is the largest source of ROS origin through the electron transport chain. For this, $5x10^3$ cells/well received the appropriate treatments previously described *(pre-stimulus* and *post-stimulus*). After treatment, the supernatant was removed and the wells washed with PBS. 190µL of DMEM and 10µL of the MTT solution were added to the wells and the plate was again placed in an oven at 37°C for 1 hour. Then, the supernatant and 100µL of DMSO was added to dissolve generated formazan crystals and absorbance was read at 570 nm in microplate reader (Thermo Plate). The calculation used to evaluate the percentage of cell reducing capacity was: absorbance of treated cells / absorbance of the control x 100.

Chemiluminescence Assay: The quantitative ROS determination was performed in a luminol-dependent chemiluminescence assay, according to Horta *et al*. (2005) with some adaptations. First, the cells received the appropriate treatments *(pre-stimulus* and *post-stimulus*). After that, 100µL of cells and 10µL of luminol (10^{-4}M) were added to the specific tubes. The final volume was adjusted to 700µL with PBS. Each tube was immediately placed in a Luminometer (Lumat, LB 9501, EG&G Berthold, Germany) and the reading was performed in 10 minutes. The results were expressed in relative light units (RLU)/minute.

Quantification of Nitric oxide (NO) and Peroxynitrite (ONOO-): For the quantification of NO, the Griess Reaction was performed (Griess, 1864). Initially, the cells received the appropriate treatments *(pre-stimulus* and *post-stimulus*). After that, the supernatant was collected and used to measure nitrite. For this, 100µl of supernatant and 100µl of Griess solution (formed from 1% sulfanilamine in 2.5% phosphoric acid and 0.1% naphthylenediamine in 25% phosphoric acid, in the proportion 1: 1) were added. The absorbance was read at 540 nm in microplate reader (Thermo Plate). The nitrite concentration was calculated by linear regression, using the standard curve obtained from a 1 mM sodium nitrite solution and DMEM culture medium.

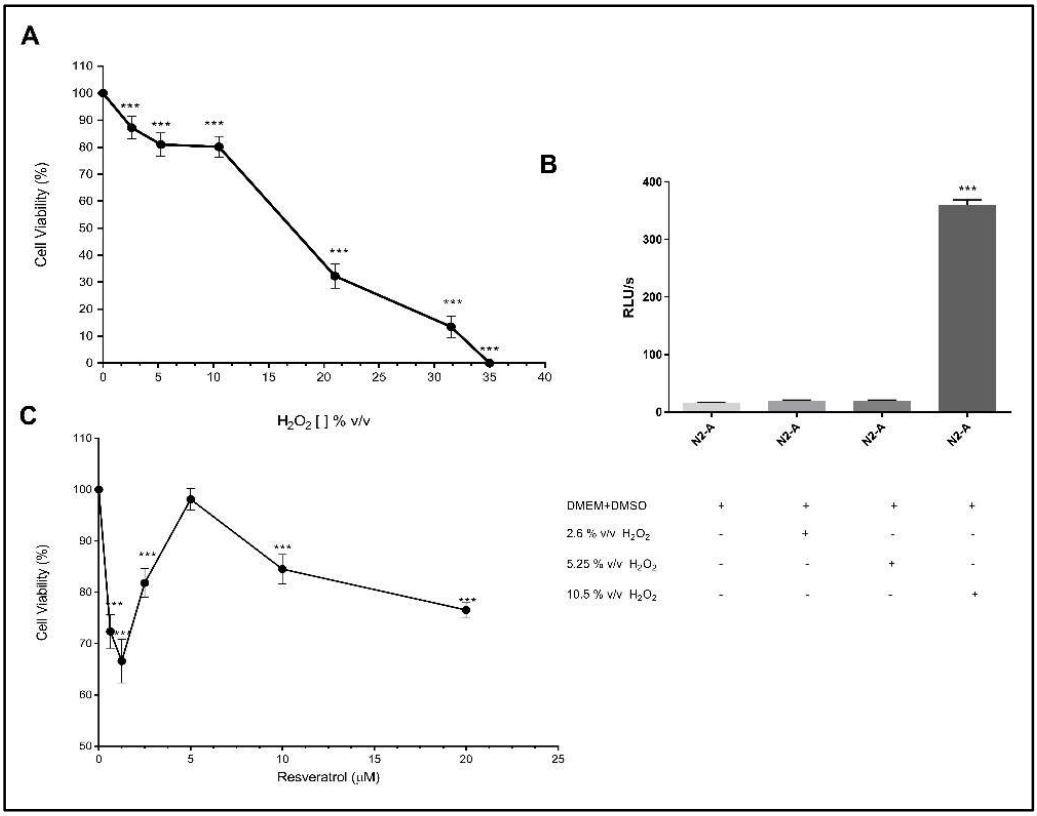

Figure 1. Padronization of H_2O_2 and Resveratrol concentrations used in the study. (A) Concentration-Response curve of H_2O_2 in N2-A. N2-A were stimulated by increasing concentrations of H_2O_2 for 20 minutes (2.6%v/v; 5.25%v/v; 10,5%v/v; 21%v/v; 31,5%v/v and 35%v/v) and viability was tested by the Trypan Blue assay. (B) Evaluation of H2O2-induced ROS production in the Chemiluminescence assay. ROS generation was expressed in RLU/min. (C) Dose-Response-Curve of resveratrol. N2-A were stimulated by increasing concentrations of resveratrol (0.63 µM; 1.25 µM; 2.5 µM; 5 µM; 10 µM and 20 µM) and absorbance to verify stimulation in cells was measured at 570 nm. The concentration chosen was 5 µM which showed an absorbance peak and corresponds to physiological dosage. *** p<0.0001 significant result by ANOVA analysis followed by Dunn's test. The tests were performed in ten repetitions for each concentration.

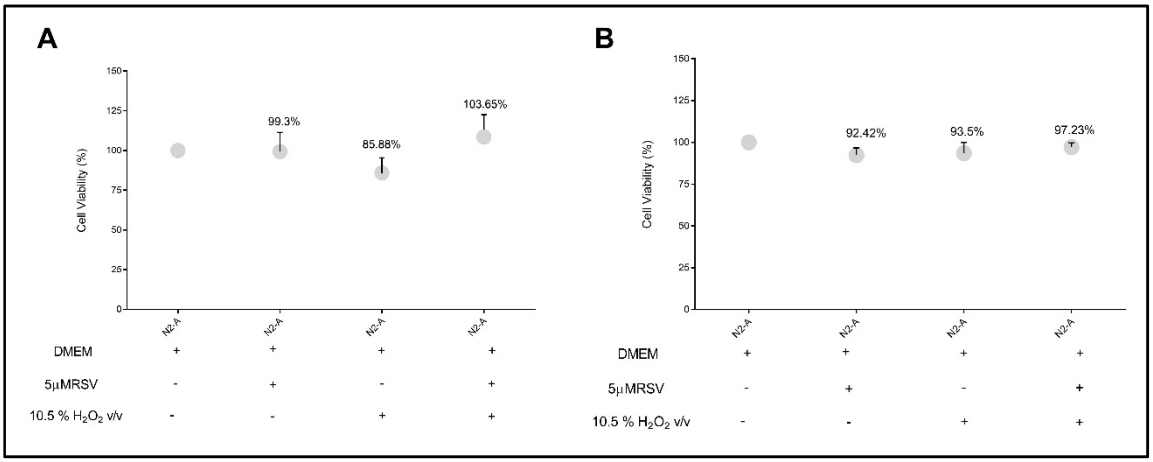

Figure 2. Evaluation of the viability of N2-A exposed to pre-stimulus (A) and post-stimulus (B) with resveratrol and H_2O_2. Cell viability was expressed in % of living cells. The tests were performed in ten repetitions for each concentration

Peroxynitrite quantification was evaluated according to the technique of Hughes & Nicklin (1996). For that, 5×10^5 cells/well were added in 6-well plates. The final volume was adjusted to 2 ml with DMEM + FBS 10% and incubated at 37°C for 24 hours. After that, the supernatant was removed and treatments were carried out (*pre-stimulus* and *post-stimulus*). The plate was again incubated for 16 hours. After this period, the supernatant was discarded, 2 mL of H_2O MilliQ was added to the cell pellets and vortexed for 1 minute. The content was measured and quantified on a plate spectrometer at a wavelength of 302 nm. The results were expressed as mean ± SE.

Dosage of enzymes Catalase (CAT) and Superoxide Dismutase (SOD)

After the cells received the appropriate treatments *(pre-stimulus* and *post-stimulus*), the supernatant was collected and the procedures described in the specific kits used for the measurement of CAT activity (EnzyChromTM Catalase Assay Kit (ECAT-100) - Bioassay systems) and SOD (EnzyChromTM Superoxide dismutase Assay Kit (ESOD-100) - Bioassay systems). The calculations were performed by analyzing the slope of the curve and linear regression and the results were expressed as mean ± SE.

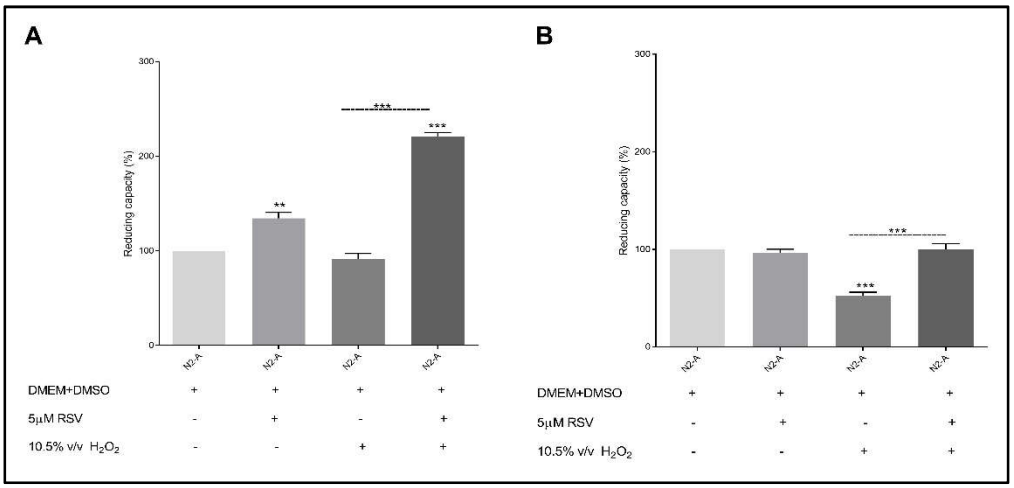

Figure 3. Effect of resveratrol on the N2-A reducing capacity. Being (A) pre-stimulus and (B) post-stimulus. The cellular reducing capacity was expressed in %. ** p <0.001; *** p <0.0001 significant by ANOVA-Kruskal-Wallis test, Dunn's Multiple post-test Comparasion. Experiments performed in triplicate. The tests were performed in six repetitions for each concentration

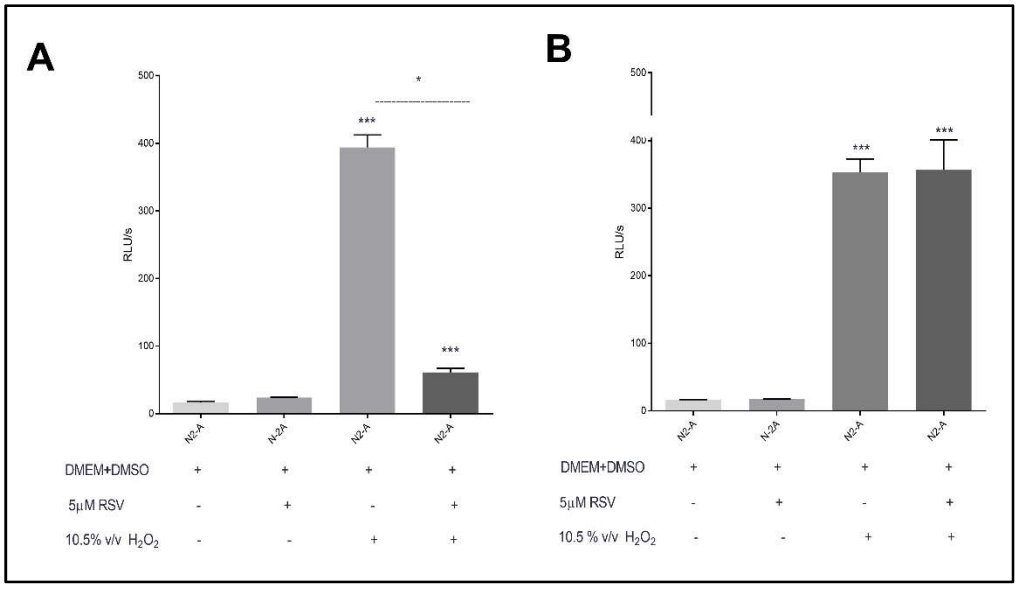

Figure 4. Evaluation of the antioxidant effect of resveratrol on N2-A cells in the chemiluminescence assay. Resveratrol effect on ROS production in H_2O_2-induced, being (A) pre-stimulus and (B) post-stimulus. The generation of ROS was expressed in RLU/min. *p <0.01 ; ** p <0.001; *** p <0.0001 significant by ANOVA-Kruskal-Wallis test, Dunn's Multiple Comparison post-test. The tests were performed in ten repetitions for each concentration

Statistical analysis: All data were analyzed by the Kolmogorov – Smirnov normality test. Univariate analysis of variance (one-way ANOVA) was used, followed by Dunnett's or Bonferroni's test when the samples had normal distribution and Dunns' test when they did not follow the normal distribution. Differences were considered significant for $p < 0.05$. All analyzes were performed using GraphPad Prism software version 5.0 (San Diego, California, USA).

RESULTS

The first stage of the study was to standardize the concentrations of H_2O_2 and Resveratrol. The results are shown in Figure 1. Analyzing the viability curve of N2-A cells in different concentrations of H_2O_2, it is possible to observe that from the concentration of 21% v/v the cells did not remain within the acceptable limit of viability (Figure 1A). From there, the cells were stimulated with the three concentrations of H_2O_2 which remained viable and subjected to the chemiluminescence assay. Such an assay is able to show which concentrations induce an oxidative stress environment. From the result shown in Figure 1B, we determined of the 10.5% v/v concentration as a stimulus dose for N2-A cells. Such concentration significantly increased the production of ROS and the cells remained viable (80.12%). Then, the N2-A cells were stimulated with increasing concentrations of resveratrol. From the Dose-Response-Curve obtained in Figure 1C, the concentration at which it showed an absorbance peak (5μM) was chosen. This concentration was in accordance with the literature, suggesting that it corresponds to the physiological cellular levels (Kursvietiene *et al*. 2016). In addition, Resazurin colorimetric assay was also used to evaluate N2-A viability against treatments (*pre-stimulus* and *post-stimulus*) (Figure 2). In general, all treatments allowed cells to remain viable and they did not promote significant cytotoxicity. After H_2O_2 and resveratrol concentrations were defined, and cell viability was proven, we assessed the influence of resveratrol on cellular reducing capacity. The results shown in Figure 3 show that in both *pre-stimulus* (Figure 3a) and *post-stimulus* (Figure 3b)

treatments, resveratrol + H_2O_2 was able to increase the N2-A cellular reducing capacity, in the scope of performance of NADPH, in relation to the group treated only with H_2O_2. This increase was 2.4 times in the *pre-stimulus* and 1.9 times in the p*ost-stimulus*. We also observed that, only in the *pre-stimulus* condition, there was an increase in the reducing capacity when treated only with resveratrol. Then, the chemiluminescence assay was performed on N2-A cells (Figure 4). When H_2O_2 was added, there was an increase of almost 25 times in the production of ROS in relation to the group without any stimulus. However, *pre-stimulation* with resveratrol reduced ROS production 6 times, still remaining 3 times above baseline production (Figure 4a). However, in the *post-stimulus*, we observed that resveratrol was not able to decrease the production of ROS caused by H_2O_2 (Figure 4b).

In order to quantify the production of NO and $ONOO^-$, important components of oxidative stress together with ROS, N2-A cells were again stimulated under the conditions of pre and post-stimulation with resveratrol. The results are shown in Figure 5. Analyzing NO production, both in the *pre-stimulus* (Figure 5a) and in the *post-stimulus* (Figure 5b), it is noticed that the cells were treated with resveratrol + H_2O_2 generated an increase in NO compared to those that did not receive any treatment. (baseline group): 3 times in the *pre-stimulus* and 1.5 times in the *post-stimulus*. When compared to the H_2O_2 group, the increase is 3 times for the *pre-stimulus* and 5 times for the *post-stimulus*.Analyzing the production of $ONOO^-$ it is observed that resveratrol was able to increase the reducing capacity of N2-A cells in relation to the group treated with H_2O_2 only.

The observed increase was 2.5 times in the *pre-stimulus* (Figure 5c) and 2 times in the *post-stimulus* (Figure 5d). Then, two enzymes well known for their antioxidant activities were dosed. They are: Catalase (CAT) and Superoxide Dismutase (SOD). The results are shown in Figure 6. CAT activity was increased when N2-A cells were treated with H_2O_2, confirming that such enzyme is affected in an oxidative stress environment. When cells received the *pre-stimulus* with resveratrol, there was no difference in the levels of the enzyme, in addition to the stimulus that had been caused by H_2O_2 (Figure 6a).When there was *post-stimulation* with resveratrol, a decrease in the enzyme was observed compared to the group that received only H_2O_2(Figure 6b). Analyzing the results of the SOD, it is possible to perceive the same performance profile of resveratrol in the two scenarios: *pre-stimulus* (Figure 6c) and *post-stimulus* (Figure 6d) but with different intensities. Only resveratrol (before or after the addition of H_2O_2) was able to increase the activity of SOD in N2-A cells, compared to cells in the baselinestate. H_2O_2 decreased the enzyme activity in both situations. Finally, treatment with resveratrol increased SOD activity compared to cells that only with H_2O_2.

DISCUSSION

The understanding of aging is still quite limited. As a complex biological process, it certainly involves a variety of factors. In modern society, neurodegenerative diseasesassociated with aging, such as Alzheimer's and Parkinson's disease, Frontotemporal dementia, have been a growing lethal threat to humans.

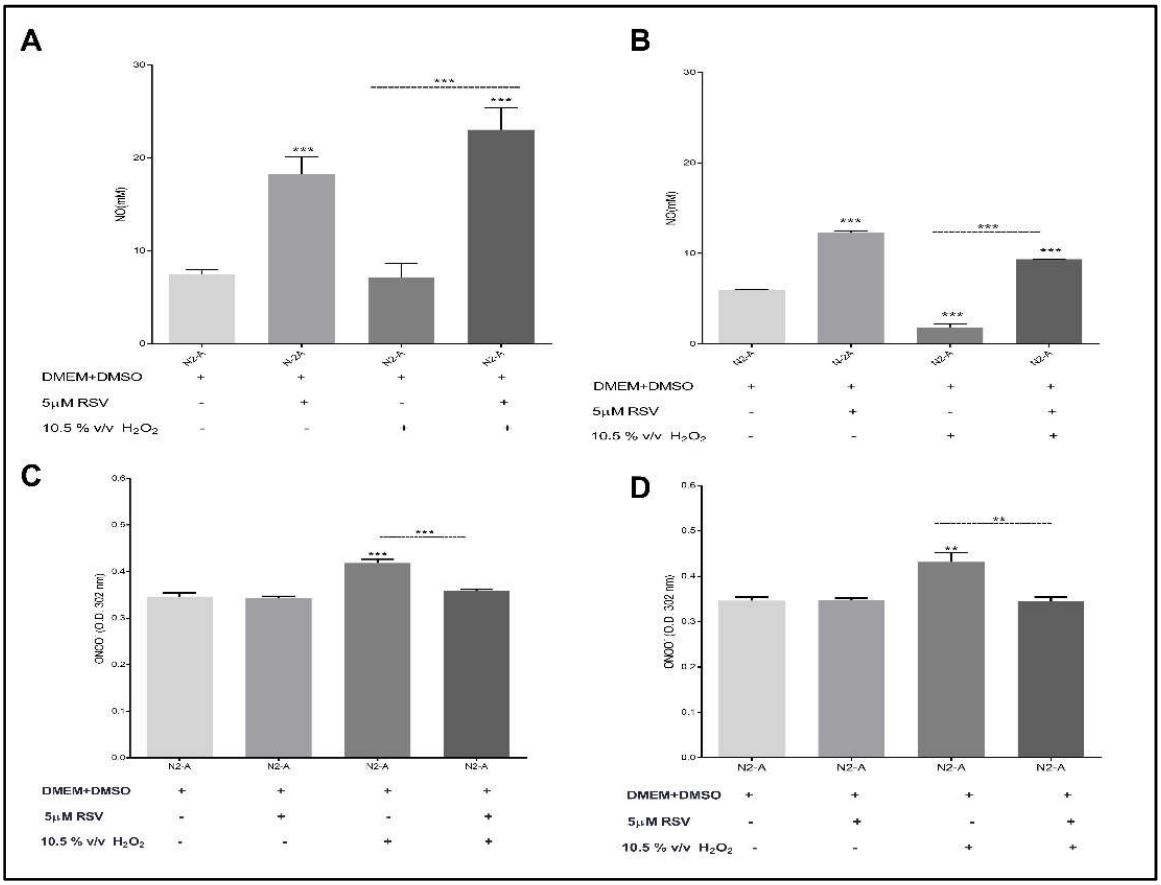

Figure 5. Effect of resveratrol on NO and $ONOO^-$ production in N2-A cells. NO generation was expressed in mM, being (A) pre-stimulus and (B) post-stimulus. $ONOO^-$ generation was expressed as O.D. (Optical Density), being (C) pre-stimulus and (D) post-stimulus. ** p <0.001; *** p <0.0001 significant by ANOVA test, post-test of Bonferroni´s Multiple Comparison. The tests were performed in ten repetitions for each concentration

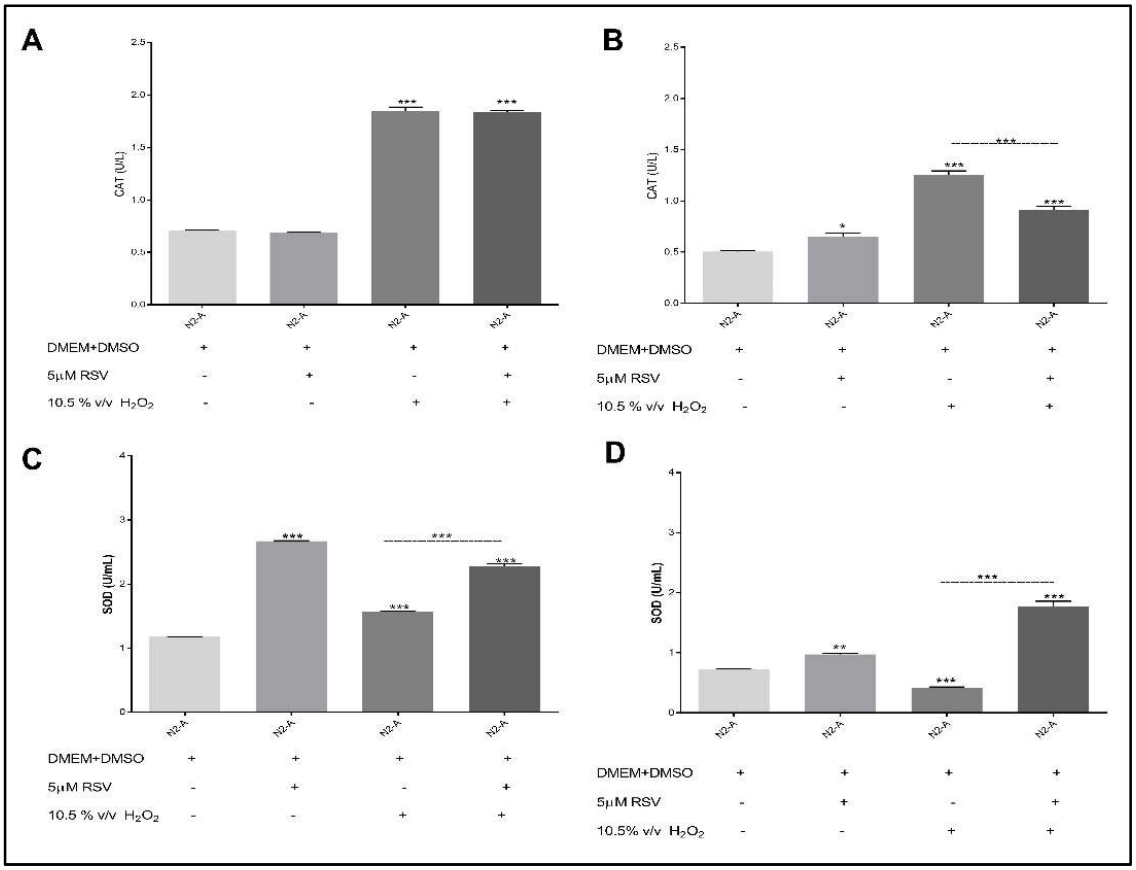

Figure 6. Effect of resveratrol on the activity of antioxidant enzymes in N2-A cells. CAT activity, being (A) pre-stimulus and (B) post-stimulus. SOD activity, being (C) pre-stimulus and (D) post-stimulus. Activity was measured in U/L (Atomic mass unit/liter). ** p <0.001 *** p <0.0001 significant by ANOVA test, post-test of Bonferroni´s Multiple Comparison. The tests were performed in six repetitions for each concentration

Cell survival depends on the balance between oxidative species generated in metabolic processes and the antioxidant system present in that system. The excess of these oxidative species causes the oxidation of biomolecules, with consequent homeostatic imbalance. This manifests itself in pathological, metabolic and degenerative effects. (Denver *et al*. 2018; Tan *et al*. 2014; Freitas *et al*. 2017). In this context, our study aimed to verify the efficiency of RSV as a neuroprotective agent in pre and post-stimulus conditions in N2-A cells. In these conditions, we mainly aim to resemble what happens in neural senescence, generating additional knowledge for clinical management. The resveratrol concentration used was 5µM, obtained by the Concentration-Response curve and corroborates with a study by Simao *et al*. (2012), where resveratrol (5µM) promoted angiogenesis in brain endothelial cells. Demonstrating its role as a neural modulator/protector in this concentration. The resveratrol molecule was always tested from two perspectives on the neuronal cells in question: *pre-stimulus* (before exposure to H_2O_2, a senescence-inducing agent), seeking to evaluate the use of polyphenol in preventing damage possibly caused by oxidative stress/aging. And under a *post-stimulus* scenario (which had already been exposed to the oxidizer), examining its neurodegenerative capabilities. We know that cellular antioxidant mechanisms can be enzymatic and non-enzymatic. In this context, we started our research evaluating the cellular reducing capacity through the MTT assay. In this evaluation, our results showed that resveratrol increased the cell reducing capacity both at baseline and when N2-A cells were stimulated by H_2O_2 in the pre-stimulus condition. When analyzing the results after the post-stimulus, we verified that they were significant only in the condition where resveratrol was added to N2-A cells after treatment with H_2O_2. Our results corroborate the findings by Toth *et al*. (2018), where resveratrol (5µM) was able to increase the reducing/antioxidant capacity of N2-A cells under both stimulus perspectives (pre and post-stimulus), being moreintense when used under a preventive design. Resveratrol has a direct effect on NADPH, inhibiting NADPH oxidase while reducing NAD, causing it to shift from the oxidized state ($NADP^+$) to the reduced state (NADPH). In order to verify whether resveratrol (5µM) would be able to act in the production of ROS in N2-A cells in the pre and post-stimulus conditions, the luminol-dependent chemiluminescence assay was used. Our results demonstrated that resveratrol decreased the formation of ROS when used as a *pre-stimulus*, which did not occur in the *post-stimulus*. This answer gives us evidence of a preventive antioxidant capacity of resveratrol. Such results corroborate the findings by Lin *et al*. (2014), which showed that the pre-treatment of astrocytes with resveratrol (5µM) was able to suppress the generation of ROS and the rate of cell death. In addition, studies by Bobermim *et al*. (2012) showed that pre-incubated with resveratrol in SH-SY5Y cells (human neuroblastoma lineage) decreased ammonia-induced ROS production. Evaluating the NO production, our results showed an increase in its production, in both treatments. NO mediates most brain endothelium-dependent responses, while resveratrol can reduce oxidative stress and increase production of NO as occurred in findings by Huang *et al*. (2014), in rat cortical neurons: a profile similar to that found in our study in N2-A cells. When analyzing the production of ONOO- our results show that resveratrol (5µM) was able to decrease its production in N2-A cells when used preventively and as a

treatment. However, we can see that this decrease was more effective when resveratrol was used under pre-stimulus. Our results are in agreement with the findings by Olas *et al*. (2008) that proved that this polyphenol was able to decrease the generation of ONOO- in peripheral blood cells.

There are some antioxidant defense groups in cells: enzymes (SOD and CAT, for example), low molecular weight antioxidants with indirect action (chelating agents, for example) and compounds with direct action (NADPH and exogenous dietary sources, such as resveratrol) (Sas *et al*. 2018). Our results did not show an increase in CAT activity when there was a pre-stimulus with resveratrol, but contrary to the literature findings, it decreased when used in the form of treatment and stimulated with H_2O_2. Analyzing the SOD enzyme, our results showed that in the pre-stimulus, resveratrol was more efficient than in the post-stimulus condition. Once again, these results corroborate those of Tung *et al*. (2015), which revealed that resveratrol protects muscle, heart and liver membranes in elderly mice by inducing endogenous antioxidant systems in these organs, mainly affecting SOD activity. Other studies also demonstrated the role of resveratrol in increasing protein expression and SOD activity in the brain of diabetic rats (Wang *et al*. 2014). In addition, the increase in the performance of SOD in neuronal cells of rats is an importantmechanism of neural protection of resveratrol against apoptosis of these cells induced by oxidative mitochondrial stress (Wang *et al*. 2014; Truong *et al*. 2018). The results of the present study provide us with evidence of the antioxidant and neuroprotective capacity of resveratrol in N2-A cells, in a more efficient condition when used as a preventive method for neural cells. This condition is important for conducting appropriate clinical protocols.

Acknowledgements

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