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ORIGINAL ARTICLE

Neostigmine treatment induces neuroprotection against oxidative stress in cerebral cortex of asthmatic mice

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Abstract

During chronic inflammatory disease, such asthma, leukocytes can invade the central nervous system (CNS) and together with CNS-resident cells, generate excessive reactive oxygen species (ROS) production as well as disbalance in the antioxidant system, causing oxidative stress, which contributes a large part to neuroinflammation. In this sense, the aim of this study is to investigate the effects of treatment with neostigmine, known for the ability to control lung inflammation, on oxidative stress in the cerebral cortex of asthmatic mice. Female BALB/cJ mice were submitted to asthma model induced by ovalbumin (OVA). Control group received only Dulbecco's phosphate-buffered saline (DPBS). To evaluate neostigmine effects, mice received 80 μg/kg of neostigmine intraperitoneally 30 min after each OVA challenge. Our results revealed for the first time that treatment with neostigmine (an acetylcholinesterase inhibitor that no crosses the BBB) was able to revert ROS production and change antioxidant enzyme catalase in the cerebral cortex in asthmatic mice. These results support the communication between the peripheral immune system and the CNS and suggest that acetylcholinesterase inhibitors, such as neostigmine, should be further studied as possible therapeutic strategies for neuroprotection in asthma.

Keywords Asthma · Oxidative stress · Neostigmine · Acetylcholinesterase inhibitor · Neuroprotection

Introduction

Asthma is a chronic inflammatory airway disease that affects 300 million people worldwide. Allergic asthma is clinically defined by variable airway obstruction that causes recurrent periods of shortness of breath, chest tightness, wheezing, and coughing. These clinical symptoms are a consequence of a dysregulated inflammation orchestrated by adaptive $CD4^+$ T helper 2 (Th2) that classically produce specific cytokines such as interleukins 4, 5, and 13 (IL-4, IL-5, and IL-13) which induces B cells to produce immunoglobulin E (IgE), mucus overproduction, and infiltration of neutrophils, macrophages, lymphocytes, and eosinophils in the airway, as well as increased levels of reactive oxygen species (ROS) (Deckers et al. 2017).

According to the literature (Chou et al. 2004; Obermeier et al. 2013) studies have shown that in chronic inflammatory disease, leukocytes can invade the central nervous system (CNS) parenchyma and together with CNS-resident cells promotes ROS production. Under normal conditions, ROS is neutralized by antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). However, defects in antioxidant mechanisms and excessive ROS production leads to oxidative stress and tissue damage.

Remarkably, excessive ROS production is a major mechanism causing damage in brain tissue through ability to modify DNA, lipids, and proteins (Haider et al. 2011; Hunter et al. 1985; Lu et al. 2000). In sepsis, a severe inflammatory disease was demonstrated that ROS production played an important role in the progression of neuroinflammation and brain

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dysfunction (Danielski et al. 2017). Recently the excessive ROS production also was observed in the cerebral cortex of asthmatic mice (Duan et al. 2018). Therefore, it does make sense to pay attention to the ROS production that occurs during neuroinflammation.

Recent studies demonstrated that acetylcholinesterase inhibitors are an important tool for therapeutic intervention in many diseases. Inhibition of acetylcholinesterase by physostigmine and neostigmine reduced neuroinflammation and degeneration in the cortex and hippocampus of a surgery stress rat model (Kalb et al. 2013). Recently, Odorcyk and colleagues showed that galantamine administration upregulates the antioxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia (Odorcyk et al. 2017). In a recent study was revealed that neostigmine (which cannot cross BBB) treatment provided airway protection against oxidative damage and attenuates lung inflammation in asthmatic mice (Antunes et al. 2019). Thus, we decided to investigate whether neostigmine, known for the ability to control lung inflammation, could revert oxidative stress in the cerebral cortex of asthmatic mice.

Materials and methods

Animals and experimental groups

This study was performed with adult female mice (BALB/cJ with 6–8 weeks old) given that during adulthood asthma affects more women than man (Naeem and Silveyra 2019). The mice were obtained from the Center for Experimental Biological Models (CeMBE, PUCRS). The animals were fed with a balanced chow diet with access to water ad libitum, housed in cages and maintained on a 12/12-h light/dark cycle. Female mice were randomized in three groups: DPBS: control; OVA: animals submitted an experimental model of asthma; and OVA+Neostigmine: animals submitted an experimental model of asthma and treated with neostigmine (acetylcholinesterase inhibitor).

Sensitization, airway challenge and neostigmine treatment

The animals were sensitized by subcutaneous injections of 20 μg ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis, USA), diluted (200 μL) in Dulbecco's phosphate-buffered saline (DPBS), on days 0 and 7, followed by three intranasal challenges with 100 μg of OVA, diluted in DPBS (50 μL), on days 14, 15, and 16 of the protocol. The control group received only DPBS in the sensitization and intranasal challenges. To evaluate neostigmine effects on the oxidative stress in the cerebral cortex, the mice received 80 μg/kg of neostigmine treatment (Normastig, União Química, São Paulo, Brazil) intraperitoneally (Hofer et al. 2008) once a day during three consecutive days (14, 15, and 16) 30 min after of OVA challenge. On day 17 of the protocol, animals were anesthetized by intraperitoneal injection solution of ketamine (0.4 mg/g) and xylazine (0.2 mg/g) followed euthanasia by heart puncture exsanguination. Bronchoalveolar lavage (BAL), lung tissue and cerebral cortex for analyzes were collected. The study protocol is illustrated in Fig. 1.

Bronchoalveolar lavage (BAL)

Mice was anesthetized with an injection containing ketamine (0.4 mg/g) and xylazine (0.2 mg/g) and tracheostomized by a steel cannula. The lungs were washed twice with 1 mL of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS).

BAL cellularity

BAL collected was centrifuged at 420 g, for 5 min, at 4 °C and the pellet was resuspended in 350 μL of PBS containing 2% FBS. Total cells count (TCC) was determined by the trypan blue exclusion test, with a Neubauer chamber (BOECO, Hamburg, Germany). For the count of eosinophil, macrophage, lymphocyte, and neutrophil, BAL suspension was centrifuged through a cytospin (FANEM, São Paulo, Brazil), and slides were stained with hematoxylin and eosin (H&E) (Panótico Rápido - Laborclin, Brazil). Four hundred cells were counted under light microscopy BMX 43 (Olympus, Tokyo, Japan).

Histopathologic analysis of lung tissue

The lungs were perfused with 10% buffered formalin on a gravity column (20 mmHg), removed and the specimens were embedded in paraffin blocks, cut into 5 μm sections and stained with H&E (Cytological Products Soldan, Brazil) for assess inflammation infiltrate. Images of the sections were captured through a BMX 43 microscope equipped with a digital camera DP73 (Olympus, Tokyo, Japan). For the peribronchial and perivascular infiltrate quantification, ten measurements (μm) were performed in each of the evaluated regions using the imaging software CellSens Standard (Olympus, Tokyo, Japan). At least 5 fields were evaluated for each animal and the mean was calculated for analysis.

Oxidative stress in the cerebral cortex

To evaluate oxidative stress parameters, after euthanasia the cerebral cortex was removed and homogenized in 10 volumes (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The cerebral cortex homogenates were centrifuged at 750 g for 10 min at 4 °C. The pellet was

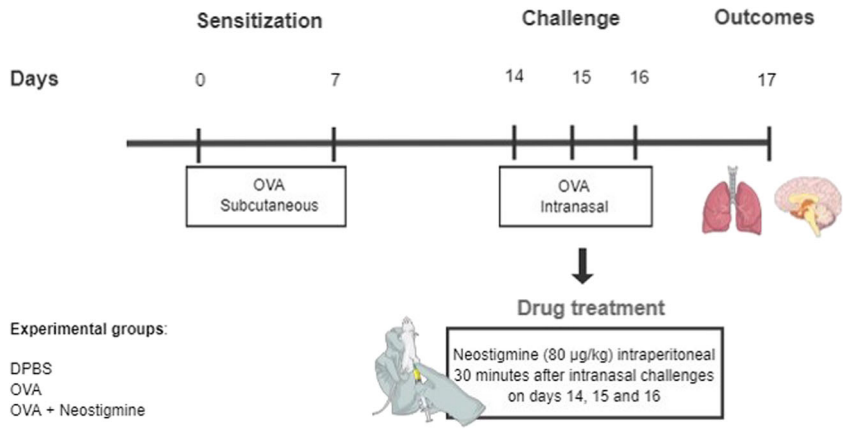


Fig. 1 Protocol used to induce an experimental model of asthma and treatment with neostigmine. OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

discarded and the cerebral cortex supernatant was immediately separated and used for the analysis of ROS production and antioxidant enzyme activities (SOD, CAT, and GPx).

Dichlorofluorescein oxidation activity

Reactive species production was measured by method based in the oxidation of 2′7′-dichlorofluorescein (H2DCF) (LeBel et al. 1992). The sample was incubated in a medium containing 100 μM of 2′7′-dichlorofluorescein diacetate (H2DCF-DA) solution. The reaction produces the fluorescent compound dichlorofluorescein (DCF), which is measured at $\lambda_{em} = 488$ nm and $\lambda_{ex} = 525$ nm; results were represented as nmol DCF/mg protein.

Superoxide dismutase (SOD) activity

The SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD. Thus, the supernatant of cerebral cortex was mixed in a solution containing 1 mM EDTA, 50 mM Tris, 80 U/ml catalase (CAT), and 0.8 mM pyrogallol and whose activity was indirectly measured at 420 nm (Greenwald 2018). The results were represented as SOD units/mg protein.

Catalase (CAT) activity

The CAT activity is based on the disappearance of H_2O_2 at 240 nm. Thus, cerebral cortex supernatant was incubated in 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/ml (Aebi 1984). Each CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the results were presented as CAT units/mg protein.

Glutathione peroxidase (GPx) activity

Was measured GPx activity by the method that using tert-butyl-hydroperoxide as substrate (Wendel 1981). The cerebral cortex supernatant was incubated in medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. NADPH disappearance was monitored in a spectrophotometer at 340 nm. Each GPx units defined as 1 μmol of NADPH consumed per minute. The specific activity was plotted as GPx units/mg protein.

Na^+, K^+-ATPase activity

To measure the Na^+,K^+-ATPase activity, the cerebral cortex was homogenized with 10 volumes of 0.32 mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 3000 g RPM for 10 min at 4 °C. The pellet was discarded and the supernatant was immediately separated for the measurement. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 170 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described (de Souza Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (Chan et al. 1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Ellman and colleagues (Ellman et al. 1961) with modifications. The cerebral cortex were homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and

centrifuged for 10 min at 1000 g. The supernatants were used for the enzymatic AChE analyses. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 μL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s).

Protein determination

Protein was measured according to Bradford, using bovine serum albumin as standard (Bradford 1976).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism (GraphPad Software, LA Jolla, CA, USA). The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey post hoc test. Differences were considered significant at $*p < 0.05$ and results are expressed as the mean ± SD.

Results

Neostigmine treatment decreases leukocyte recruitment in the BAL of asthmatic mice

First, we confirm if neostigmine could improve the inflammatory response in the lung of mice. The OVA group had a significant increase in the TCC when compared with the control group ($P < 0.001$, Fig. 2a). However, in the group that received neostigmine is possible to observe a reduced in the TCC ($P < 0.001$, Fig. 2a). Moreover, the asthma group presented a significant increase in absolute count of macrophages ($P < 0.001$, Fig. 2b), lymphocytes ($P < 0.01$, Fig. 2c), neutrophils ($P < 0.001$, Fig. 2d), and eosinophils ($P < 0.001$, Fig. 2e) when compared to the control group. On the other hand, we showed that the neostigmine treatment was able to reduce the number of macrophages ($P < 0.05$, Fig. 2b), lymphocytes ($P < 0.01$, Fig. 2c), neutrophils ($P < 0.001$, Fig. 2d), and eosinophils ($P < 0.001$, Fig. 2e) in BAL when compared to the asthma group.

Treatment with neostigmine improves leukocyte infiltrate in the lung tissue of asthmatic mice

The histological quantification of the lung sections demonstrated that, in comparison with the control group, animals submitted to a model of allergic asthma had an increased peribronchial ($P < 0.01$, Fig. 3a and b) and perivascular ($P < 0.001$, Fig. 3a and c) cells infiltrate. These results corroborate our previous finding in BAL cellularity and confirm that our asthma model was effective in inducing an allergic pulmonary response. Figure 3 also shows that neostigmine treatment reduced peribronchial ($P < 0.05$, Fig. 3a and b) and perivascular ($P < 0.001$, Fig. 3a and c) infiltration when compared to the asthma group, demonstrating the therapeutic role of acetylcholinesterase inhibitor in control of the recruitment of inflammatory cells.

Neostigmine treatment reverts ROS production in the cerebral cortex of asthmatic mice

After confirming the treatment efficiency in control of inflammatory cells recruitment in the lung tissue, we investigate whether neostigmine can provide a protector effect in ROS production in the cerebral cortex of mice. We evaluated the neostigmine treatment in ROS production by DCF formed from the oxidation of H2DCF. We observed a significant increase in ROS production ($P < 0.05$, Fig. 4a) in the cerebral cortex of asthma group when compared to the control group. Interestingly, we verified that the neostigmine administration reverted ROS production ($P < 0.01$, Fig. 4a) in the cerebral cortex when compared with asthma group.

Neostigmine treatment altered antioxidant enzymes in the cerebral cortex of asthmatic mice

Antioxidant defenses were also measured by determination of antioxidant enzymatic activities (SOD, CAT, and GPx) in the cerebral cortex of mice submitted to asthma model. In SOD and GPx did not alter activity between the groups. However, is possible observed that CAT activity increased ($P < 0.01$, Fig. 4c) in asthma group when compared to the control group. On the other hand, Fig. 4 showed that treatment with neostigmine significantly decreased CAT activity ($P < 0.001$, Fig. 4c) when compared with asthma group. The antioxidant enzyme SOD promotes the dismutation of superoxide anion (O2•–) in hydrogen peroxide (H_2O_2) whereas CAT and GPx catalyze the reduction of H_2O_2 to H_2O. Unbalanced SOD/CAT ratio suggest an increase of hydrogen peroxide production and accumulation, increasing oxidative stress. In this sense, a ratio between SOD and CAT enzyme activities were also analyzed. The SOD/CAT ratio was decreased ($P < 0.05$, Fig. 4e) in asthmatic mice group when compared to the control group. On the other hand, neostigmine treatment promoted SOD/CAT ratio increased ($P < 0.05$, Fig. 4e) when compared to the asthma group.

Treatment with neostigmine did not alter Na^+,K^+-ATPase activity in the cerebral cortex of asthmatic mice

We also investigated the effect of neostigmine treatment in Na^+,K^+-ATPase activity. Figure 5 shows that mice subjected to asthma model showed a significant reduction of Na^+,K^+-

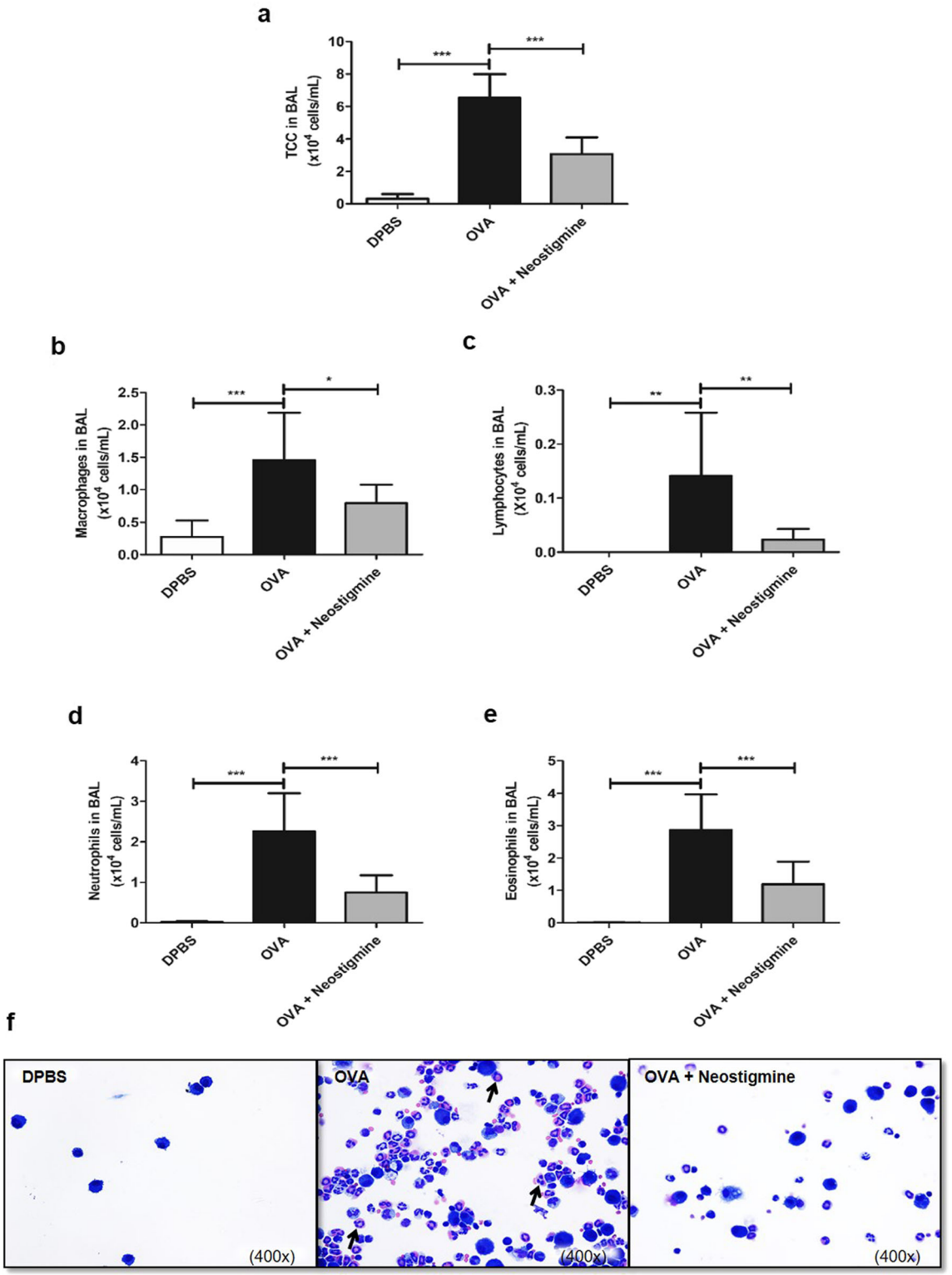


Fig. 2 Mice treated with neostigmine decreased leukocytes recruitment in the BAL. (**a**) absolute total cells count, (**b**) absolute macrophages count, (**c**) absolute lymphocytes count, (**d**) absolute neutrophils count, (**e**) absolute eosinophils count. (**f**) Representative image of the differential cells count (H&E, 400x magnification). Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p < 0.001$, **$p < 0.01$ different from OVA group *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (One-way ANOVA followed by Tukey test). BAL: bronchoalveolar lavage; OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

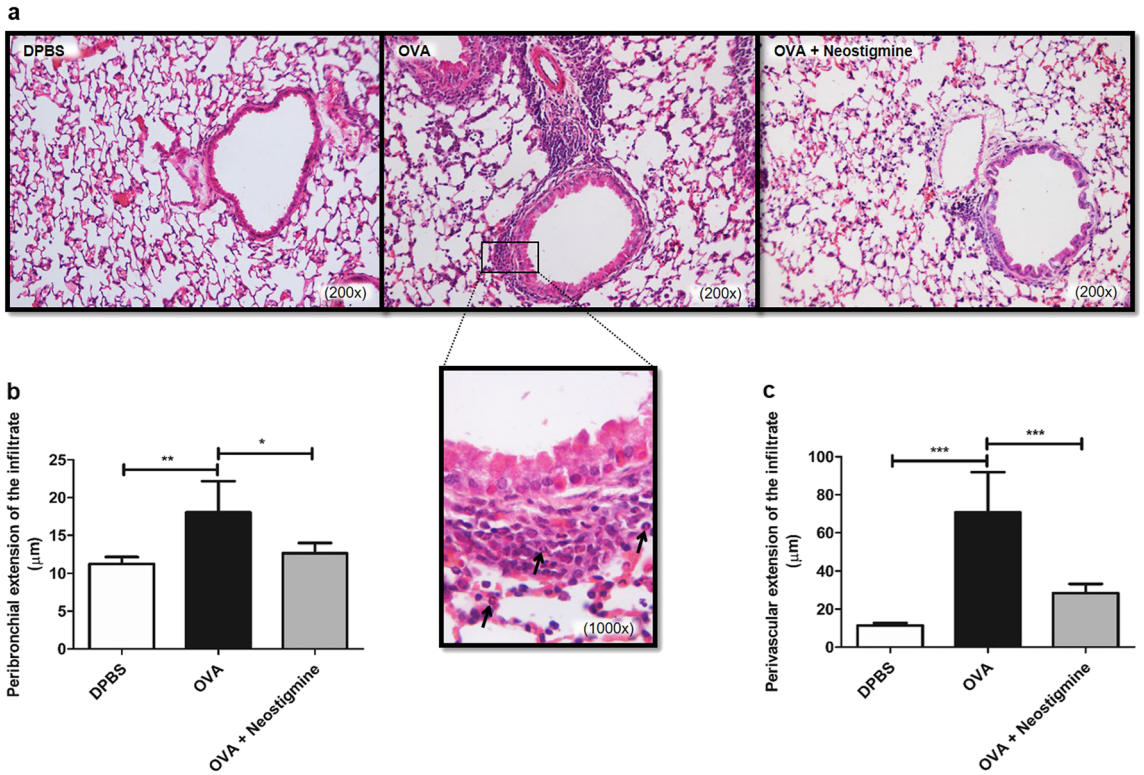


Fig. 3 Neostigmine treatment reduced leukocytes infiltrate in the lung. (**a**) Representative lung sections stained with H&E (200x and 1000x magnification). Histological quantification of (**b**) peribronchial and (**c**) perivascular infiltrate. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group $^{**}p < 0.01$, $^{***}p < 0.001$, different from OVA group $^{*}p < 0.05$, $^{***}p < 0.001$ (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

ATPase activity ($P < 0.001$, Fig. 5) when compared to the control group. However, this figure also showed that neostigmine did not alter the Na^+,K^+-ATPase activity when compared to the asthma group.

Effects of neostigmine treatment on AChE activity in cerebral cortex of asthmatic mice

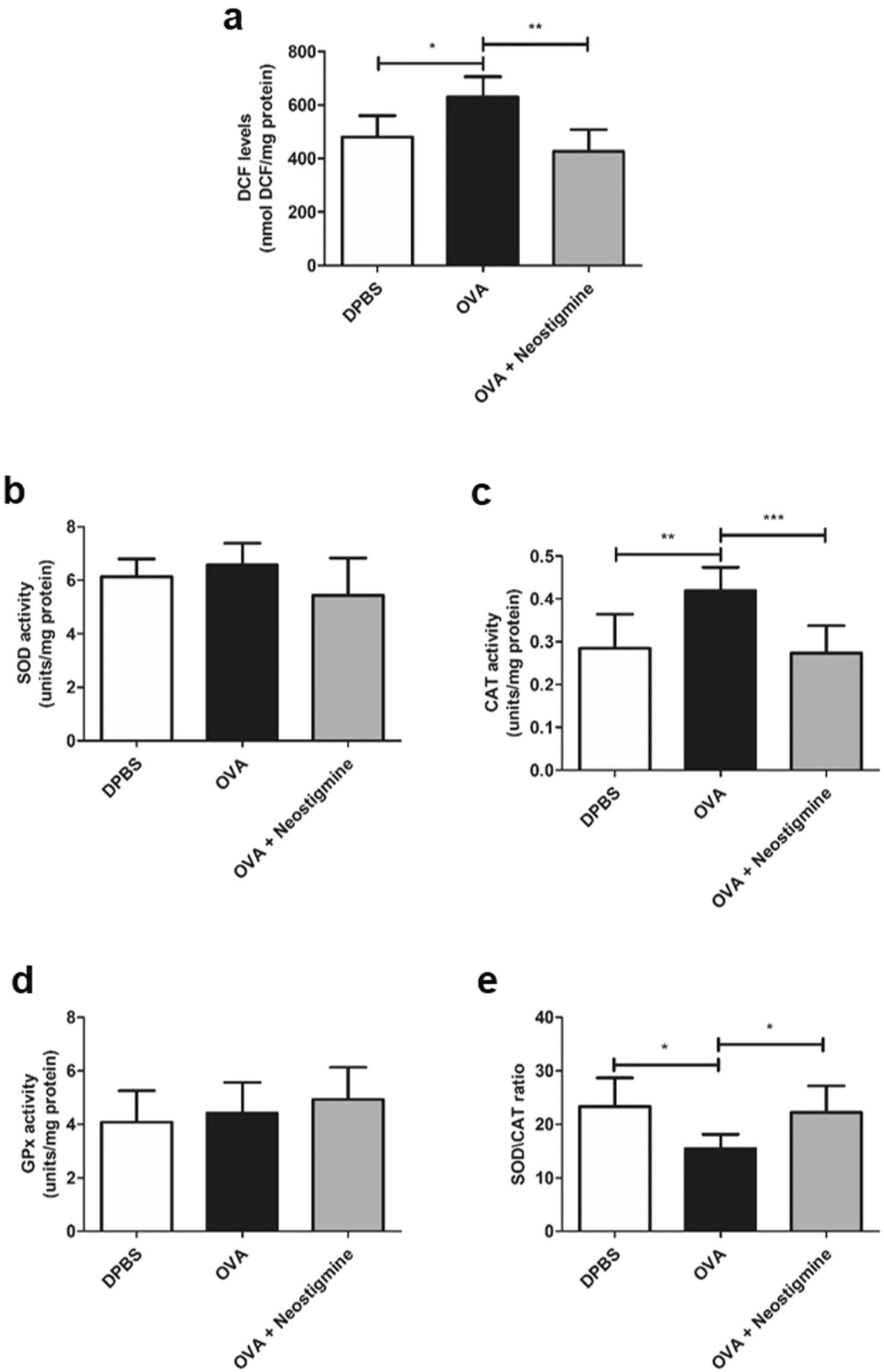
Finally, we investigated the neostigmine treatment on AChE activity. Asthmatic mice showed a significant increase in the AChE activity ($P < 0.05$, Fig. 6) when compared to the control group. However, the neostigmine-treated group was not reduced the AChE activity when compared to the asthma group.

Discussion

Asthma is characterized by chronic inflammatory in the lung driven by leukocytes and Th2 lymphocytes. The brain is highly sensitive to peripherals inflammation, which can result in neuroinflammation. In fact, Xia and colleagues showed that asthma not only induced lung inflammation but results in an increase of IL-1β and TNFα in the mice hippocampus and prefrontal cortex, causing neuroinflammation (Xia et al. 2014). However, this study also revealed that treatment with inhaled budesonide, an anti-inflammatory used for asthma treatment, was able reverts neuroinflammation by a decrease in IL-1β and TNFα in hippocampus and prefrontal cortex. Cytokines are important biomarkers of neuroinflammation. However, it is known that oxidative stress also associated strongly with neuroinflammation and tissue damage. In a recent work was observed the excessive ROS production in the cerebral cortex of asthmatic mice (Duan et al. 2018). In this sense, we decided to investigate whether neostigmine, a known inhibitor of lung inflammation could reverse oxidative stress in the cerebral cortex of asthmatic mice.

Initially, we resolved to confirm whether treatment with acetylcholinesterase inhibitor could decrease recruitment of leukocytes in the airways. TCC showed a decrease in the number after neostigmine treatment. In the differential cells count it was evident the neostigmine ability to reduce recruitment of inflammatory cells, especially of eosinophils that the

Fig. 4 Treatment with neostigmine improves oxidative stress parameters in the cerebral cortex. (**a**) Reactive species production by DCF, (**b**) superoxide dismutase activity, (**c**) catalase activity, (**d**) glutathione peroxidase activity (**e**) SOD/CAT ratio. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group $^{*}p < 0.05$, $^{**}p < 0.01$, different from OVA group $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline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DCF levels (nmol DCF/mg protein)
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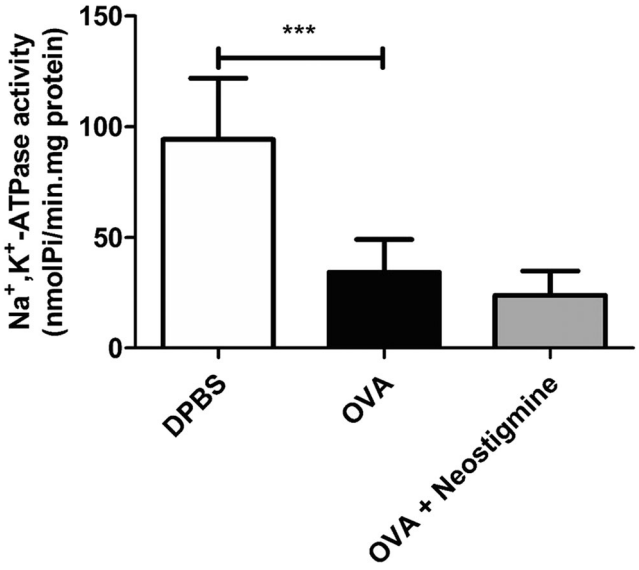

Fig. 5 Effect of neostigmine treatment on Na^+,K^+-ATPase activity in the cerebral cortex. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p < 0.001$ (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

hallmark of type 2 inflammation. Indeed, our histopathologic analysis of lung tissue showed a decreased peribronchial and perivascular infiltrate in mice that received neostigmine treatment. Corroborating with our results, Kanashiro and colleagues also observed the ability of neostigmine to reduce recruitment of leukocytes in inflammatory diseases (Kanashiro et al. 2016).

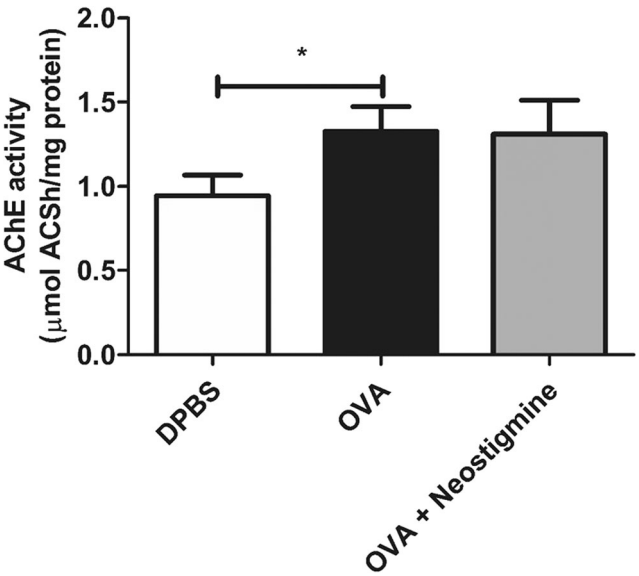

Fig. 6 Effect of treatment with neostigmine on AChE activity in the cerebral cortex. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group *$p < 0.05$ (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

Then, we began to investigate the neostigmine effects on the oxidative stress parameters in the cerebral cortex of mice submitted an allergic asthma model. The brain tissues have unique characteristics that make them especially susceptible to free radicals damage, have a high rate of oxygen consumption, low antioxidant levels, has a high content of lipids, and presents a high metabolic activity, being, therefore, a tissue more sensitive to oxidative damages when compared with other tissues (Netto et al. 2018). Neuroinflammation has been reported to be associated with oxidative stress caused by exacerbated ROS production (Hritcu and Ciobica 2013). The ROS consist of free radicals of oxygen and associated molecules, including $O_2•^-$, H_2O_2, and hydroxyl radical ($OH•^-$) (Kumar et al. 2012). A ROS increase was observed, as measured through the DCF fluorescence assay in the OVA group, demonstrating that lung inflammation can induce oxidative damage in the cerebral cortex. In contrast, our results showed that neostigmine treatment decreased ROS formation. Thus, we reported that asthma-induced lung inflammation results in ROS formation in the cerebral cortex and we showed, for the first time, that neostigmine treatment can effectively revert this parameter.

To protect against the oxidative damage, cells possess several enzymatic and nonenzymatic antioxidant defenses that interrupt the cascade of oxidative reactions, limiting the extent of oxidative damage. Given that neostigmine treatment prevented ROS production, we also evaluated the neostigmine effects on enzymatic antioxidant systems. SOD is a metalloenzyme that catalyzes the dismutation of $O_2•^-$, forming H_2O_2. One molecule of the formed H_2O_2 is reduced to H_2O and the other is oxidized to O_2 by dismutation of CAT. GPx also acts convert H_2O_2 to H_2O.

SOD activity did not alter between the groups, indicating that the increase in the production of reactive species is not by $O_2•^-$ formation. However, we observed an increase in CAT activity in the OVA group, probably is a compensatory mechanism to minimize the excess production H_2O_2, since GPx activity did not alter between the groups. Furthermore, the mice treated with neostigmine showed a reduce CAT activity probably because neostigmine reduces the production of reactive species and the consequent formation of H_2O_2.

SOD and CAT work in sequence and an unbalanced SOD/CAT ratio suggest an accumulation of hydrogen peroxide, leading to oxidative stress. Thus, we evaluated a ratio between SOD and CAT enzyme activities. The cerebral cortex SOD/CAT ratio was decreased in OVA group when compared to control group. Interestingly, mice submitted to allergic asthma and treated with neostigmine showed enhance in SOD/CAT ratio. Thus, we suggest that the ROS production is being neutralized through the antioxidant system by neostigmine effect. Odorcyk and colleagues showed similar results, galantamine (acetylcholinesterase inhibitor) administration promoted upregulates in the anti-oxidant system (Odorcyk et al. 2017).

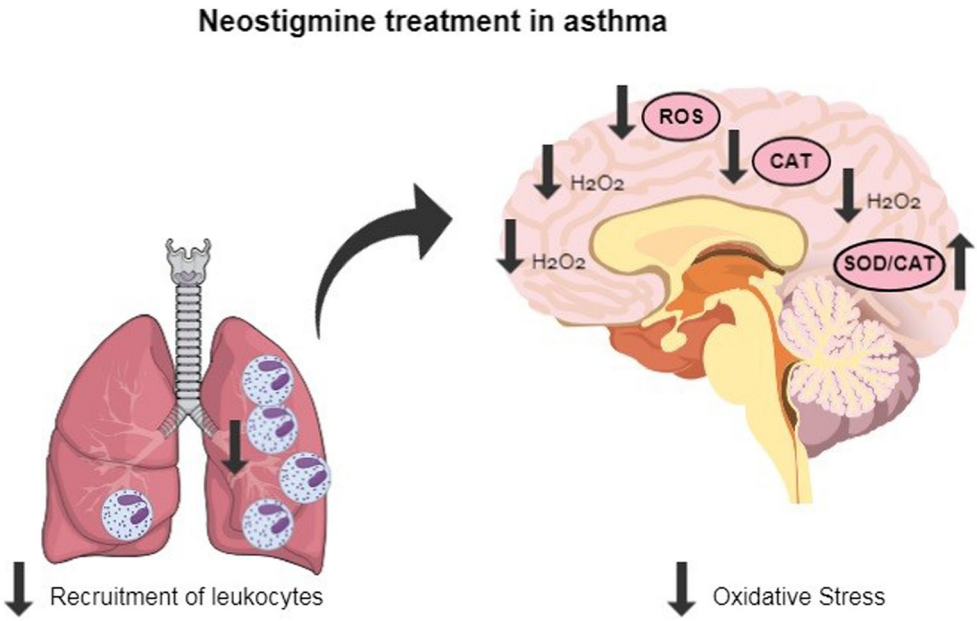

Fig. 7 Summary of the neostigmine effects that lead to neuroprotection against oxidative stress in asthmatic mice. CAT: catalase; SOD: superoxide dismutase; ROS: reactive oxygen species, H_2O_2: hydrogen peroxide

Studies have been showed that ROS increase and exacerbate oxidative stress response and these processes may be associated with brain energy metabolism impairment. However, the mitochondrial ATP production is a fundamental function for cellular energy metabolism (Biasibetti-Brendler et al. 2018). About 40–50% of the ATP generated is consumed by Na^+,K^+-ATPase, a crucial enzyme that is responsible for the generation of the membrane potential necessary to maintain neuronal excitability and cellular volume control. The Na^+,K^+-ATPase is present in high concentration in cell membranes and is crucial for brain development and function (Jeremias et al. 2012). Impairment in Na^+,K^+-ATPase activity has been associated with diverse diseases, including cerebral ischemia and neurodegenerative disorders (Wyse et al. 2000; Yu 2003). Corroborating with this results, our data also showed an impairment in Na^+,K^+-ATPase activity in the cerebral cortex of the allergic asthma group. However, neostigmine treatment was not able to revert the impairment in Na^+,K^+-ATPase activity. Machado and colleagues showed that rats subjected to hyperhomocysteinemia (Hcy) presented a significant reduction of Na^+, K^+-ATPase. However, vitamin C, per se, did not alter the Na^+,K^+-ATPase activity, but when administered concomitantly with Hcy, it was able to prevent the damage caused by Hcy (Machado et al. 2011), suggest that our results can be explained by a moment of neostigmine administration.

In this article, we observed that neostigmine administration promoted neuroprotection by decreased ROS production and upregulation in the antioxidant system. We believe that these results are due to the mechanism of action of the drugs that act by inhibiting acetylcholinesterase and thus, upregulation acetylcholine (ACh). ACh has been recognized as an important component of the cholinergic anti-inflammatory pathway (CAP) that leads to the inflammation control (Borovikova et al. 2000; Kanashiro et al. 2016; Wang et al. 2003). In order, ACh is synthesized from choline and acetyl Co-A by choline acetyltransferase (ChAT) and pumped into storage vesicles by the vesicular acetylcholine transporter (VAChT). ACh is released by exocytosis and either binds to receptors (muscarinic and/or nicotinic) and after release, ACh is degraded by AChE to non-active choline (Gwilt et al. 2007). In this way, we observed that mice submitted to a model of asthma had an increased AChE activity when compared to the control group. On the other hand, we did not observe alterations in activity after neostigmine administration. We hypothesized that this result may be due to the time of cerebral cortex collection for analysis. The AChE activity was measured twenty-four hours after the last administration of neostigmine. However, the half-life of the drug is approximately one hour. Interestingly, even with a short half-life, is evident that neostigmine provided neuroprotection by decreased ROS production and upregulation in the antioxidant system. Thus, we summarize our results in Fig. 7.

In conclusion, the present study revealed for the first time that treatment with neostigmine, known for the ability to control lung inflammation, was able to revert ROS production and change anti-oxidant enzyme catalase in the cerebral cortex in asthmatic mice. These results support the communication between the peripheral immune system and the CNS and suggest that acetylcholinesterase inhibitors, such as neostigmine, should be further studied as possible therapeutic strategies for neuroprotection in asthma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiments were conducted in accordance with the Brazilian Society of Laboratory Animal Science (SBCAL), using fewer animals and adequate management of pain and suffering, during the study procedures and euthanasia. This study was approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA, 7934).

References

Aebi, H. (1984). [13] catalase in vitro *Methods in enzymology* (Vol. 105, pp. 121-126): Elsevier

Antunes GL, Silveira JS, Kaiber DB, Luft C, da Costa MS, Marques EP et al (2019) Cholinergic anti-inflammatory pathway confers airway protection against oxidative damage and attenuates inflammation in an allergic asthma model. J Cell Physiol

Biasibetti-Brendler H, Schmitz F, Pierozan P, Zanotto BS, Prezzi CA, de Andrade RB, Wyse AT (2018) Hypoxanthine induces neuroenergetic impairment and cell death in striatum of young adult Wistar rats. Mol Neurobiol 55(5):4098–4106

Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR et al (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405(6785):458

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254

Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+−stimulated ATPase activity. Anal Biochem 157(2):375–380

Chou WH, Choi DS, Zhang H, Mu D, McMahon T, Kharazia VN et al (2004) Neutrophil protein kinase Cδ as a mediator of stroke-reperfusion injury. J Clin Invest 114(1):49–56

Danielski LG, Della Giustina A, Badawy M, Barichello T, Quevedo J, Dal-Pizzol F, Petronilho F (2017) Brain barrier breakdown as a cause and consequence of neuroinflammation in sepsis. Mol Neurobiol:1–9

de Souza Wyse AT, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na+, K+-ATPase activity after brain ischemia. Neurochem Res 25(7):971–975

Deckers J, De Bosscher K, Lambrecht BN, Hammad H (2017) Interplay between barrier epithelial cells and dendritic cells in allergic sensitization through the lung and the skin. Immunol Rev 278(1):131–144

Duan J, Kang J, Qin W, Deng T, Liu H, Li B et al (2018) Exposure to formaldehyde and diisononyl phthalate exacerbate neuroinflammation through NF-κB activation in a mouse asthma model. Ecotoxicol Environ Saf 163:356–364

Ellman GL, Courtney KD, Andres V Jr, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7(2):88–95

Greenwald, R. A. (2018). *Handbook Methods For Oxygen Radical Research*: CRC press

Gwilt CR, Donnelly LE, Rogers DF (2007) The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? Pharmacol Ther 115(2):208–222

Haider L, Fischer MT, Frischer JM, Bauer J, Höftberger R, Botond G et al (2011) Oxidative damage in multiple sclerosis lesions. Brain 134(7): 1914–1924

Hofer S, Eisenbach C, Lukic IK, Schneider L, Bode K, Brueckmann M et al (2008) Pharmacologic cholinesterase inhibition improves survival in experimental sepsis. Crit Care Med 36(2):404–408

Hritcu L, Ciobica A (2013) Intranigral lipopolysaccharide administration induced behavioral deficits and oxidative stress damage in laboratory rats: relevance for Parkinson's disease. Behav Brain Res 253:25–31

Hunter M, Nlemadim B, Davidson D (1985) Lipid peroxidation products and antioxidant proteins in plasma and cerebrospinal fluid from multiple sclerosis patients. Neurochem Res 10(12):1645–1652

Jeremias IC, Scaini G, Constantino L, Vuolo F, Ferreira AK, Scherer EBS et al (2012) The decrease on Na+, K+-ATPase activity in the cortex, but not in hippocampus, is reverted by antioxidants in an animal model of sepsis. Mol Neurobiol 46(2):467–474

Kalb A, von Haefen C, Sifringer M, Tegethoff A, Paeschke N, Kostova M et al (2013) Acetylcholinesterase inhibitors reduce neuroinflammation and-degeneration in the cortex and hippocampus of a surgery stress rat model. PLoS One 8(5):e62679

Kanashiro A, Talbot J, Peres RS, Pinto LG, Bassi GS, Cunha TM, Cunha FQ (2016) Neutrophil recruitment and articular hyperalgesia in antigen-induced arthritis are modulated by the cholinergic anti-inflammatory pathway. Basic & clinical pharmacology & toxicology 119(5):453–457

Kumar H, Lim HW, More SV, Kim BW, Koppula S, Kim IS, Choi DK (2012) The role of free radicals in the aging brain and Parkinson's disease: convergence and parallelism. Int J Mol Sci 13(8):10478–10504

LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′, 7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5(2):227–231

Lu F, Selak M, O'Connor J, Croul S, Lorenzana C, Butunoi C, Kalman B (2000) Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. J Neurol Sci 177(2):95–103

Machado FR, Ferreira AG, da Cunha AA, Tagliari B, Mussulini BHM, Wofchuk S, Wyse AT (2011) Homocysteine alters glutamate uptake and Na+, K+-ATPase activity and oxidative status in rats hippocampus: protection by vitamin C. Metab Brain Dis 26(1):61–67

Naeem A, Silveyra P (2019) Sex differences in Paediatric and adult asthma. EMJ 4(2):27–35

Netto MB, de Oliveira Junior AN, Goldim M, Mathias K, Fileti ME, da Rosa N et al (2018) Oxidative stress and mitochondrial dysfunction contributes to postoperative cognitive dysfunction in elderly rats. Brain Behav Immun 73:661–669

Obermeier B, Daneman R, Ransohoff RM (2013) Development, maintenance and disruption of the blood-brain barrier. Nat Med 19(12): 1584

Odorcyk F, Nicola F, Duran-Carabali L, Figueiró F, Kolling J, Vizuete A et al (2017) Galantamine administration reduces reactive astrogliosis and upregulates the anti-oxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia. Int J Dev Neurosci 62:15–24

Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S et al (2003) Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. Nature 421(6921):384

Wendel, A. (1981). [44] glutathione peroxidase *Methods in enzymology* (Vol. 77, pp. 325-333): Elsevier

Wyse AT, Streck EL, Barros SV, Brusque AM, Zugno AI, Wajner M (2000) Methylmalonate administration decreases Na+, K+-ATPase activity in cerebral cortex of rats. Neuroreport 11(10):2331–2334

Xia MX, Ding X, Qi J, Gu J, Hu G, Sun XL (2014) Inhaled budesonide protects against chronic asthma-induced neuroinflammation in mouse brain. J Neuroimmunol 273(1–2):53–57

Yu SP (2003) Na+, K+-ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death. Biochem Pharmacol 66(8): 1601–1609