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Anthocyanins as a potential pharmacological agent to manage memory deficit, oxidative stress and alterations in ion pump activity induced by experimental sporadic dementia of Alzheimer's type

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Abstract

Anthocyanins (ANT) are polyphenolic flavonoids with antioxidant and neuroprotective properties. This study evaluated the effect of ANT treatment on cognitive performance and neurochemical parameters in an experimental model of sporadic dementia of Alzheimer's type (SDAT). Adult male rats were divided into four groups: control (1 mL/kg saline, once daily, by gavage), ANT (200 mg/kg, once daily, by gavage), streptozotocin (STZ, 3 mg/kg), and STZ *plus* ANT. STZ was administered via bilateral intracerebroventricular (ICV) injection (5 µL). ANT were administered after ICV injection for 25 days. Cognitive deficits (short-term memory and spatial memory), oxidative stress parameters, acetylcholinesterase (AChE) and Na^{+}-K^{+-} ATPase activity in the cerebral cortex and hippocampus were evaluated. ANT treatment protected against the worsening of memory in STZ-induced SDAT. STZ promoted an increase in AChE and Na^{+}-K^{+-}ATPase total and isoform activity in both structures; ANT restored this change. STZ administration induced an increase in lipid peroxidation and decrease in the level of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in the cerebral cortex; ANT significantly attenuated these effects. In the hippocampus, an increase in reactive oxygen species (ROS), nitrite and lipid peroxidation levels, and SOD activity and a decrease in CAT and GPx activity were seen after STZ injection. ANT protected against the changes in ROS and antioxidant enzyme levels. In conclusion, the present study showed that treatment with ANT attenuated memory deficits, protect against oxidative damage in the brain, and restore AChE and ion pump activity in an STZ-induced SDAT in rats.

Keywords: anthocyanins; Alzheimer disease; oxidative stress; acetylcholinesterase; Na^{+}-K^{+-}ATPase; streptozotocin

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and reduced cognitive capacity [1]. It is the most common cause of dementia and its prevalence increases significantly with age [2,3]. It is estimated that 67.5 million people in the world will have AD by 2030 and this could reach 115.4 million by 2050 [4]. Sporadic AD is multifactorial and comprises the majority of cases of this disease [2,3,5,6].

The neuropathological changes underlying AD include extracellular senile plaques formed by β-amyloid peptide (Aβ) and intracellular accumulation of neurofibrillary tangles composed of hyperphosphorylated protein tau that promote synaptic dysfunction and neuronal death [7]. Furthermore, oxidative stress, neuroinflammation, altered ionic homeostasis, cholinergic dysfunction, and deficits in insulin signaling are also related with AD pathogenesis [8-10].

Oxidative stress has been associated with the initiation and progression of AD [11,12]. This is supported by the potential high vulnerability of neurons to reactive oxygen species (ROS), large amount of polyunsaturated fatty acids, high level of pro-oxidant metals, and low level of endogenous antioxidants in the brain [13,14]. Oxidative stress can induce membrane lipid damage, changes in enzymes critical to neuronal and glial function, and structural damage to DNA leading to tissue damage, synapse dysfunction, and cell death [15].

Furthermore, studies have also proposed a relationship between oxidative stress and dysfunction in acetylcholinesterase (AChE) and Na^+-K^+-ATPase activity in many diseases [16]. AChE is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) and this enzyme has been an important therapeutic target in AD since this pathology was first associated with the loss of cholinergic neurons and decrease in the level of ACh in the brain, leading to cognitive deficits [17,18]. In addition, Na^+-K^+-ATPase plays a role in intracellular ion and membrane potential homeostasis, regulating neuronal excitability. Alterations in Na^+-K^+-ATPase activity are associated with impaired synaptic responses, leading to alterations in the process of learning and memory [16].

Many studies in the literature have focused on the therapeutic potential of natural compounds aimed at the prevention of neurodegenerative diseases [19].

Anthocyanins (ANT), polyphenolic flavonoids found in various flowers, fruits, and vegetables, have demonstrated important biological activities, such as antioxidant and anti-inflammatory actions [20,21]. Our research group has reported the beneficial effects of ANT against lipopolysaccharide (LPS)-induced neuroinflammation [22], ethidium bromide-induced demyelination [23], and scopolamine-induced memory deficits [24]. Of particular importance in this study, we also demonstrated that pretreatment with ANT prevented the behavioral and neurochemical alterations caused by streptozotocin (STZ)-induced sporadic dementia of Alzheimer's type (SDAT) [25].

In this sense, considering the growing number of studies that provide evidence of the health benefits of ANT, the present study investigated the effect of ANT on protecting learning and memory ability in STZ-induced SDAT in rats. To delineate a possible mechanism of action for ANT, we also investigated the activity of key enzymes involved in cholinergic transmission, as well as oxidative and nitrosative stress markers, in the cerebral cortex and hippocampus of these animals.

2. Material and methods

2.1 Chemicals

Acetylthiocholine iodide (AcSCh), Coomassie Brilliant Blue G, ouabain, STZ, dichloro-dihydro-fluorescein diacetate (DCFH-DA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and adenosine triphosphate (ATP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). An anthocyanin-rich extract was obtained from grape skins (AC-12-R-WS-P/10120/Gin: 601412) and is commercially available from Christian Hansen A/S. All other reagents used in the detailed experiments were of analytical grade and the highest purity.

2.2 Animals

Forty adult male *Wistar* rats (2 months old) weighing 200–300 g were used in this study. The animals were kept in the Central Animal House of Federal University of Pelotas in colony cages under standard temperature (23 ± 1°C), relative humidity (45–55%), and lighting (12-h light/dark cycle) conditions for all trials. The rats had *ad libitum* access to an adequate commercial diet and water. This study was approved

by the Ethics Committee and Animal Experimentation of the Federal University of Pelotas under the protocol number CEEA 0179/2015.

2.3 Intracerebroventricular injection of STZ

For all surgical procedures, adult male rats were anesthetized with ketamine (100 mg/mL) and xylazine (20 mg/mL), administered intraperitoneally. The head of the animal was positioned in the stereotaxic apparatus and a midline sagittal incision was made in the scalp. The skull was drilled on both sides according to the stereotaxic coordinates used for the lateral ventricles. These coordinates were -0.8 mm anteroposterior and 1.5 mm mediolateral from the bregma and -4.0 mm dorsoventral to the dura with the bregma as the zero reference point [25,26]. The animals were divided into four groups: control (C), ANT, STZ, and STZ + ANT. The animals in the STZ and STZ + ANT groups received intracerebroventricular injection (ICV) of 3 mg/kg STZ dissolved in a citrate buffer (pH 4.5), delivered through the holes drilled in the skull [27]. The other groups received vehicle solution (citrate buffer) ICV. Both STZ and vehicle were injected using a 10 µL 28-gauge Hamilton® syringe and each ventricle received 5 µL of solution at a rate of 1 µL/min for five minutes.

2.4 Treatment with anthocyanin

Three days after the ICV injection of STZ or vehicle, the animals in the ANT and STZ + ANT groups were treated with 200 mg/kg ANT dissolved in saline daily by gavage for 25 days (see Figure 1). Animals in the Control and STZ groups received saline (1 mL/kg) also by gavage during the same time. The dose of ANT administered was selected according to findings from previous studies [25].

2.5 Behavioral tests

2.5.1 Open field test

After twenty-six days of STZ injection, the animals underwent an open field test. In this test, the animals were placed in the apparatus consisting of a square arena measuring 56 × 40 × 30 cm with the floor divided into twelve squares (12 × 12 cm each). Rats were placed individually in one of the four corners of the apparatus

and remained within the setup for five minutes. During this time, total crossing and rearing response were analyzed. The arena was cleaned with 40% ethanol after each individual session. This test allows quantification of locomotor activity and identification of motor disabilities and altered exploratory activity [25,28].

2.5.2 Object recognition test

Twenty-four hours after the open field test that was also used as habituation to the apparatus, the animals underwent an object recognition test to evaluate short-term memory. First, the animals were placed individually in the arena with two identical objects (object A and B) for five minutes and allowed to explore them freely (training session). After two hours, in the test session, the animals were placed again in the arena for five minutes but one of the previous objects (B) was replaced by a new object (object C). The time spent exploring the familiar and novel object was recorded. The objects used were pairs of plastic mounting blocks and were placed in a symmetrical position inside the arena. The arena and objects were cleaned between trials with 40% ethanol to remove residues and smells. Exploration was considered only when the rats were sniffing or touching the objects with their nose and/or forepaws. Results were analyzed using the recognition index for each animal calculated as follows, recognition index = TC/(TA+TC), where TA is the time taken to explore familiar object A and TC is the time taken to explore new object C [29].

2.5.3 Y-maze Test

Twenty-eight days after the STZ injection, the animals underwent a Y-maze test to evaluate spatial memory. This test used a Y-maze that had three arms, with each arm randomly designated as either the start arm (A), novel arm (B), or other arm (C). This test had a training session where the animal was placed in the apparatus on the start arm and was free to explore only the start arm and other arm for five minutes. The novel arm remained blocked throughout the training session. After two hours, the test session was performed with an open novel arm and the animal could freely explore all three arms over a five-minute period. The apparatus was cleaned with 40% ethanol after each session. The time spent in each arm was determined and the results were expressed as the percentage of time spent and number of entries on the new arm [30]. Twenty-four hours after the Y-maze test, the

animals were euthanized; and brain was removed and the hippocampus and cerebral cortex were dissected for evaluation using biochemical assays. Blood also was collected for analysis of glucose levels.

2.6 AChE activity determination

Samples of cerebral cortex and hippocampus were homogenized on ice in a glass potter with 10 mM Tris–HCl solution (pH 7.4). Aliquots of brain structure homogenates were stored at -80 °C until utilization. The protein content was determined using the Coomassie blue method, with bovine serum albumin as the standard solution [31].

The AChE enzymatic assay was determined using a spectrophotometric method [32]. This method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, which was measured by absorbance at 412 nm, over a 2-min period at 25°C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM AcSCh. All samples were tested twice and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

2.7 Determination of total ATPase, Na^+-K^+-ATPase, and isoform activities

Na^+-K^+-ATPase activity was measured in the cerebral cortex and hippocampus supernatant as previously described [22]. Briefly, the assay medium consisted of 30 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 120 µg of protein in the presence or absence of ouabain (3 µM or 4 mM) to reach a final volume of 200 µL. The reaction was started by the addition of ATP to a final concentration of 3 mM. After 30 minutes at 37°C, the reaction was stopped by the addition of 50 µL of 50% (w/v) trichloroacetic acid (TCA). Saturating substrate concentrations were used, and the reaction rate was linear with respect to protein content and time. Appropriate controls were included in the assays for the non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described [33], using KH_2PO_4 as the reference standard. The absorbance at 630 nm was measured. The specific Na^+-K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and was expressed in nmol of Pi/mg of protein/min.

Different concentrations of ouabain were used to evaluate the activity of different Na^+-K^+-ATPase isoforms. A classical pharmacological approach based on the isoform-specific sensitivity to ouabain was used [34]. The experiments were performed as previously described [35]. To determine if treatments alter ouabain-sensitive ATPase activity, 3 µM or 4 mM ouabain was used (so as to only inhibit the Na^+-K^+-ATPase isoforms containing subunits α2/α3, or to every inhibit isoform, respectively).

2.8 Oxidative stress determination

2.8.1. Brain tissue preparation

The cerebral cortex and hippocampus were homogenized in sodium phosphate buffer (20 mM) containing KCl (140 mM), pH 7.4. The homogenates were centrifuged at 2500*g* for ten minutes at 4°C and the supernatant was separated to analyze oxidative stress parameters. The samples were stored at -80°C until utilization. Protein content was determined using the Lowry method [36], with bovine serum albumin as the standard solution.

2.8.2 Reactive oxygen species (ROS) determination

ROS formation was determined as previously described [37] with some modifications. The method was based on the oxidation of DCFH-DA to fluorescent dichlorofluorescein (DCF). DCF fluorescence was measured using excitation at 485 nm and emission at 520 nm 30 minutes after the addition of DCFH-DA to the sample. ROS levels were expressed as µmol DCF/mg of protein.

2.8.3 Nitrite levels quantification

Nitrite (NO_2^-) content was measured using the Griess reaction, as previously described [38]. Briefly, 50 µL of sample plus 50 µL of sulphanilamide in 5% phosphoric acid were incubated for 10 minutes at room temperature. Next, 50 µL of N-(1-naphthyl) ethylenediamine dihydrochloride was added and the mixture was incubated for 10 minutes at room temperature while protected from illumination. Absorbance at 540 nm was measured in a 96-well microplate reader. A sodium nitrite

solution was used as the reference standard and the results were expressed as µmol NO_2^- /mg of protein.

2.8.4 Thiobarbituric acid reactive substances (TBARS) level method

TBARS was used to determine lipid peroxidation through the malondialdehyde (MDA) levels according to a method described previously [39]. Briefly, 100 µL of tissue homogenate was mixed with 15% TCA and 0.67% thiobarbituric acid. This mixture was heated at 95°C for 30 minutes and cooled (4°C) after for 10 minutes. The absorbance was measured at 532 nm and the results were expressed as nmol TBARS/mg of protein.

2.8.5 Total thiol level assay

The total level of thiol groups was measured using the DTNB method according to a modified, previously described method [40]. Briefly, 10 µL of sample was mixed with 145 µL of PBS–EDTA (1 mM), pH 7.5. For the reaction, 10 µL of DTNB (10 mM) in PBS was added to the mixture. After one hour of incubation at room temperature, the absorbance was read at 412 nm and the results were expressed as nmol TNB/mg of protein.

2.8.6 Superoxide dismutase (SOD) activity assay

The activity of SOD was determined using a method that is based on measuring the inhibition of the autoxidation of adrenaline as described previously [41]. For this assay, the medium contained catalase (10 µM), 10 µL of sample, glycine buffer (50 mM, pH 10.2) and adrenaline (60 mM). SOD levels were assayed using the sample absorbance at 480 nm. The results were expressed as Units/mg of protein. One unit of SOD was defined as the amount of enzyme necessary to inhibit 50% of adrenaline autoxidation.

2.8.7 Catalase (CAT) activity assay

CAT activity was measured according to a previously described method [42]. For this analysis, 10 µL of sample was mixed with Triton X-100 (1:10 w/v) and potassium phosphate buffer (pH 7.0). The reaction was started with the addition of hydrogen peroxide (H_2O_2) in the reaction medium. This method is based on the ability

of the CAT to decompose H_2O_2 and is measured by the decrease in absorbance at 240 nm. One unit of CAT was defined as one μmol of H_2O_2 consumed per minute and the specific activity was expressed as Units/mg of protein.

2.8.8 Glutathione peroxidase (GPx) activity assay

The activity of GPx was analyzed using a commercial kit from Randox Laboratories Ltd. (United Kingdom) according to the manufacturer's instructions. This method is based on that previously described [43] in which GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is instantly converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The resultant decrease in absorbance at 340 nm was measured. One GPx unit was defined as 1 μmol of NADPH consumed per minute and the specific activity was recorded as Units/mg of protein.

2.9 Determination of glucose levels

Glucose levels were determined in serum using a commercial kit (Labtest® Diagnostica S.A. MG, Brazil), according with the manufacturer's instructions.

2.10 Statistical analysis

Statistical analysis of test results was carried out using one or two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* testing for multiple comparisons. $P < 0.05$ was considered to represent a significant difference in all experiments. All data were expressed as the mean ± SEM.

3. Results

3.1 Anthocyanins protect impairment of non-spatial memory induced by STZ administration

Figure 2 shows the effects of ANT treatment on the behavioral results from the open field, object recognition, and Y-maze tests. In the open field test, it was observed that STZ did not alter the number of total crossings or rearing responses,

suggesting that neither STZ nor ANT affected the locomotor or exploratory capacity of the animals (Fig. 2A and B).

Our results also demonstrated that STZ injection induced a memory impairment in the object recognition task and ANT treatment effectively attenuated the memory deficits [$F_{(1,23)}$ = 7.72; $P < 0.05$; Fig. 2C] . In this study, we also evaluated the effects of the treatment with ANT and STZ on spatial memory in Y-maze apparatus. STZ decreased both the percentage of time spent [$F_{(1,32)}$ = 11.18; $P < 0.01$; Fig. 2D] and number of entries [$F_{(1,36)}$ = 24.19; $P < 0.001$; Fig. 2 E] into the novel arm. ANT treatment did not increase the time spent in the novel arm nor the number of entries into the novel arm.

3.2 Anthocyanins protect against alterations in AChE activity in both the cerebral cortex and hippocampus induced by STZ administration

Figure 3 shows the effect of ANT (200 mg/kg) and ICV-STZ (3 mg/kg) on AChE activity in the cerebral cortex and hippocampus of rats. STZ injection promoted a significant increase in AChE activity in both structures and ANT administration was able to protect against this increase in the cerebral cortex [$F_{(1,40)}$ = 7.73; $P < 0.01$; Fig. 3A] and hippocampus [$F_{(1,39)}$ = 12.67; $P < 0.01$; Fig. 3B].

3.3 Anthocyanins protect against alterations in Na^+-K^+-ATPase total and isoform activity in the cerebral cortex and hippocampus induced by STZ administration

Figures 4 and 5 shows the effect of ANT (200 mg/kg) and ICV-STZ (3 mg/kg) on the total ATPase, total Na^+-K^+-ATPase, and α2,3 and α1 isoforms activities in the cerebral cortex and hippocampus, respectively. STZ administration caused a significant increase in ATPase and Na^+-K^+-ATPase activity in the cerebral cortex and ANT treatment was able to protect against these changes [$F_{(1,20)}$ = 5.33; $P < 0.01$; Fig. 4A] [$F_{(1,20)}$ = 5.02; $P < 0.01$; Fig. 4B]. ANT treatment also protect against the increase induced by ICV-STZ administration in Na^+-K^+-ATPase α2,3 [$F_{(1,20)}$ = 14.06; $P < 0.001$; Fig. 4C] and α1 [$F_{(1,20)}$ = 14.70; $P < 0.001$; Fig. 4D] isoform activities.

Similar results were observed in the hippocampus. ICV-STZ also caused an increase in the total ATPase and Na^+-K^+-ATPase activities and ANT treatment was able to protect against these changes [$F_{(1,20)}$ = 5.24; $P < 0.05$; Fig. 5A] [$F_{(1,17)}$ = 4.97; $P < 0.05$; Fig. 5B]. ANT treatment also protect against the increase induced by ICV-

STZ administration in Na^+-K^+-ATPase α2,3 activity [$F_{(1,16)}$ = 5.71; $P < 0.05$; Fig. 5C]. Regarding the Na^+-K^+-ATPase α1 isoform activity, no alterations were observed in any group evaluated in this study [$F_{(1,17)}$ = 0.1058; P =0.7490; Fig. 5D].

3.4 Anthocyanins protect against the changes in antioxidant responses in the cerebral cortex and hippocampus induced by STZ administration

Figure 6 shows the oxidative stress parameters in the cerebral cortex of rats that underwent STZ administration and were treated with ANT (200 mg/kg) for 25 days. First, it was observed that neither STZ nor ANT modified the ROS levels (Fig. 6A). However, STZ administration caused an increase in nitrite levels [$F_{(3,23)}$ = 5.39; $P < 0.05$; Fig. 6B] that was not restore by ANT. STZ administration led to an increase in TBARS level, an effect attenuated by ANT treatment [$F_{(1,19)}$ = 6.21; $P < 0.05$; Fig. 6C]. The sulfhydryl content did not significantly changed in this structure in any group (Fig. 6D). Regarding the antioxidant enzymes, it was observed that ICV-STZ injection promoted a reduction in their activity and ANT treatment was restored this effect on SOD [$F_{(1,15)}$ = 11.47; $P < 0.01$; Fig. 6E] and CAT [$F_{(1,15)}$ = 7.29; $P < 0.05$; Fig. 6F]. ANT also was able to protect against the decrease in GPx activity induced by STZ injection [$F_{(1,14)}$ = 8.89; $P < 0.01$; Fig. 6G]).

Figure 7 shows that ANT treatment was able to attenuated the increase in ROS levels ($F_{(1,15)}$ = 5.86; $P < 0.05$; Fig. 7A] in the hippocampus. Moreover ICV-STZ induced an increase in nitrite [$F_{(1,23)}$ = 8.27; $P < 0.01$; Fig. 7B] and TBARS levels [$F_{(1,20)}$ = 43.14; $P < 0.001$; 7C] however, ANT did not protect against this effect. No changes were observed in the total sulfhydryl content in any group (Fig. 7D). Regarding the antioxidant enzymes, it was observed that STZ injection increased SOD and decreased CAT and GPx activities. ANT administration protected against the changes in SOD [$F_{(1,15)}$ = 7.35; $P < 0.05$; Fig. 7E], CAT [$F_{(1,15)}$ = 6.33; $P < 0.05$; Fig. 7F], and GPx activity [$F_{(1,15)}$ = 7.50; $P < 0.05$; Fig. 7G].

3.5 Treatment with anthocyanins and/or STZ did not alter the blood glucose levels

Figure 8 shows that the levels of blood glucose was not altered in any of the experimental groups evaluated in this study [$F_{(1,18)}$ = 1.47; $P > 0.05$].

4. Discussion

The present study investigated the neuroprotective potential of ANT in an STZ-induced model of SDAT in rats. This model promotes multiple alterations similar to those found in patients with AD, such as a decrease in brain glucose metabolism, oxidative stress, reduction in cholinergic signaling, neuroinflammation, neuronal loss, and impairment of learning and memory [44-47]. A recent study showed that pathological alterations in this model were dependent on the time since the STZ injection, with up to one month after injection considered an acute response, between one and three months a compensatory phase, and between six and nine months a decompensatory phase with a chronic progressive decline [48].

Our findings demonstrate that STZ impairs memory acquisition (short-term memory) and spatial memory. In addition, no difference between groups was observed in the open field test, excluding the possibility of STZ interfering with locomotor activity in the memory tests. These findings are in accordance with previous studies that used this rodent model [25,49-51-56].

The damage caused by ICV-STZ administration can reach septal and corpus callosum regions [57], and beyond, causing changes at the level of the fornix, anterior hippocampus, and periventricular structures [58]. These structures are involved in memory function, with the perirhinal cortex most strongly related to object recognition after short intervals and the hippocampus linked with spatial memory and long-term object recognition [59]. Moreover, the septum has cholinergic projections on to cortical regions and the hippocampus, which are also involved in memory acquisition [57,60], and it is suggested that ICV-STZ administration disrupts these connections, altering memory consolidation [57].

Treatment with ANT was able to protect against memory deficits induced by ICV-STZ only in the object recognition test. This finding is in accordance with previous studies that also showed that an ANT-rich diet could have learning and memory enhancing effects in mice with trimethyltin-induced neurotoxicity [61], improved short-term and working memory in old rats [62], and spatial learning and memory in D-galactose-treated rats [63] and transgenic AD murine model [64]. Furthermore, a recent study with humans showed that intake of an ANT-rich cherry juice for 12 weeks could have beneficial effects on cognitive performance in older adults with mild to moderate dementia [65].

In addition, our results also showed that ANT treatment restores AChE activity in the cerebral cortex and hippocampus in an experimental model of STZ-induced SDAT. It is well established in the literature that the cholinergic system plays a pivotal role in the regulation of learning and memory and shows changes during aging and AD progression [66,67]. In fact, cholinesterase inhibitors act by inhibiting the degradation of ACh, improving cholinergic transmission and reducing temporarily the cognitive deficit of symptomatic patients with AD [68]. The increase in AChE activity may lead to a decrease in ACh levels, contributing to memory deficits. This finding is in accordance with previous studies using STZ-induced SDAT [25,27,69,70].

Treatment with blueberries (*Vaccinium corymbosum* L.) for two months reduced AChE activity in D-galactose-treated rats [71]. In an in vitro assay, an ANT-rich grape skin extract inhibited AChE activity in a dose-dependent manner with an IC_{50} of 363.61 µg/mL [72]. Another study demonstrated that oral pretreatment with pelargonidin (10 mg/kg) attenuated the hippocampal increase in AChE activity in a model of AD induced by amyloid β_{25-35} peptide in rats [73]. Previous findings from our research group also demonstrated that ANT treatment protects against alterations in AChE activity and memory deficits caused by scopolamine administration [24].

The Na^+-K^+-ATPase enzyme plays a crucial role in maintaining the Na^+ and K^+ gradient across the plasma membrane. Three isoforms are expressed in the brain: the α1 isoform is found in many cell types, the α2 isoform is predominantly expressed in astrocytes, and the α3 isoform is exclusively expressed in neurons [74]. Data from the literature have demonstrated that dysfunctions in specific isoforms alter spatial learning and motor activity [74,75] and a decrease in Na^+-K^+-ATPase activity in the brain is associated with memory impairment [76,77].

In the present study, it was observed that STZ induced an increase in Na^+-K^+-ATPase activity, including the α1 and α2/α3 isoforms. A previous study reported a reduction in Na^+-K^+-ATPase activity 14 days after STZ injection, during the initial phase of the establishment of the SDAT model [25]. Other evidence has also showed that there is a reduction in the activity of this enzyme in different brain structures 21 days after STZ administration [78]. Studies have associated the reduction in Na^+-K^+-ATPase activity with markers of oxidative stress since this enzyme has -SH residues which makes it susceptible to oxidation. On the other hand, evidence shows that this enzyme has several amino acid residues, such as serine, tyrosine, and threonine,

that are targets of phosphorylation by cellular kinases. Glucose and insulin, for example, seem to play an important role in regulating Na^+-K^+-ATPase activity. It was described that 1, 2, and 3 hours after insulin administration there was a decrease in activity [79]. Moreover, the activation of insulin receptors results in reversible covalent modification of the catalytic subunits of intracellular signaling pathways that are utilized by insulin in controlling Na^+-K^+-ATPase activity, including phosphatidylinositol-3-kinase [80], AKT/ERK [81], PKA, and PKC [82,83]. Thus, is plausible suggest the metabolic dysfunctions caused by STZ administration mainly affect insulin receptor-dependent signaling pathways, impairing the regulation of Na^+-K^+-ATPase. However, our results showed an increase in Na^+-K^+-ATPase in brain after STZ administration demonstrating that other mechanisms besides insulin signaling may be involved in the alterations of this enzyme in this pathological condition [84,85].

Interestingly, it was demonstrated that pretreatment with ANT (200 mg/kg) for seven days prevented the decrease in the Na^+-K^+-ATPase in the cerebral cortex and hippocampus induced by scopolamine in rats [24]. It was also shown that ANT prevent the impairment of Na^+-K^+-ATPase in brain of experimentally demyelinated rats [22]. The beneficial effects of the ANT on brain function in learning and memory deficits and age-related neurodegeneration [21,86] include antioxidant and anti-inflammatory activities [20,87], modulation of neuronal signaling pathways and gene expression that are important to control synaptic plasticity [86], and improvement of cerebral blood flow [21]. Thus, considering that alterations in the Na^+-K^+-ATPase directly compromises axonal impulse transmission, our findings demonstrate that ANT also can contribute to the restoration of ATP levels in the brain.

Our results showed changes in oxidative stress parameters in the cerebral cortex and hippocampus after 28 days of ICV-STZ administration. It is well established that administration of ICV-STZ induces oxidative stress through ROS and reactive nitrogen species (RNS) generation [45]. ROS formation is involved with cellular injury in AD and is related in the initiation and development of memory impairments in rats [53,88]. Moreover, STZ treatment causes depletion of antioxidant systems, increase in the TBARS and protein carbonylation levels, decline in ATP level, and mitochondrial dysfunction [45,47].

Interestingly, the oxidative-nitrative damage caused by ICV-STZ was detected in the brain of rats 1 to 8 weeks after the injection [47]. The upregulation of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) protein and mRNA expression promoted by STZ injection may be an enzymatic source of the elevated nitrite level in the cortex and hippocampus in this model. Once expressed, iNOS produces a high level of nitric oxide (NO) constantly, having a toxic effect on neurons [86]. Moreover, the excess of superoxide ($O_2^{\bullet-}$) can react rapidly with NO, producing peroxynitrite ($ONOO^-$), which reacts with several biological molecules promoting damage and neuronal cell death [27,89-91].

Corroborating with our findings, other studies also showed a decrease in enzymatic antioxidant defense after ICV-STZ administration [27,92,93]. SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2, which can be neutralized through the action of other enzymes such as CAT and GPx [14]. It has been documented that ICV-STZ can reduce the GSH level. This is critical in eliminating H_2O_2 and organic peroxides by GPx since this enzyme depends on GSH as an electron donor for the reduction of peroxides [12,92,94]. The decrease in SOD, GPx, and CAT activity may impair $O_2^{\bullet-}$ and H_2O_2 removal and this can generate hydroxyl radicals and lipid peroxidation, triggering alterations in the biological properties of membranes [14,53,95]. In this study, lipid peroxidation was significantly increased in the ICV-STZ group as demonstrated by the TBARS content. Curiously, the higher SOD activity found in the hippocampus could be a compensatory mechanism in response to the increased $O_2^{\bullet-}$ accumulation. The hippocampus is especially vulnerable to the pathological alterations present in AD and the pyramidal neurons in the CA1 region is particularly susceptible to oxidative stress [96,97]. Furthermore, it has been shown that the neurons in this region contain higher levels of $O_2^{\bullet-}$ and ROS production [97]. Besides, there is a high demand for ROS/RNS as signaling molecules and this fact can be observed in CA1 neurons that require $O_2^{\bullet-}$ for long-term potentiation [97]. Evidences suggest that this scenario could lead a copper zinc superoxide dismutase (CuZnSOD) upregulation to facilitate the removal of the radicals and an increase in manganese superoxide dismutase (MnSOD) expression within the pyramidal neurons of the hippocampus [96,98] On the other hand, the overexpression of SOD may promote an increase in H_2O_2 formation that can induce a harmful oxidation of cell components [96,99].

In addition, ANT treatment was able to protect against the oxidative damage caused by ICV-STZ, suggesting that this effect may be associated with the improvement of dysfunctional memory. ANT have a direct free radical-scavenging activity due to the hydrogen donation capacity that is dependent on its chemical structure, especially the presence of hydroxyl groups in ring B [87,100]. The antioxidant action of ANT is further evidenced by indirect pathways, such as he modulation of antioxidant enzymes, reducing the formation of DNA oxidative abducts and endogenous ROS by inhibiting NADPH oxidase and xanthine oxidase, or by modifying mitochondrial respiration and arachidonic metabolism [87].

It is important to consider that ANTs are capable of crossing the blood brain barrier (BBB), and are found in several regions of the brain, including regions related to memory and learning such as the cortex and hippocampus [101-103]. In an in vitro study the transport of ANT across a BBB cell model was evaluated and suggested that these compounds and their metabolites were able to cross due to their lipophilicity [104].

In conclusion, the present study demonstrated that ANT treatment was able to protect against memory deficits in an experimental model of STZ-induced SDAT. These beneficial effects may be related with its antioxidant capacity and modulation of AChE and Na^+-K^+-ATPase activity in brain regions (Fig. 9).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Legends of figures

Figure 1 - Scheme for induction protocol of experimental model of sporadic dementia of Alzheimer's type and anthocyanins (200 mg/kg) treatment in rats.

Figure 2 - Effects of treatment with anthocyanins (200 mg/kg) on the results of behavioral tests of animals that were administered intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. The locomotor activity behavior was analyzed using the open field task on the number of total crossings (A) and rearing responses (B). The non-spatial memory and spatial memory was analyzed respectively using the object recognition task (C) and Y-maze apparatus: percentage of time spent (D) and number of entries (E) into the novel arm. Data are expressed as mean ± S.E.M. *Denotes significant difference from the vehicle group for $P < 0.05$; ***$P < 0.001$. $^{\#}$ Denotes significant difference from the STZ group for $P < 0.05$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 3 - Acetylcholinesterase (AChE) activity in the cerebral cortex (A) and hippocampus (B) in animals treated with intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. **Denotes significant difference from the vehicle group for $P < 0.01$. $^{\#\#}$Denotes a significant difference compared with the STZ group for $P < 0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 4 - Total ATPase (A), Na^+-K^+-ATPase (B), Na^+-K^+-ATPase α2,3 (C) and Na^+-K^+-ATPase α1 (D) isoform activity in the cerebral cortex in animals treated with intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. *Denotes significant difference from the vehicle group for $P < 0.05$; ** $P<0.01$; ***$P < 0.001$. $^{\#}$ Denotes a significant difference compared with the STZ group for $P < 0.05$; $^{\#\#}P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 5 - Total ATPase (A), Na^+-K^+-ATPase (B), Na^+-K^+-ATPase α2,3 (C) and Na^+-K^+-ATPase α1 (D) isoform activity in the hippocampus in animals treated with

intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. *Denotes significant difference from the vehicle group for $P < 0.05$; ** $P<0.01$. # Denotes a significant difference compared with the STZ group for $P < 0.05$; ## $P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 6 - Effects of anthocyanins treatment (200 mg/kg) on reactive oxygen species level (A); nitrite level (B); TBARS level (C); sulfhydryl content (D); superoxide dismutase activity (E); catalase activity (F); and glutathione peroxidase activity (G) in the cerebral cortex of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M. *Denotes significant difference from the vehicle group for $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. #Denotes significant difference from the STZ group for $P < 0.05$; ## $P < 0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 7 - Effects of anthocyanins treatment (200 mg/kg) on reactive oxygen species level (A); nitrite level (B); TBARS level (C); sulfhydryl content (D); superoxide dismutase activity (E); catalase activity (F); and glutathione peroxidase activity (G) in the hippocampus of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M. *Denotes significant difference from the vehicle group for $P < 0.05$; ** $P < 0.01$. # Denotes significant difference from the STZ group for $P < 0.05$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 8 - Effects of anthocyanins treatment (200 mg/kg) blood glucose levels in serum of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M.

Figure 9 - Neuroprotective potential of anthocyanins against damage caused by the administration of intracerebroventricular streptozotocin in an experimental model of sporadic dementia of Alzheimer's type.

Ethics Committee protocol number
CEEA 0179/2015

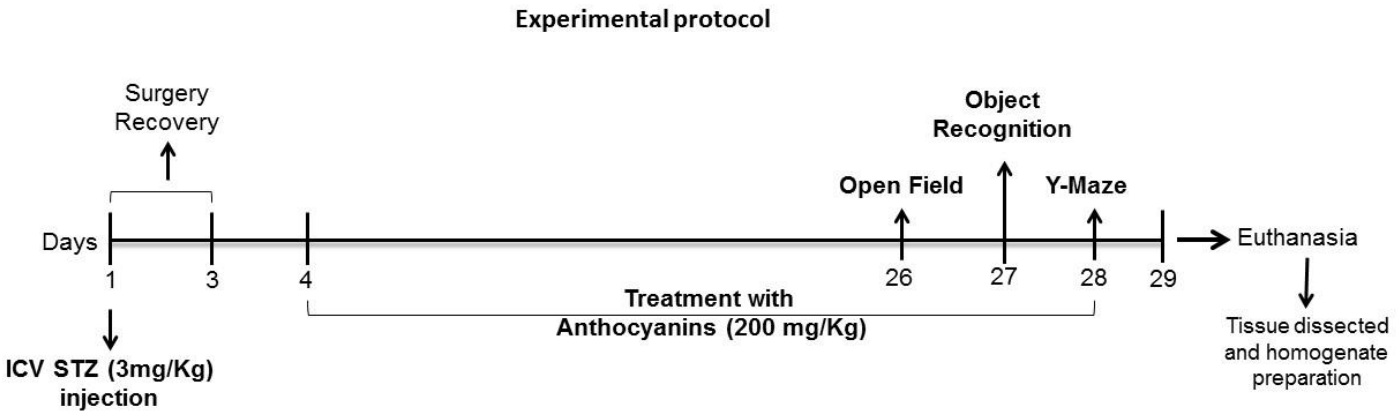
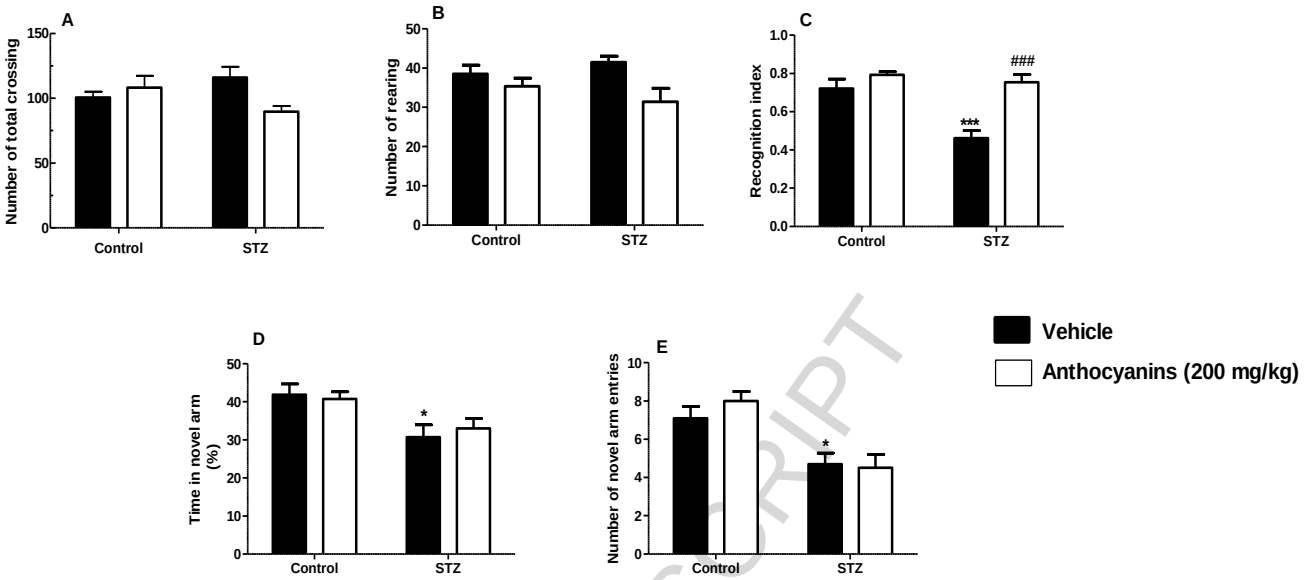


Figure 1



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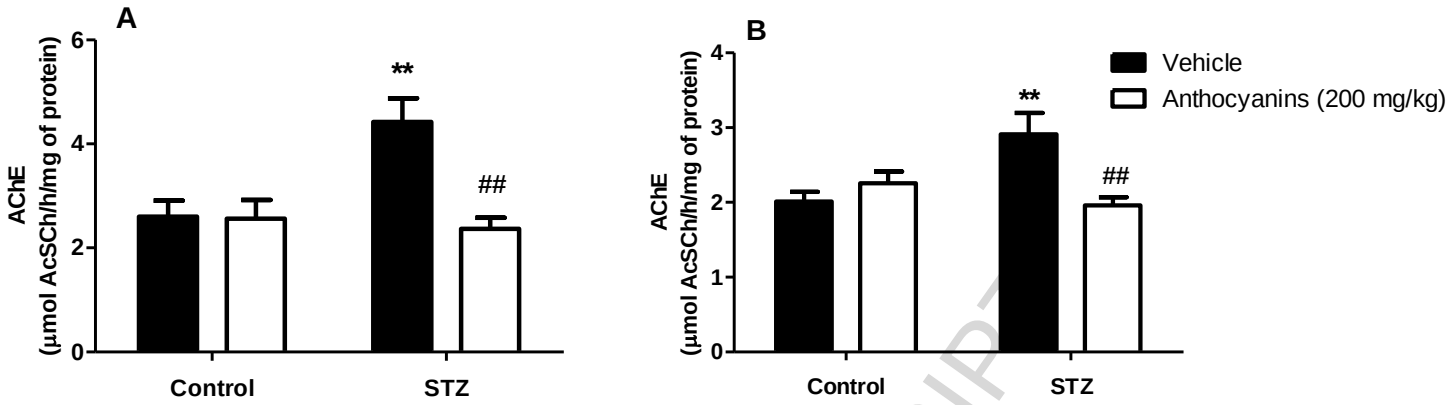

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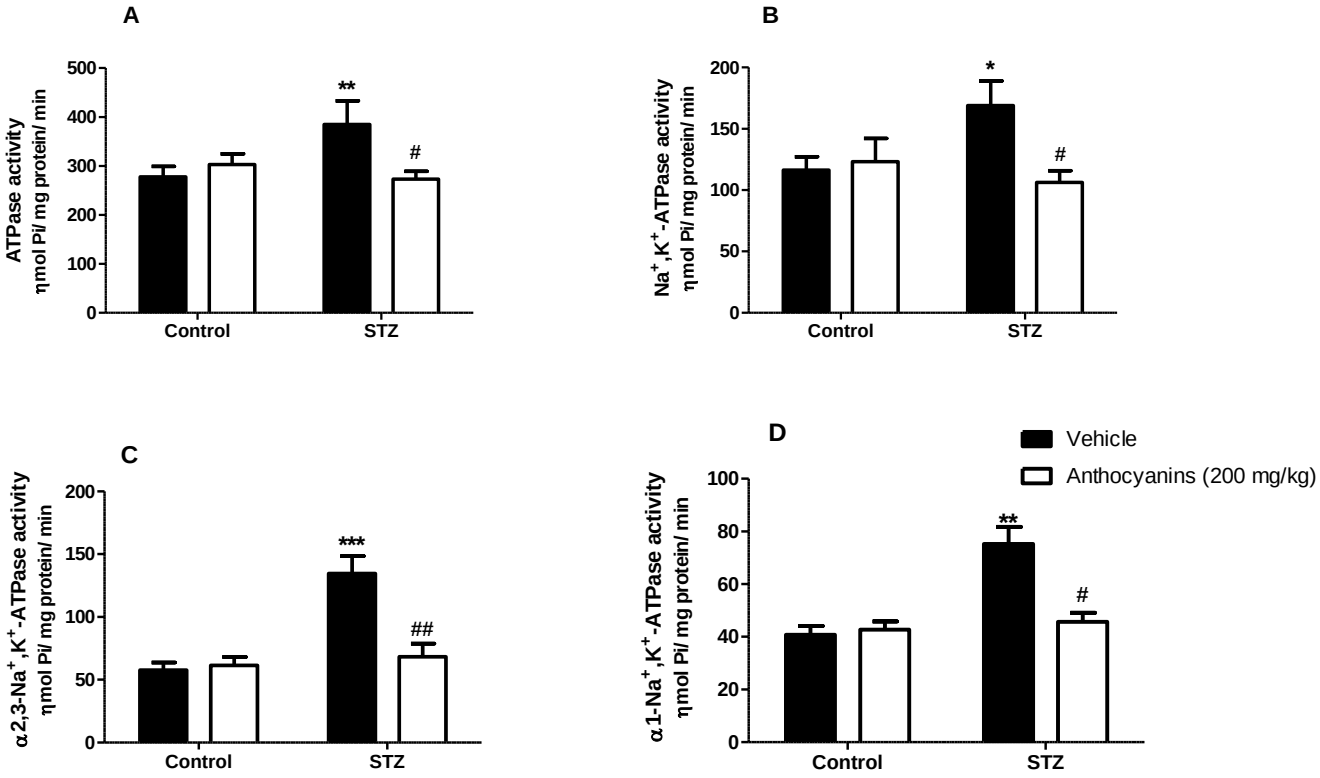
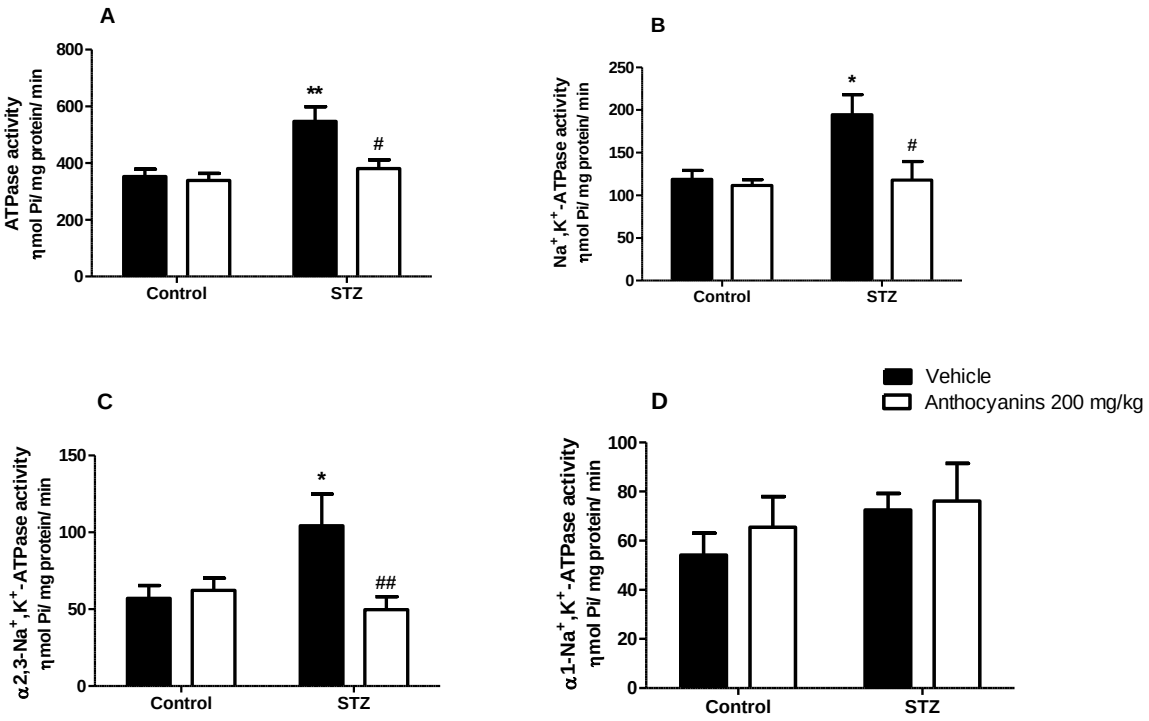


Figure 4



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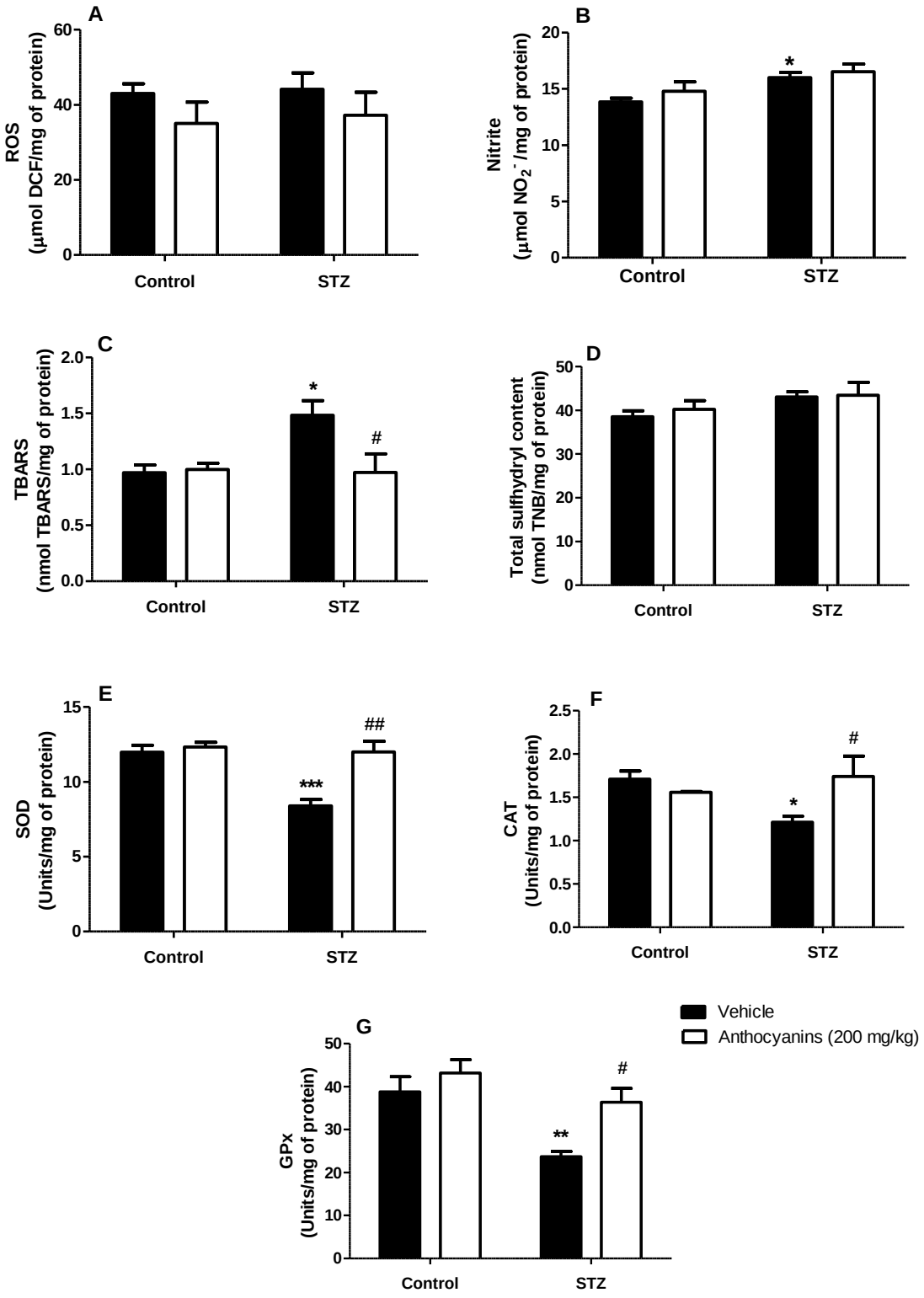

Figure 6

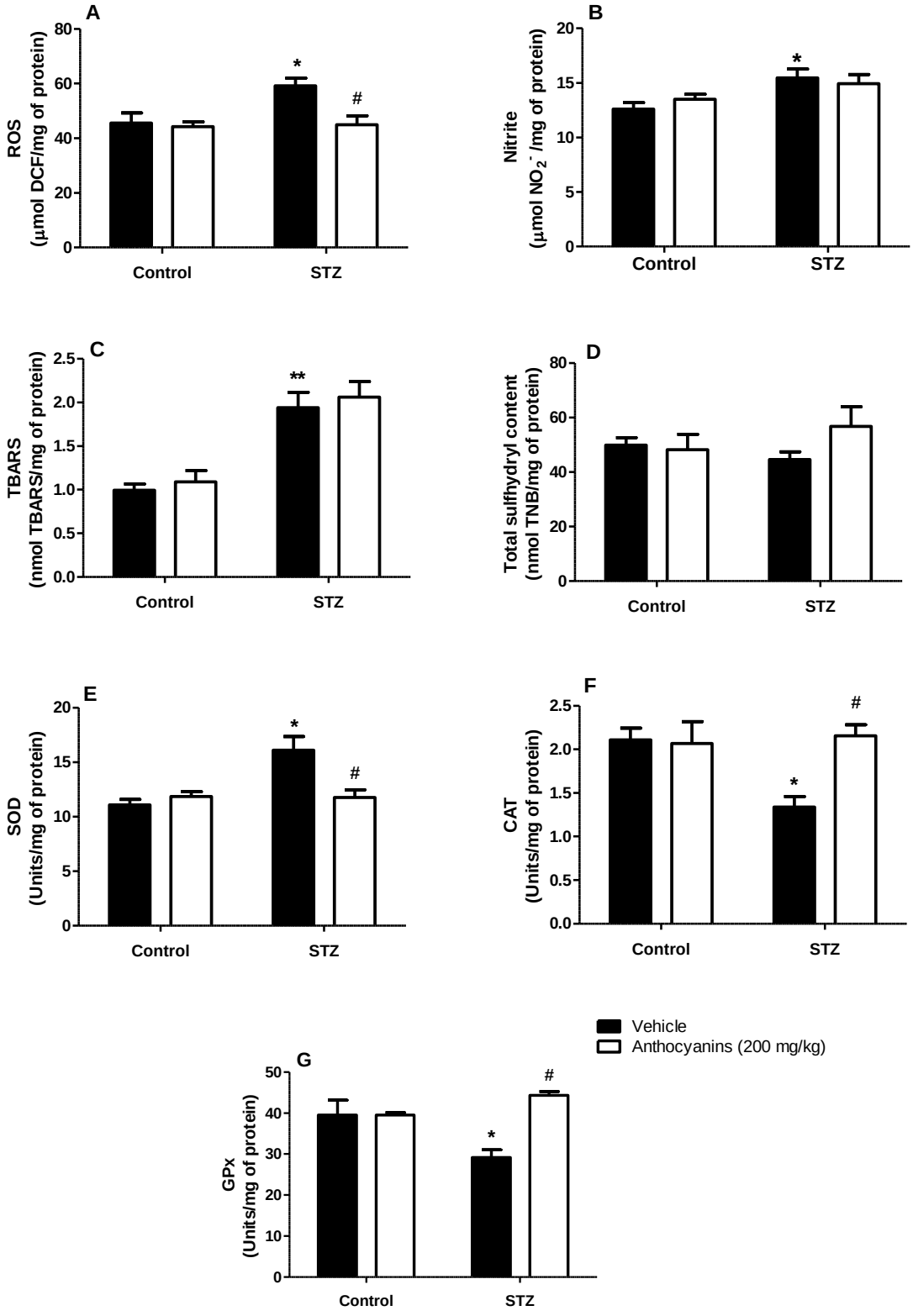

Figure 7

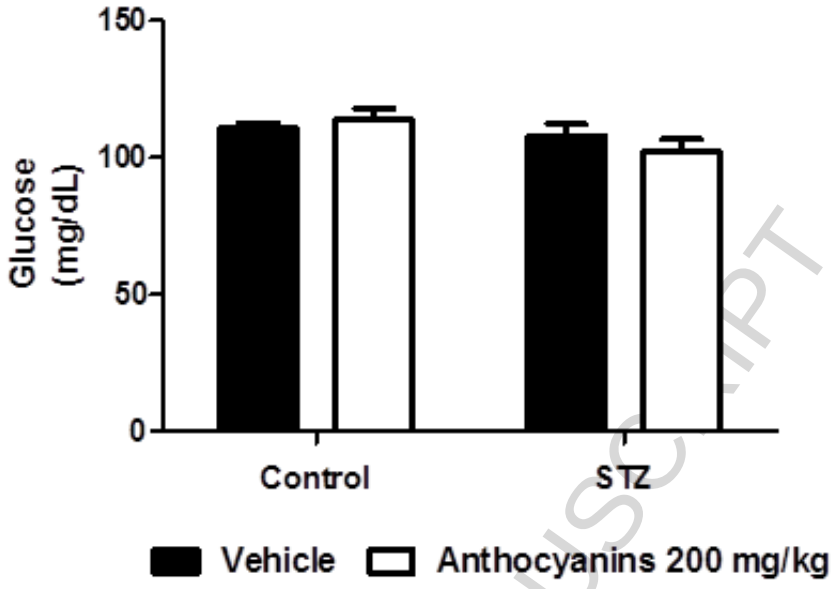

Figure 8

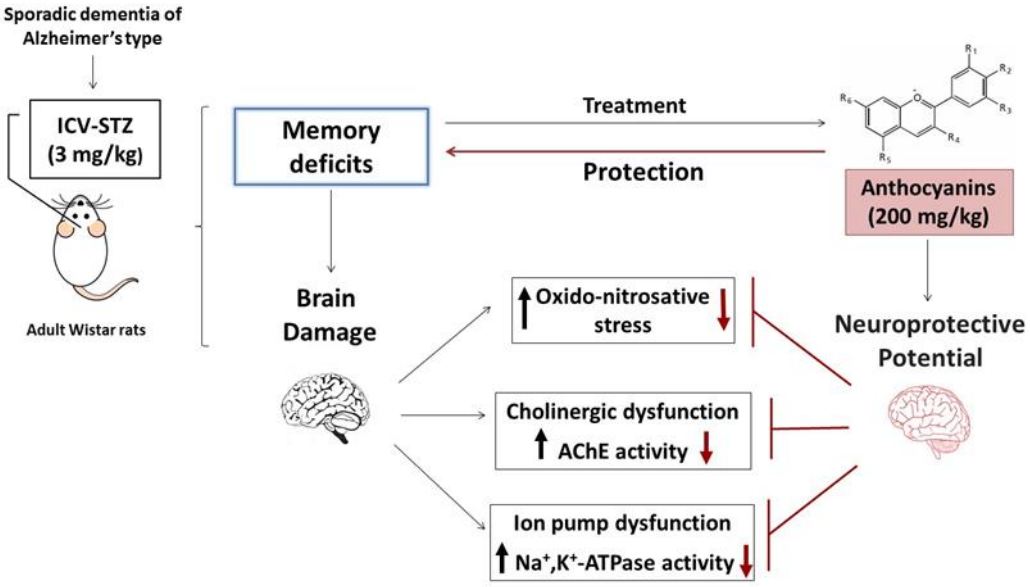

Figure 9