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Highlights

- Anthocyanins are flavonoid with neuroprotective properties;
- Anthocyanins prevented scopolamine-induced memory deficits;
- Anthocyanins are able to prevent the AChE upregulation in brain of scopolamine-treated rats
- Anthocyanins protect against impairment of membrane bound ATPases induced by scopolamine.

Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats

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Abstract

Anthocyanins are a group of natural phenolic compounds responsible for the colour to plants and fruits. These compounds might have beneficial effects on memory and have antioxidant properties. In the present study we have investigated the therapeutic efficacy of anthocyanins in an animal model of cognitive deficits, associated to Alzheimer's disease, induced by scopolamine. We evaluated whether anthocyanins protect the effects caused by SCO on nitrite/nitrate (NOx) levels and Na^+,K^+-ATPase and Ca^{2+}-ATPase and acethylcholinesterase (AChE) activities in the cerebral cortex and hippocampus (of rats. We used 4 different groups of animals: control (CTRL), anthocyanins treated (ANT), scopolamine-challenged (SCO), and scopolamine+anthocyanins (SCO+ANT). After seven days of treatment with ANT (200mg/kg; oral), the animals were SCO injected (1mg/kg; IP) and were performed the behavior tests, and submitted to euthanasia. A memory deficit was found in SCO group, but ANT treatment prevented this impairment of memory (*P<0.05*). The ANT treatment *per se* had an anxiolitic effect. AChE activity was increased in both in cortex and hipoccampus of SCO group, this effect was significantly attenuated by ANT (*P<0.05*). SCO decreased Na^+,K^+-ATPase and Ca^{2+}-ATPase activities in hippocampus, and ANT was able to significantly (*P<0.05*) prevent these effects. No significant alteration was found on NOx levels among the groups. In conclusion, the ANT is able to regulate cholinergic neurotransmission and restore the Na^+,K^+-ATPase and Ca^{2+}-ATPase activities, and also prevented memory deficits caused by scopolamine administration.

Keywords: Anthocyanins; Scopolamine; Acetylcholinesterase; Memory; Anxiety-like behaviour.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by a progressive deterioration of memory and of other cognitive functions that lead to dementia (Scarpini and Cogiamanian, 2003; Scarpini et al., 2003). The neuropathological features of this disease include: the extracellular deposition of amyloid plaques, the development of intraneuronal neurofibrillary tangles, neuroinflammation and neuronal loss in limbic cortical regions such as the hippocampus (Lacor, 2007; Palop and Mucke, 2010). Although multiple neurotransmitter systems appear to be affected in AD, the cholinergic dysfunctions have received particular attention and most of the therapies for this disease are directed to this system. The acetylcholinesterase (AChE) is an important enzyme that rapidly hydrolyses acetylcholine (ACh), regulating the levels of this neurotransmitter in the synaptic cleft, thus being involved in cognitive function of learning and memory (Gron et al., 2006; Hut and Van der Zee, 2011). Although AChE has a major role in the regulation of cognitive functions, this enzyme is not limited to cholinergic transmission (Blokland, 1995; Paleari et al., 2008)., it is also implicated in several non-cholinergic actions including cell proliferation (Appleyard, 1994) and neurite outgrowth (Chacon et al., 2003). In this way, the AChE activity has been the target of emerging therapeutic strategies for diseases associated to cognitive deficits; and the consumption of red wine with high content in polyphenols has been noted to be beneficial for neurodegenerative diseases, like AD (Ibach and Haen, 2004; Musial et al., 2007).

Anthocyanins (ANT) are flavonoids found in grape juice and red wine, with phenolic groups present in their chemical structure (Veitch and Grayer, 2008; Williams and Grayer, 2004; Yoshida et al., 2009). It is known that ANT are potent antioxidants (Kahkonen and Heinonen, 2003; Kahkonen et al., 2001) and have neuroprotective properties (Del Rio et al., 2010), being beneficial for animal models of Parkinson's (Kim et al., 2010) and Alzheimer's diseases (Shih et al., 2010). In fact, it was shown that ANT improves memory of aged rats,

(Andres-Lacueva et al., 2005) and also of elderly humans (Krikorian et al., 2010b).

The Na^+,K^+-ATPase and Ca^{2+}-ATPase are crucial enzymes involved in the control of ionic homeostasis, generation of membrane potential and synaptic neurotransmission. Na^+,K^+-ATPase is responsible for the active transport of Na^+ and K^+ and maintains the ionic gradient for neuronal excitability (Jorgensen et al., 2003; Kaplan, 2002). Moreover, Na^+,K^+-ATPase might play a relevant role in neuronal and synaptic plasticity (Glushchenko and Izvarina, 1997; Scuri et al., 2007) and decreased enzyme activity or expression directly impairs signaling, with deleterious consequences on memory and anxiety in rats (dos Reis et al., 2002; Moseley et al., 2007), increases Ca^{2+} influx in brain slices (Fujisawa et al., 1965) and causes death in rats (Lees et al., 1990). Ca^{2+}-ATPase is responsible for control of intracellular Ca^{2+} homeostasis. Furthermore, the decreased activity of Ca^{2+}-ATPase has been associated with production of reactive oxygen species and neurodegenerative diseases (Clarke and Fan, 2011; Kodavanti, 1999; Skou and Esmann, 1992).

Changes in the activity of Na+,K+-ATPase and Ca2+-ATPase, which are crucial enzymes involved in the control of ionic homeostasis and synaptic transmission, were shown to underlie alterations in memory and anxiety (dos Reis et al., 2002; Moseley et al., 2007) and also with neurodegenerative processes related with excessive production of reactive oxygen species (ROS) and Ca^{2+} homeostasis deregulation (Ashmore et al., 2009; Giacomello et al., 2013).

Scopolamine (SCO) is a non-selective muscarinic receptor antagonist used to induce memory deficits in animal models (Klinkenberg and Blokland, 2010). It was also reported that SCO reduces frontal cortex perfusion in young humans (Honer et al., 1988) and impairs the energetic metabolism, reducing the ATP levels in cerebral cortex of rats (Blin et al., 1994; Ray et al., 1992). Mitochondrial dysfunction and ATP levels reduction are pathological events associated with neurodegenerative diseases, linked to cognitive decline, like AD (Ferrer, 2009; Hauptmann et al., 2009).

In this context, since ANT has an important function as antioxidant and neuroprotective compound, in this study we investigated whether this natural

compound is able to prevent memory deficits found in animals administrated intraperitoneally with SCO. Moreover, we evaluated the nitrite/nitrate (NOx) levels, as well as the activities of enzymes important for neurotransmission such as AChE, Na^+,K^+-ATPase and Ca^{2+}-ATPase, which are known to be altered in Alzheimer's disease.

Material and Methods

Chemicals

Acetylthiocholine, Trizma Base, Acetonitrile, Percoll, Coomassie Brilliant Blue G and Scopolamine (SCO) were purchased from Sigma Chemical Co (St Luis, MO, USA). Anthocyanins was purified from grape skin (AC-12-R-WS-P/10120/Gin:601412) and are commercially available by Christian Hansen A/S. All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Male Wistar rats (3 month year old) weighing 350–400 g were used in this study. They were kept in the Central Animal House of Federal University of Santa Maria in colony cages at an ambient temperature of 25±2 °C and relative humidity 45–55% with 12 h light/dark cycles, with free access to standard rodent pelleted diet and water *ad libitum*. All procedures were carried out according to NIH Guide for Care and Use of Laboratory Animals, and Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of Federal University of Santa Maria (23081.003601/2012-63).

Drug administration

The animals were divided into two groups of analysis; the first analysis consisted in treat 7-10 animals per group with anthocyanins (200mg/kg body weight; by gavage around 10 a.m) for 7 days, and in last day the animals received anthocyanins 30 min before the training in inhibitory avoidance apparatus. Scopolamine (1mg/kg) was dissolved in saline and injected

intraperitoneally (i.p) 30 min after the training in inhibitory avoidance apparatus, as previously described (Ali and Arafa, 2011; Marisco et al., 2013); the second group of animals were submitted to same treatment and sacrificed two hours post training, with seven animals per group (see Scheme 1). The dose of anthocyanins used was chosen on the basis of previous studies indicating neuroprotection (Gutierres et al., 2012b; Manach et al., 2004; Saija et al., 1990; Varadinova et al., 2009). In addition, the daily intake of anthocyanins in residents of the United States is estimated to be about 200 mg or about 9-fold higher than that of other dietary flavonoids, and this also served as a basis for this study (Manach et al., 2004; Wang and Stoner, 2008).

Behavioral analysis

Inhibitory avoidance task

In the last day of treatment with anthocyanins (7^{th} day), the animals were trained in a step-down inhibitory avoidance apparatus, as previously described (Marisco et al., 2013; Rubin et al., 2000b), and 30 min after this training received scopolamine (1 mg/kg; IP). Twenty four later the memory perforemace of animals were evaluated in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25×25×35-cm box with a grid floor whose left portion was covered by a 7×25-cm platform, 2.5 cm high. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3-s 0.4-mA shock was applied to the grid. Retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 300s was established.

Open field

Immediately after the inhibitory avoidance test session, the animals were transferred to an open-field measuring 56×40×30 cm, with the floor divided into 12 squares measuring 12×12 cm each. The open field session lasted for 5 min and during this time, an observer, who was not aware of the

pharmacological treatments, recorded the number of crossing responses and rearing responses manually. This test was carried out to identify motor disabilities, which might influence inhibitory avoidance performance at testing.

Elevated plus maze task

Anxiolytic-like behavior was evaluated using the task of the elevated plus maze, as previously described (Frussa-Filho et al., 1999; Rubin et al., 2000a). The apparatus consists of a wooden structure raised to 50 cm from the floor. This apparatus is composed of 4 arms of the same size, with two closed-arms (walls 40 cm) and two open-arms. Initially, the animals were placed on the central platform of the maze in front an open arm. The animal had 5 minutes to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

Foot shock sensitivity test

Reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed and was used to determine the flinch and jump thresholds in experimentally naïve animals (Berlese et al., 2005; Rubin et al., 2000a). The animals were placed on the grid and allowed for a 3 min habituation period before the start of a series of shocks (1s) delivered at 10 s intervals. Shock intensities ranged from 0.1 to 0.5 mA with 0.1 mA increments. The adjustments in shock intensity were made in accordance with each animal's response. The intensity was raised by one unit when no response occurred and lowered by one unit when a response was made. A flinch response was defined as withdrawal of one paw from the grid floor, and a jump response was defined as withdrawal of three or four paws. Two measurements of each threshold (flinch and jump) were made, and the mean of each score was calculated for each animal.

Brain tissue preparation

The animals were anesthetized under halothane atmosphere before being killed by decapitation and brain were removed and separated into cerebral cortex and hippocampus and placed in a solution of Tris–HCl 10mM, pH 7.4, on ice (Gutierres et al., 2012c). The brain structures were gently homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −80°C until utilization. Protein was determined previously in a strip that varied for each structure: cerebral cortex (0.7 mg/ml) and hippocampus (0.8 mg/ml), as determined by the Coomassie blue method as previously described (Bradford, 1976), using bovine serum albumin as standard solution.

Synaptosomes Preparation

Synaptosomes were isolated essentially as previously described (Nagy and Delgado-Escueta, 1984), using a discontinuous Percoll gradient. The cerebral cortex, hippocampus and were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1,000xg for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an isosmotic solution consisting of 320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA by centrifugation at 15,000 g to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.4-0.6 mg/ml. Synaptosomes were prepared fresh daily and maintained at 0º-4º throughout the procedure and used to measure AChE activity.

Assay of Lactate Desydrogenase (LDH)

The integrity of the synaptosomes preparations was confirmed by determining the lactate dehydrogenase (LDH) activity which was obtained after

synaptosome lysis with 0.1 % Triton X-100 and comparing it with an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brasil).

Determination of AChE activity in brain

The AChE enzymatic assay was determined using a spectrophotometric method (Ellman et al., 1961) with minor modifications (Gutierres et al., 2012a). 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, during 2min 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 acetylthiocholine iodide (AcSCh). All samples were run in triplicate and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

Na^+,K^+-ATPase activity measurement

Na^+,K^+-ATPase activity was measured as previously described (Carvalho et al., 2012). Briefly, assay medium consisted of (in mM) 30 Tris-HCl buffer (pH 7.4), 0.1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$ and 50 µg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 µL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 3 mM. After 30 min at 37ºC, the reaction was stopped by the addition of 70 µL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol of Pi/min/mg of protein.

Ca^{2+}-ATPase activity measurement

Ca^{2+}-ATPase activity was measured as previously described (Rohn et al., 1993) with minor modifications (Trevisan et al., 2009). Briefly, the assay medium consisted of (in mM) 30 Tris-HCl buffer (pH 7.4), 0.1 EGTA, 3 $MgCl_2$ and 100 µg of protein in the presence or absence of 0.4 $CaCl_2$, in a final volume of 200 µL. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 3 mM. After 60 min at 37ºC, the reaction was stopped by the addition of 70 µL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used, and reaction was linear with protein concentration and time. Appropriate controls were included in the assays to assess non-enzymatic ATP hydrolysis . The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as a reference standard. The Ca^{2+}-ATPase activity was determined by subtracting the activity measured in the presence of Ca^{2+} from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA) and expressed in nmol of Pi/min/mg of protein.

Assay of NOx (NO_2 plus NO_3) as a marker of NO synthesis

For NOx determination, an aliquot (200 µl) was homogenized in 200mM Zn_2SO_4 and acetonitrile (96%, HPLC grade). Then, the homogenate was centrifuged at 16,000 xg for 20min at 4°C , and the supernatant was collected for analysis of NOx content as previously described (Miranda et al., 2001). The resulting pellet was suspended in NaOH (6 M) for protein determination.

Statistical analysis

The statistical analysis of test step-down latencies was carried out by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (nonparametric two-way ANOVA). The training latency, open field, binding assay and foot shock sensitivity were analyzed by *one-way ANOVA* following by student Newman-Keuls. The other tests were analyzed by *two-way ANOVA*, followed by Tukey test, and considered $P<0.05$ or $P<0.001$ as a significant difference in all experiments.

Results

Behavioral tests

Anthocyanins prevent the impairment of memory induced by scopolamine.

In this study we used 4 groups of animals: control (CTRL), anthocyanins (ANT), scopolamine (SCO), and scopolamine plus anthocyanins (SCO+ANT). Table 2 shows the effect of the treatment with ANT on the SCO-induced memory deficits, in the step-down latencies. Statistical analysis of Scheirer–Ray–Hare test (*nonparametric two-way ANOVA*) showed a significant saline or SCO (1mg/kg; IP) *vs* saline or ANT (200mg/kg) interaction, revealing that treatment with SCO decreased the test latency (s) indicating significantly impairment of memory. However, the ANT+SCO group showed a significantly increased in the test latency (s) suggesting that ANT restore the impairment of memory induced by SCO (Table 2). Statistical analysis of training showed no difference between groups (Table 2). However, motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether SCO or ANT affected shock threshold, or locomotor ability of the animals. Statistical analysis of open-field data (*one-way ANOVA*) revealed that SCO did not alter the number of crossing [F (3,36)=0.99, *P*>0.05; Table 3] or rearing [F (3,36)=0.13, *P*>0.05; Table 3] responses in a subsequent open-field test session, suggesting that neither SCO nor ANT caused gross motor disabilities at testing. Moreover, SCO did not alter foot shock sensitivity, as demonstrated by the similar flinch and jump thresholds exhibited by the animals. These data suggest that neither treatment with SCO+ANT administered before nor SCO administered after training of inhibitory avoidance caused motor disabilities or altered foot shock sensitivity: flinch [F (3,36)= 1.30; *P>0.05*], jump [F (3,36)= 0.48; *P>0.05*] and vocalization [F (3,36)= 1.11; *P*>0.05] (Table 3).

Effect of anthocyanins treatment on anxiolytic-like behavior

Although there are studies showing that flavonoids have anxiolytic proprieties, there are no studies showing that ANT act as compounds possessing these properties. Thus we decided to investigate the effect of ANT or SCO treatments on anxiolytic-like behavior in the elevated plus maze task (Figure 1). Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or ANT (200 mg/kg) interaction to Time in Closed Arms [F (1,36)= 14.780; *P<0.0001;* Figure 1B], revealing that treatment with ANT had an anxiolytic effect *per se*. However, we did not observed significant difference between ANT or SCO treatments on % Time in Open Arms [F (1,36)= 0.001; *P>0.05;* Figure 1A] and Nº of Entries in Closed Arms [F (1,36)= 0.132; *P>0.05;* Figure 1C] or Nº of Entries in Open Arms [F (1,36)= 0.846; *P>0.05;* Figure 1D].

Enzymatic activities

Anthocyanins prevent the increase in AChE activity induced by scopolamine.

Since there are evidences showing that memory impairment in AD come from studies that report alterations in AChE activity, the sequence of experiments we investigated whether ANT restores AChE activity in the pharmacological model of cognitive induced by SCO. Figure 2 shows the effect of ANT and SCO on the activity of AChE in cerebral cortex and hippocampus of rats, both in supernatant (S1) and synaptosomes of rats. Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200m/kg) interaction, suggesting that the ANT treatment prevents the increase in AChE activity in synaptosomes of cerebral cortex [F= (1,28)= 6.135; *P<0.05;* Figure 2A] and hippocampus [F= (1,28)= 7.515; *P<0.05;* Figure 2A] induced by SCO.

Statistical analysis of testing (*two-way ANOVA*) also showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment prevented the increase in AChE activity induced by SCO in S1 fraction of cerebral cortex [F= (1,28)= 6.322; *P<0.05;* Figure 2B] and hippocampus [F (1,28)= 5. 447; *P<0.05;* Figure 2B]..

Anthocyanins prevent the decrease of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities induced by scopolamine in hippocampus.

Na^+,K^+-ATPase and Ca^{2+}-ATPase are enzymes involved in the control of neurotransmission, since regulating membrane potential and intracellular Ca^{2+}concentrations, respectively. Figure 3 shows the effect of ANT and SCO on the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase in cerebral cortex and hippocampus of rats. Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment prevented the decrease in Na^+,K^+-ATPase activity induced by SCO in cerebral cortex [$F(1,28)= 7.781$; $P<0.05$] and hippocampus [$F(1,28)= 5.866$; $P<0.05$] (Figure 3).

Additionally, *two-way ANOVA* showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment also prevented the decrease of Ca^{2+}-ATPase activity in the hippocampus [$F(1,28)= 4.803$; $P<0.05$] (Figure 3B). However, we did not observed significant differences between groups in the activity of this enzymes in cerebral cortex [$F(1,28)= 1.080$, $P>0.05$]

NOx levels determination

Anthocyanins are described to possess antioxidant effects, at this set of experiments we investigated if ANT affect the levels of nitrite plus nitrate (NOx) in the brain of rats. Figure 4 shows the effect of ANT and SCO on the NOx levels production in cerebral cortex and hippocampus of rats. Statistical analysis of testing (*two-way ANOVA*) showed no significant interactions between groups in cerebral cortex [$F(1,28)= 1.149$; *P>0.05*] and hippocampus [$F(1,28)= 0.009$; *P>0.05*]

Discussion

Ageing-associated disorders include immune dysfunction (Candore et al., 2006; Sansoni et al., 2008), cognition degeneration (Barzilai et al., 2006; Mehta, 2007), cardiovascular disease (Dominguez and Barbagallo, 2007) and metabolic syndrome (Maggi et al., 2008). Increasing evidence suggests that ageing increases the risk of degeneration of the nervous system, which mostly affects the moral and physiological life of the elderly. As a result of the development of medical science and health care, the average human life span is increasing; however, the future socioeconomic burden of the elderly must be a major of concern in developed countries (Shih et al., 2010).

A number of investigators have found that flavonoids, including some anthocyanins, possess oral bioavailability in rats (Matsumoto et al., 2001; McGhie et al., 2003; Miyazawa et al., 1999) and that they are able to cross the rat blood–brain barrier, either chronic or acute administration (Andres-Lacueva et al., 2005) suggesting that these compounds can feasibly have a direct effect on brain. Anthocyanins dietary consumption in some individuals has been estimated to be up to 200 mg/day, which is higher than that of other flavonoids (23 mg/day) such as quercetin (Frank et al., 2002; McGhie et al., 2003; Scalbert and Williamson, 2000). In the present study it was observed that the pre-administration of anthocyanins (ANT) potentiated memory retention in scopolamine (SCO) administered animals. This are in accordance with the evidences showing that ANT is able to improve memory of old rats in Morris water maze test (Andres-Lacueva et al., 2005) and of old mice in the inhibitory avoidance task (Barros et al., 2006) and also of elderly humans (Krikorian et al., 2010b). Moreover, a 2-month dietary supplementation of rats with blueberries prevented deficits in learning and the loss of CA1 pyramidal neurons induced by bilateral hippocampal injections of kainic acid (Duffy et al., 2008). It has been shown that ANT are potent antioxidants, being effective scavengers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Kahkonen and Heinonen, 2003; Kahkonen et al., 2001), with a clear neuroprotective role (Del Rio et al., 2010). These results implicate that ANT possess health benefits. Of particular interest, procyanidins as well as resveratrol are considered to be one

of the bioactive components of the red wine responsible for the cardioprotective effects, known as “French Paradox” (Nishizuka et al., 2011). If this is the case, these protective effects conferred by polyphenols of red wine might be related to the prevention of age-related cognitive deficits, since it is well recognized that populations which consume anthocyanins enriched fruits have health benefits including improvement in cognition and neuronal function with aging (Krikorian et al., 2012; Krikorian et al., 2010a).

Furthermore, shock motivated learning tests, particularly in those that investigate the effect of drugs given before the acquisition test, is whether pharmacological treatments affect locomotor activities or motivational aspects of learning, such as shock sensitivity. Immediately after inhibitory avoidance test, the animals were subjected to an open-field test which is widely used for evaluating motor abnormalities (Belzung and Griebel, 2001). The open field session revealed that the treatment with SCO or ANT did not alter spontaneous locomotor activity, the animals showed a similar number of crossing or rearing responses (Table 3). Moreover, we observed that the rats of different groups did not shows altered shock sensitivity, as verified by their similar flinch, jump and vocalization thresholds (Table 3). Our data showed that neither SCO nor ANT administration caused motor disabilities or altered foot shock sensitivity, excluding their possibility of interference in step-down latencies of inhibitory avoidance task.

Besides learning and memory evaluation, we also assessed the anxiolytic-like behavior of the rats by the elevated plus maze task, and we observed an anxiolytic effect of ANT *per se*. which are in agreement with other studies showing that ANT has an anxiolytic effects in rats and mice in the elevated-plus maze test (Barros et al., 2006; Ramirez et al., 2005). we have also investigated if ANT has affinity for $GABA_A$ receptors important targets for the control of anxiety, and in this study the ANT (100μM) exhibited affinity for $GABA_A$ receptors since it displaces by about 50% the binding of flunitrazepan to the benzodiazepine site of $GABA_A$ receptor (Gutierres et al., 2013).

The activation of muscarinic m1 receptors, which are coupled to the phosphoinositide (PI) second messenger transduction system, is the initial objective of cholinergic replacement therapy in AD (Bymaster et al., 1998a;

Bymaster et al., 1998b). These data support the use of scopolamine, since it compromises cholinergic neurotransmission and mimics the memory deficit observed in diseases characterized cholinergic dysfunction, such as AD (Christensen et al., 1992; Kopelman and Corn, 1988; Wesnes et al., 1991). The present study shows that ANT attenuated scopolamine-induced impairment in memory retention and reduction of AChE activity, indicating that ANT and cholinergic system have a close interaction. These data are in agreement with results of others (Blitzer et al., 1990; Izquierdo, 1989), which showed that muscarinic aceylcholine receptors play important roles in hippocampal-based learning, memory and neuronal plasticity (Anagnostaras et al., 2000; Messer et al., 1990). Therefore, it might be considered that ANT have a neuroprotective effect on hippocampal cholinergic system.

Our results showed that scopolamine administration significantly increases AChE activity in the cerebral cortex and hippocampus of animals, and these results are consistent with other (Choi et al., 2012; Jeong et al., 2008; Rang Oh et al., 2012). Scopolamine has been used to mimic age-related neuronal dysfunction in order to screen anti-amnesic drugs (Sakurai et al., 1998). The elevation of brain oxidative status after administration of amnesic doses of scopolamine further substantiates the value of scopolamine-induced amnesia as an animal model to test for drugs with potential therapeutic benefits in dementia (El-Sherbiny et al., 2003). In addition, the axonal transport of endogenous AChE showed impairment both of fast antero and retrograde transport (Southam et al., 1991). In vivo investigation of rats treated with scopolamine, showed that brain AChE was markedly reduced (Southam et al., 1991). Our results showed that scopolamine increased the AChE activity and this effect was prevented by the treatment with ANT. These results together with those showing that ANT improves memory deficits suggest that this compound may up regulate the cholinergic system.

AChE metabolizes ACh to choline and acetyl-CoA. AChE exists into different molecular forms, which can be distinguished on the basis of their shapes, e.g., collagen-tailed asymmetric forms and globular (G) forms (Lane et al., 2006). There are evidences that different isoforms of AChE may be differentially expressed in different brain regions (Lane et al., 2006; Malatova et

al., 1980; Zakut et al., 1985), and that these isoforms can be considered important markers for AD (Kasa et al., 1997; Lane et al., 2006; Shen, 2004). Furthermore, it is known that AChE activity in S1 corresponds to the total AChE activity (different isoforms associated), while in the synaptosomes (re-sealed nerve terminal) exist a greater amount of membrane-bound isoforms G4 (Mazzanti et al., 2006). In our study we found that SCO treatment increased AChE activity both in homogenate (S1) and synaptosomes of cerebral cortex and hippocampus of rats suggesting that all AChE isoforms were altered.

There are studies r reporting that SCO impairs energy metabolism and reduces the ATP levels in the cerebral cortex of rats (Blin et al., 1994; Ray et al., 1992), and it is known that the worsening of mitochondrial function and ATP levels reduction are pathological hallmarks found in neurodegenerative diseases, such as AD, which are closely linked to cognitive decline (Ferrer, 2009; Hauptmann et al., 2009). Other studies also show that SCO reduces the frontal cortex perfusion in young humans (Honer et al., 1988). In addition, it was also observed that intramuscular SCO administration impairs the oxygen consumption and the tissue metabolism of the cardiovascular and CNS of humans (Kirvela et al., 1994). This is in line with previous studies by Stone et al (1991) showing that glucose treatment is able to prevent deficits on the memory induced by SCO, suggesting that deleterious effects of SCO could be related to energy depletion in neurons (Stone et al., 1991); and also with our previous study that showed that SCO reduces the levels of ATP in the cerebral cortex and hippocampus of rats and ANT treatment prevents this (Gutierres et al., 2012b). A likely explain for this effect could be related to the vasodilatory capacity of anthocyanins (Mudnic et al., 2011), since that this flavonoid crosses the blood brain barrier (Youdim et al., 2003), induces vasodilation and activate endothelial oxide nitric synthase, increasing the production of nitric oxide (Edirisinghe et al., 2011; Min et al., 2011; Mudnic et al., 2011).

ATP levels into the cell have been suggested to modulate Na^+,K^+-ATPase and Ca^{2+}-ATPase activities since a reduction of intracellular ATP decreases the activity of these enzymes (Erecinska and Silver, 2001; Michaelis et al., 1983; Parsons et al., 2004; Therien and Blostein, 2000). The high energetic cost of these enzymes is crucial to the maintain the electrochemical

gradient necessary for neuronal excitability, adjustment of cell volume, osmotic balance, transport of molecules attached to the co-transport of Na^+ and intracellular Ca^{2+} homeostasis (Jorgensen et al., 2003; Kaplan, 2002; Mata and Sepulveda, 2010).

Besides alterations in the cholinergic transmission, cognitive disorders have also an impairment of the generation of membrane potential and the influx of neuronal Ca^{2+} (Berrocal et al., 2009; Mata et al., 2011). Considering that Na^+,K^+-ATPase is one of the most abundant brain enzyme, consuming about 40–60% of the ATP generated (Kaplan, 2002), it is not surprising that alterations in its activity may cause a variety of abnormalities. It has been describe that a decrease in Na^+,K^+-ATPase results in depletion of intracellular K^+, accumulation of intracellular Na^+, and, consequently, leads to membrane depolarization and increases in intracellular free Ca^{2+} due to activation of voltage-gated Ca^{2+} channels and a reversed operation of the Na^+/Ca^{2+} exchanger (Archibald and White, 1974; DiPolo and Beauge, 1991; Geering, 1997; Pavlov and Sokolov, 2000; Xiao et al., 2002). On the other hand, alterations in the intracellular Ca^{2+} concentrations are responsible for modulating the activity of Ca^{2+}-ATPase enzyme which regulates the intracellular levels of this second messenger (Mata and Sepulveda, 2010; Verkhratsky et al., 2012; Yamaguchi, 2012).

In this study we found a reduction in the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities in cerebral cortex and hippocampus of animals treated with SCO. These enzymes are sensitivities to tissue levels of ATP, it is possible that the decreased of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities induced by SCO may also be associated with the reduction of ATP levels. In line with this, reduced activity of Na^+,K^+-ATPase and of Ca^{2+}-ATPase has been suggested to play a central role in memory process (dos Reis et al., 2002; Lingrel et al., 2007; Moseley et al., 2007) and pathogenesis of neurodegenerative diseases, such as AD (Hattori et al., 1998; Mata et al., 2011) and Parkinson's disease (Grisar et al., 1992; Rose and Valdes, 1994; Zaidi, 2010).

Conclusion

In conclusion, the present study provides evidences suggesting that ANT may affects sensitivity of cholinoreceptors and protect enzymes ATP dependent. Therefore, ANT indeed has a close interaction with the cholinergic system and underlying memory retention process.

Conflicts of Interest statement

There are no conflicts of interest.

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Legends

Scheme 1. Experimental protocol design

Table 1 - Structural identification of anthocyanins

Table 2 - Effect of ANT treatment (200 mg kg^{-1}) and SCO injection (1 mg kg^{-1}) on the step down latencies (s) in inhibitory avoidance task in rats.

Table 3 - Effect of scopolamine and anthocyanin on the behavior of rats (number of crossing and rearing responses) and on foot shock sensitivity (flinch, jump and vocalization) in open field arena.

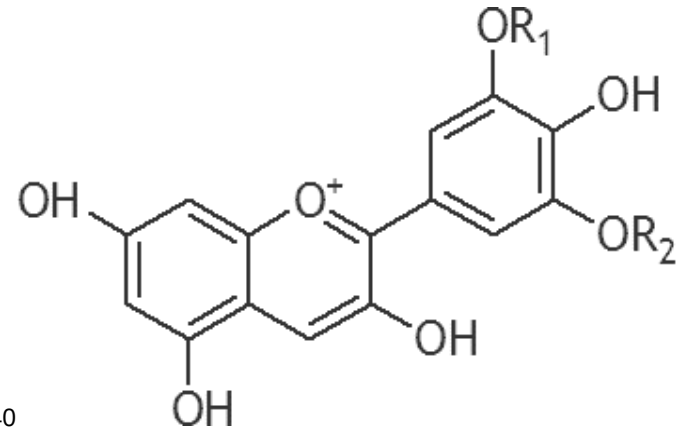
Figure 1 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on anxiety-like behavior in adult rats in the elevated plus maze task. Bars represent the mean ± SEM. * *P<0.05* represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA).

Figure 2 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on AChE activity in synaptosomes (A) and S1 (B) in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA)

Figure 3 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on Na^{+}, K^{+}-ATPase (A) and Ca^{2+}-ATPase (B) activities in cerebral cortex and hippocampus of adult rats. Bars represent the mean ± SEM. * *P<0.05* represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA)

Figure 4 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on NOx levels in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM (Two way ANOVA).

Table 1. Structural identification of anthocyanins.



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Anthocyanins	R1	R2	Formula	M.W
Cyanidin	OH	H	C15H11O6	322,72
Malvidin	OCH3	H	C16H13O6	336,74
Delphinidin	OH	OH	C15H11O7	338,72
Petunidin	OCH3	OH	C16H13O7	352,74
Malvidin	OCH3	OCH3	C17H15O7	366,77

Table 2 - Effect of ANT treatment (200 mg kg^{-1}) and SCO injection (1 mg kg^{-1}) on the step down latencies (s) in inhibitory avoidance task in rats.

	Latency of Training (s		***.atency of tes*** ***(s)***	
Groups	*Mean* ± SEM	*minimum*	*median*	*maximum*
Control	7.50 ± 1.99	69.00	175.00	300.00
ANT	8.37 ± 1.79	110.00	210.00	300.00
SCO	5.30 ± 1.59	25.00	66.50*	110.00
SCO+ ANT	8.22 ± 1.35	116.00	218.00#	300.00
Statistical Analysis	$F_{(3.31)}$= 0.77; p>0.05	-	H=9.75; p<0.01	-

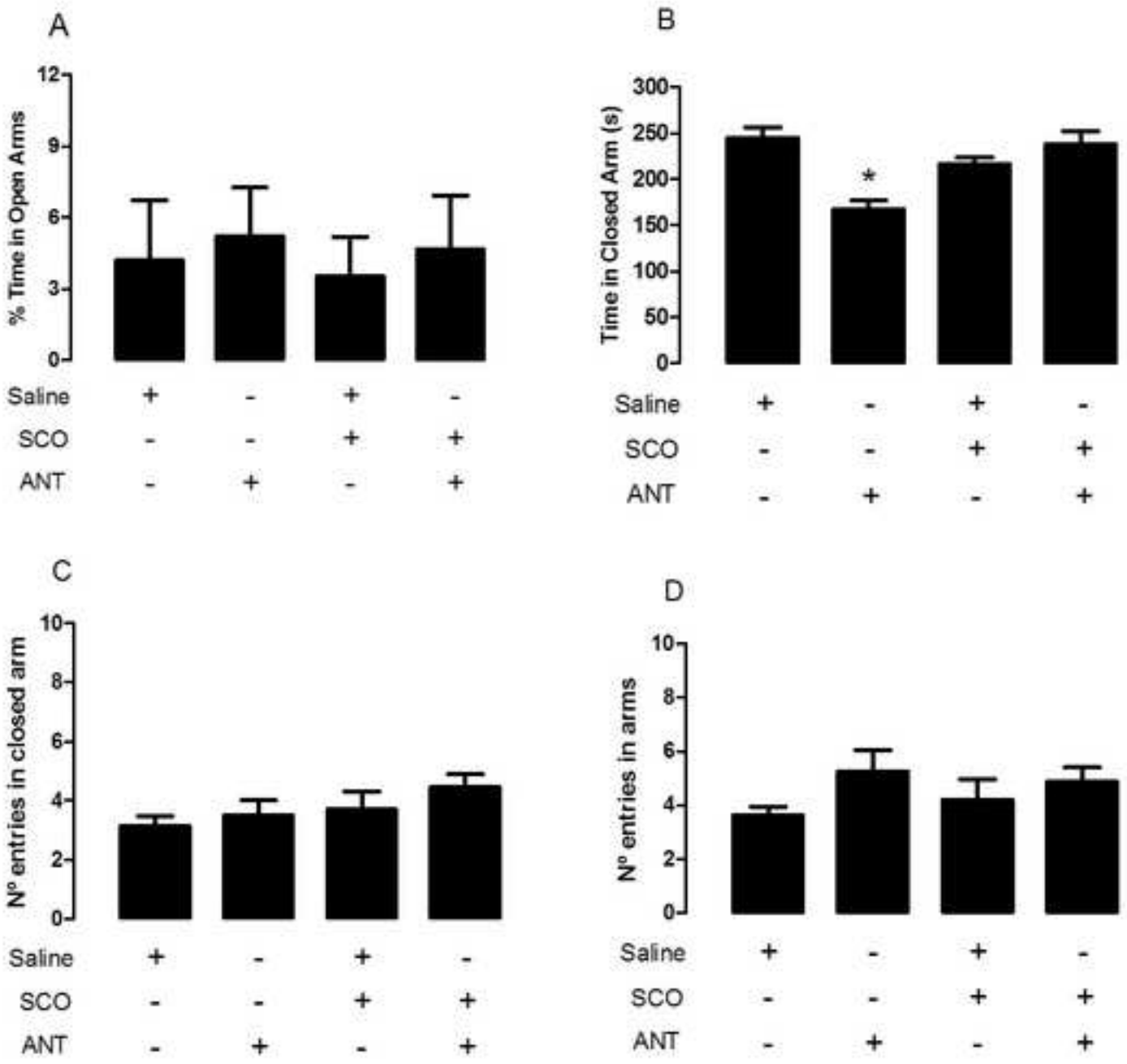
Data training are means ± SEM. Data Test are the median ± interquartile, 6-10 animals in each group. * *P<0.05* compared with the others groups. # *P<0.05* compared with SCO group by the Dunn's nonparametric multiple comparisons task (Scheirer-Ray-Hare extension of two way ANOVA, nonparametric test).

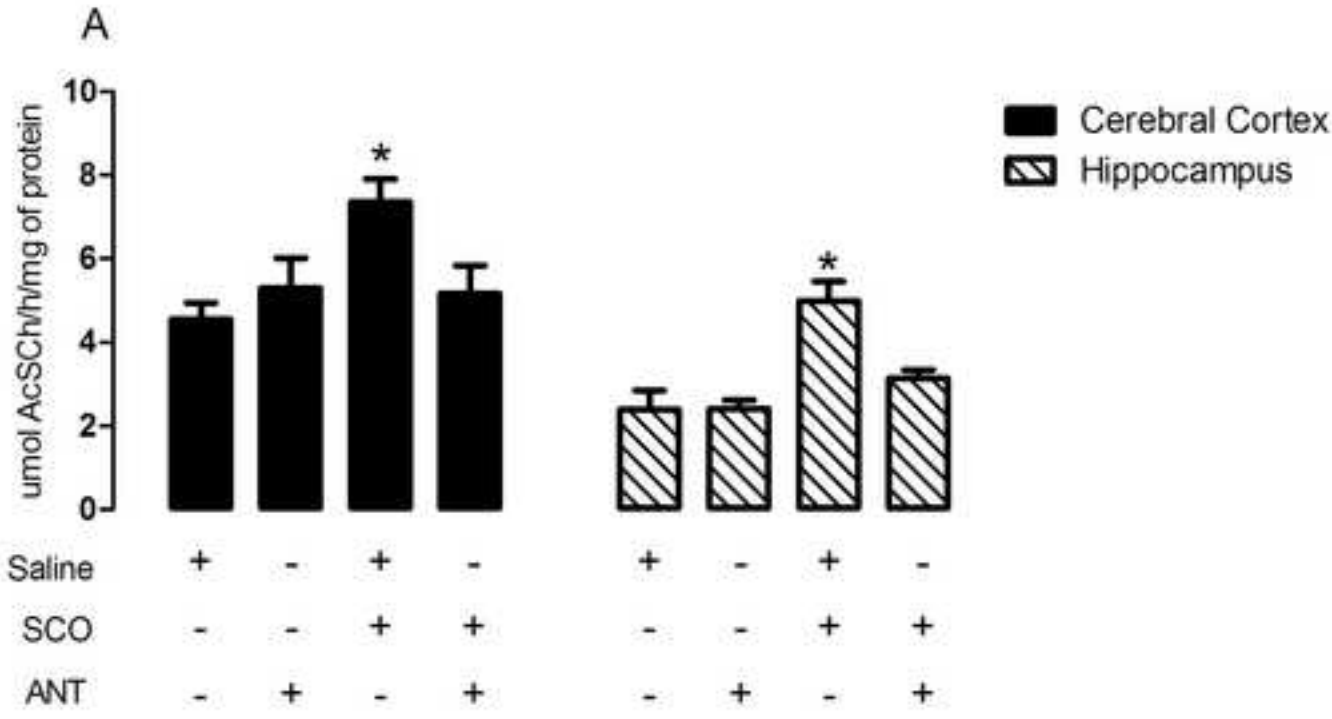
Table 3 - Effect of scopolamine and anthocyanin on the behavior of rats (number of crossing and rearing responses) and on foot shock sensitivity (flinch, jump and vocalization) in open field arena.

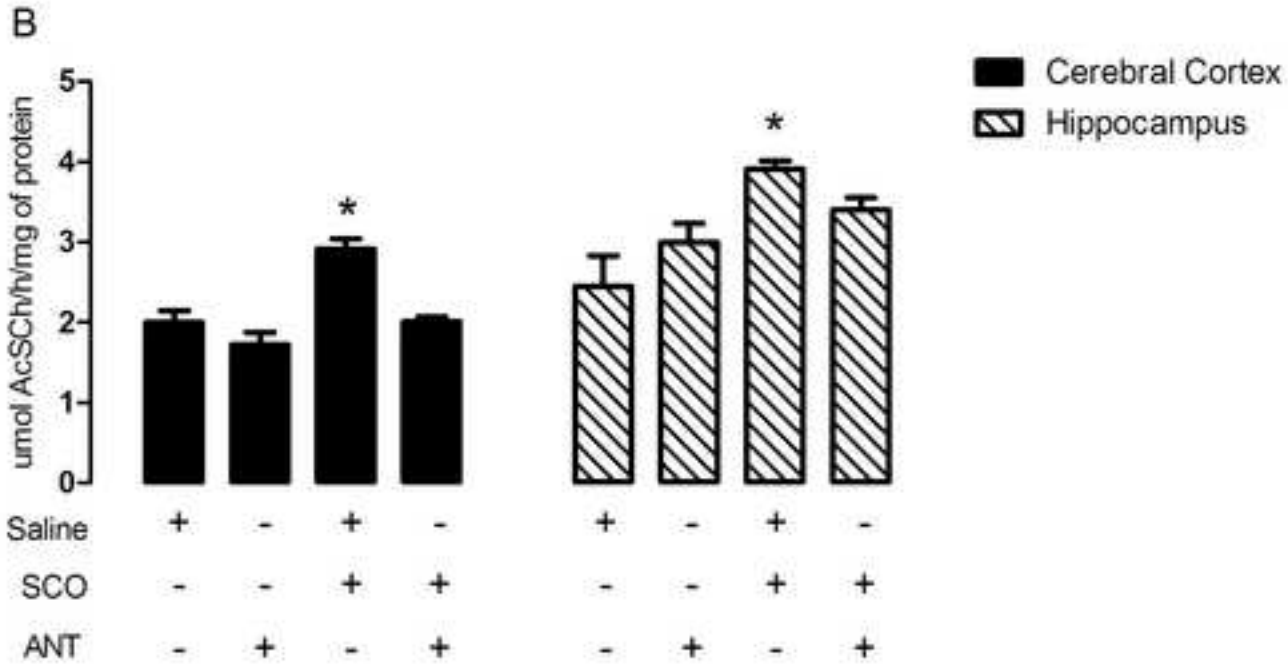
Crossing	*Rearing*	*Flinch (mA)*	*Jump (mA)*	*Vocalization (mA)*
21.75 ± 3.13	16.00 ± 2.28	0.36 ± 0.01	0.45 ± 0.02	0.35 ± 0.05
17.25 ± 2.19	13.63 ± 2.09	0.41 ± 0.03	0.36 ± 0.02	0.41 ± 0.03
22.10 ± 2.57	18.00 ± 2.96	0.34 ± 0.01	0.43 ± 0.02	0.44 ± 0.02
23.89 ± 3.01	20.22 ± 2.36	0.37 ± 0.03	0.33 ± 0.02	0.41 ± 0.03
$F_{(3.36)}$= 0.99; p>0.05	$F_{(3.36)}$= 0.13; p>0.05	$F_{(3.31)}$= 1.30; p>0.05	$F_{(3.31)}$= 4.48; p>0.05	$F_{(3.31)}$= 1.11; p>0.05

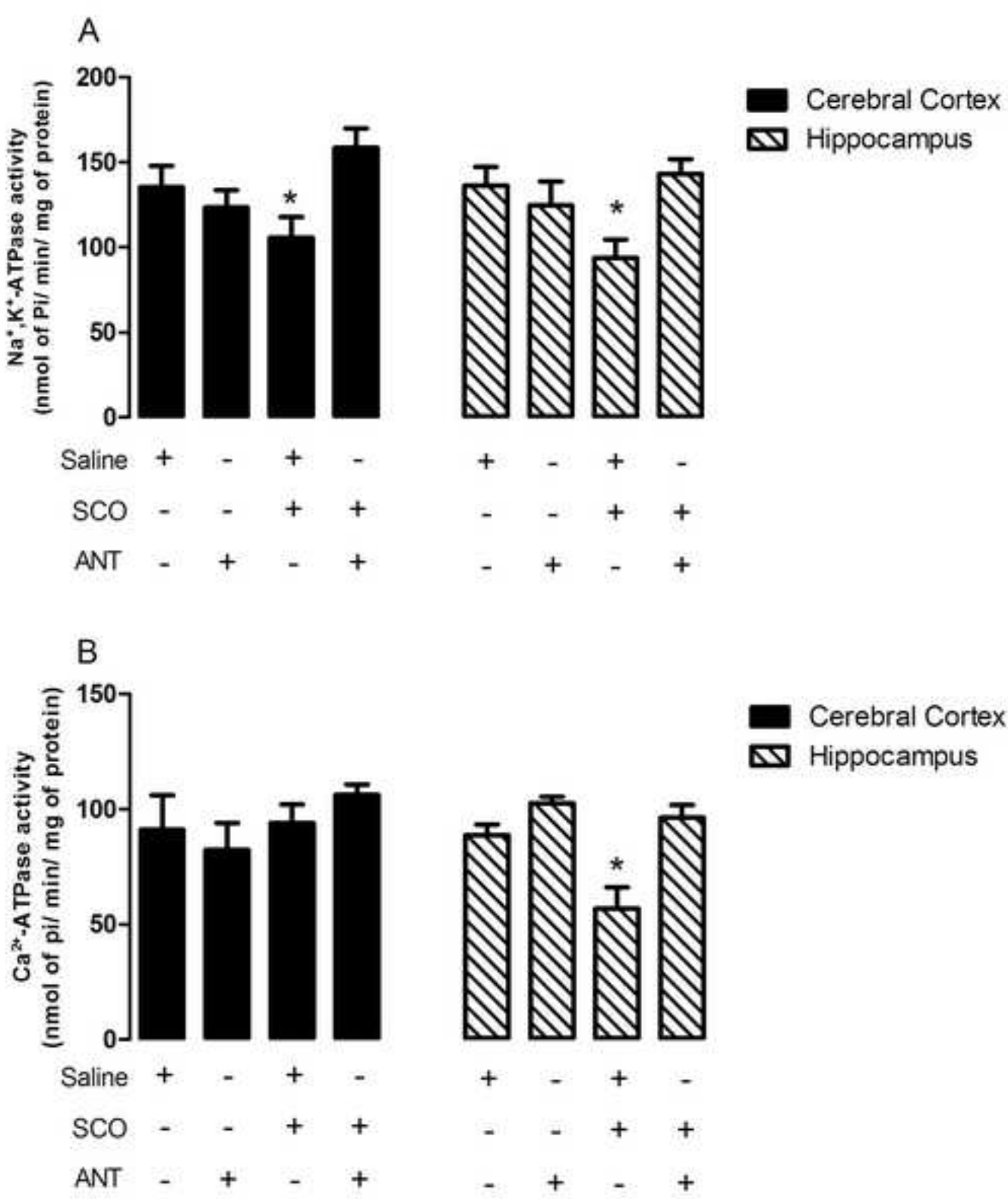
Data are means ± SEM for 6-10 animals in each group.

Figure(1)

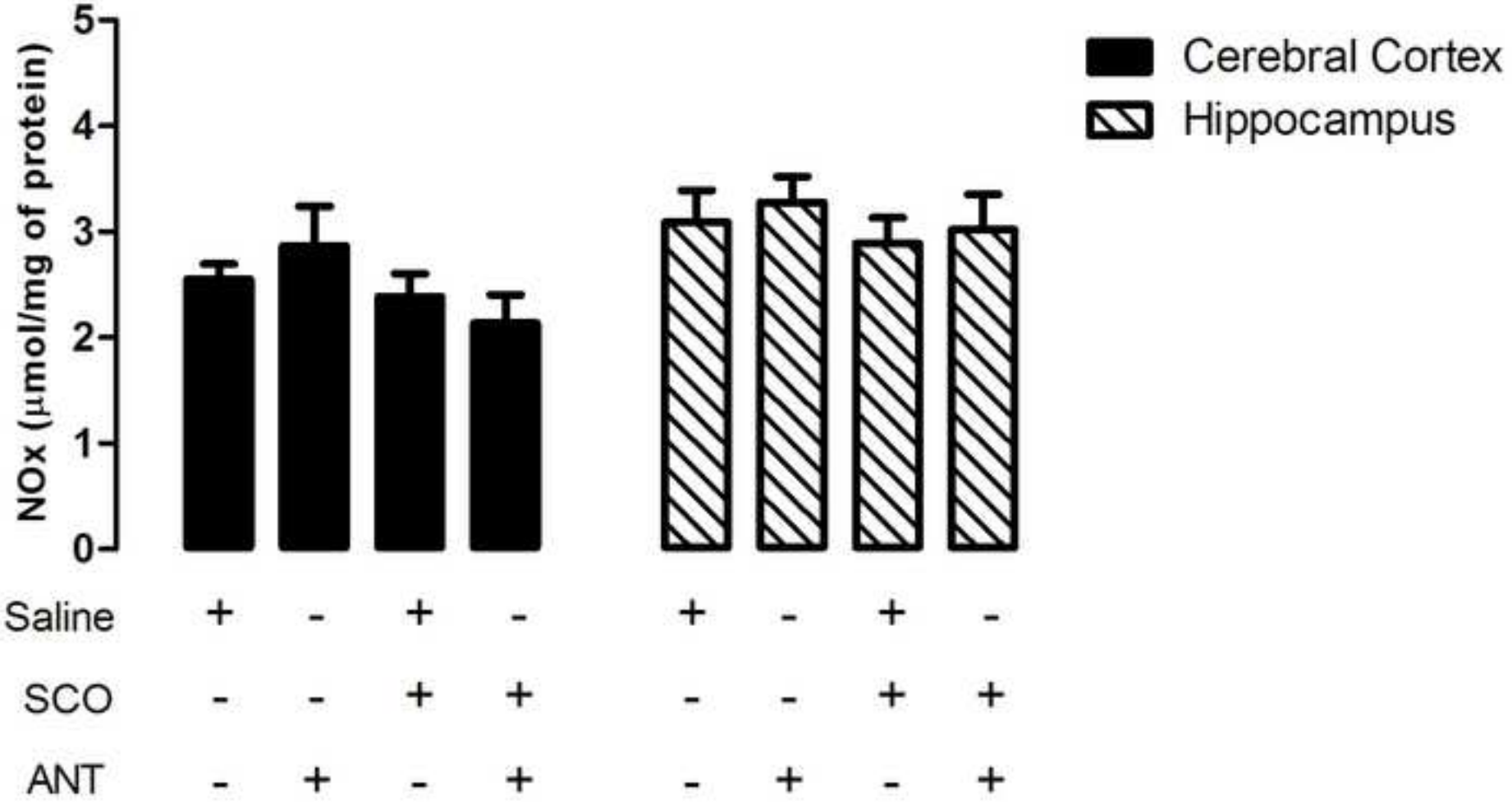

A
% Time in Open Arms
12
9
6
3
0
Saline + - + -
SCO - - + +
ANT - + - +
B
Time in Closed Arm (s)
300
250
200
150
100
50
0
*
Saline + - + -
SCO - - + +
ANT - + - +
C
Nº entries in closed arm
10
8
6
4
2
0
Saline + - + -
SCO - - + +
ANT - + - +
D
Nº entries in arms
10
8
6
4
2
0
Saline + - + -
SCO - - + +
ANT - + - +



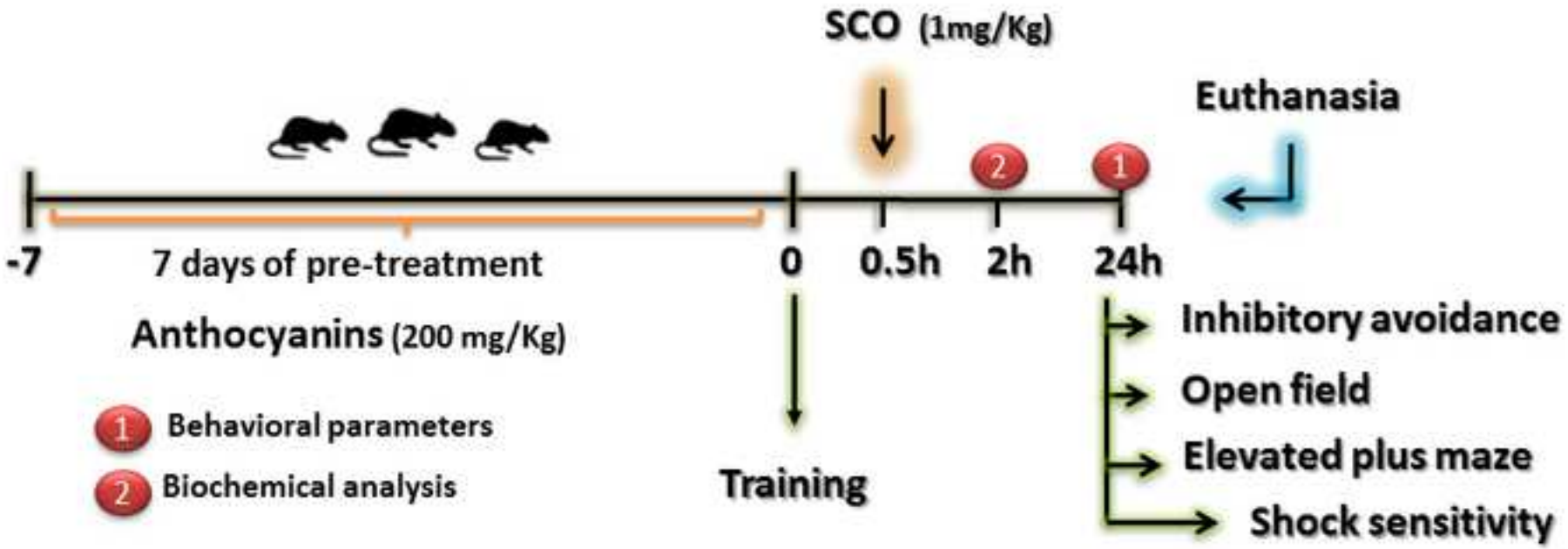




Figure(4)


NOx (μmol/mg of protein)
0
1
2
3
4
5
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +

scheme


SCO (1mg/Kg)
Euthanasia
2
1
-7
7 days of pre-treatment
0
0.5h
2h
24h
Anthocyanins (200 mg/Kg)
Inhibitory avoidance
Open field
Elevated plus maze
Shock sensitivity
1 Behavioral parameters
2 Biochemical analysis
Training