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Memory deficits and oxidative stress in cerebral ischemia–reperfusion: Neuroprotective role of physical exercise and green tea supplementation

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ABSTRACT

Ischemic stroke is a major cause of morbidity and mortality all over the world. Among impairments observed in survivors there is a significant cognitive learning and memory deficit. Neuroprotective strategies are being investigated to minimize such deficits after an ischemia event. Here we investigated the neuroprotective potential of physical exercise and green tea in an animal model of ischemia–reperfusion. Eighty male rats were divided in 8 groups and submitted to either transient brain ischemia–reperfusion or a sham surgery after 8 weeks of physical exercise and/or green tea supplementation. Ischemia–reperfusion was performed by bilateral occlusion of the common carotid arteries during 30 min. Later, their memory was evaluated in an aversive and in a non-aversive task, and hippocampus and prefrontal cortex were removed for biochemical analyses of possible oxidative stress effects. Ischemia–reperfusion impaired learning and memory. Reactive oxygen species were increased in the hippocampus and prefrontal cortex. Eight weeks of physical exercise and/or green tea supplementation before the ischemia–reperfusion event showed a neuroprotective effect; both treatments in separate or together reduced the cognitive deficits and were able to maintain the functional levels of antioxidant enzymes and glutathione.

1. Introduction

Ischemic stroke results from obstruction of a blood vessel supplying the brain, and is considered a major cause of morbidity and mortality (Tian et al., 2013). Ischemia events are known to cause learning and memory deficits (Cechetti et al., 2012).

The reperfusion after ischemia damages neuronal cells and tissues generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Sarkaki et al., 2013) contributing to the oxidative stress, which has been implicated in a variety of acute and chronic neurologic conditions (Dal-Pizzol et al., 2000, 2001). Indeed, one of the brain regions most sensitive to ischemia–reperfusion injury, the hippocampus, plays a key role in learning and memory (Collino et al., 2006).

Regular physical exercise improves hippocampus function (van Praag, 2008; Vivar, Potter, & van Praag, 2013), which helps to prevent sequels and assists in recovery (Voss, Vivar, Kramer, & van Praag, 2013). Pre-ischemia treadmill training up-regulated GLT-1 expression, decreases the extracellular glutamate concentration, reduces the cerebral infarction volume, and improves the neurobehavioral performance of rats (Yang et al., 2012). Additionally, Cechetti et al. (2012) reported positive effects of exercise performed post-hypoperfusion or either in pre- and post-hypoperfusion. In general, the benefits of physical exercise on ischemia–reperfusion are related to prevention of oxidative stress (Cechetti et al., 2012; Hamakawa et al., 2013).

In addition to exercise, the consumption of natural compounds, such as omega fatty acids or plant polyphenols benefits brain function (van Praag, 2009). The green tea (*Camellia Sinensis*) has been suggested as a potential source of antioxidants (Park et al., 2009; Wu, Hsieh, Wu, Wood, & Chen, 2012) available through diet (Wu et al., 2012; Xu et al., 2010). Green tea contains catechines (30–40% of its dry weight), which found in the green tea have potential antioxidant activity (Berube-Parent, Pelletier, Dore, & Tremblay, 2005). The epigallocatechin gallate (EGCG) is a major component of green tea and has been shown to be a neuroprotective agent

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in animal models of focal and global brain ischemia (Park et al., 2009).

The particular effects of physical exercise and green tea on memory deficits are documented in the literature (Marlatt, Potter, Lucassen, & van Praag, 2012; van Praag, 2009; Voss et al., 2013), but the association between these two interventions has not been studied. If associating physical exercise and green tea promotes neuroprotection and minimization of deficits after ischemia at a larger extent of isolated interventions, such association could be a potential neuroprotective strategy. Therefore, we investigate if physical exercise and green tea supplementation either associated or by separate have a neuroprotective effect when administered before ischemia–reperfusion.

2. Material and methods

2.1. Animals and experimental design

Male Wistar rats were bought from Central Vivarium of Federal University of Santa Maria (RS/Brazil) and housed three per cage under controlled light and environmental conditions (12 h light/12 h dark cycle at 23 ± 2 °C and 50 ± 10% humidity) with food and water or green tea *ad libitum*. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1996) and Local Institution Animal Care and Use Committee (IRB #0132012).

The weight of each rat and the liquid consumption for each cage house were measured daily. At the age of 2 months, they were randomly assigned to one of 4 experimental groups: (a) control: rats not submitted to intervention; (b) exercise: rats submitted to physical training for 8 weeks; (c) green tea supplementation: rats supplemented with green tea during 8 weeks; and (d) exercise with green tea supplementation: rats submitted to physical training and supplemented with green tea simultaneously during 8 weeks (Fig. 1).

After 8 weeks the groups were reorganized. Sham or ischemia–reperfusion surgeries were performed and groups were subdivided, as follow:

- group 1 – sham: rats submitted to the sham surgery without the occlusion of carotid arteries;
- group 2 – sham and exercise: rats submitted to physical exercise before sham surgery;
- group 3 – sham and green tea supplementation: rats supplemented with green tea before sham surgery;
- group 4 – sham and exercise with green tea supplementation: rats submitted to physical training and supplemented with green tea simultaneously before sham surgery;
- group 5 – ischemia–reperfusion: rats submitted to the surgery with temporary bilateral occlusion of carotid arteries (ischemia–reperfusion);
- group 6 – ischemia–reperfusion and exercise: rats submitted to physical training before ischemia–reperfusion surgery;
- group 7 – ischemia–reperfusion and green tea supplementation: rats supplemented with green tea before ischemia–reperfusion surgery;
- group 8 – ischemia–reperfusion and exercise with green tea supplementation: rats submitted to physical training and supplemented with green tea simultaneously before ischemia–reperfusion surgery.

After intervention, all rats were submitted to behavioral tests. When behavioral tests were finished, rats were euthanized for posterior brain tissue preparation. Biochemical analyses performed in the brain tissues permitted to quantify the concentration of glutathione (GSH) (Hissin & Hilf, 1976), catalase (Aebi, 1984), reactive oxygen species levels (ROS) (Loetchutinat et al., 2005) and thiobarbituric acid reactive substances (TBARS) (Ohkawa, Ohishi, & Yagi, 1979), and also the activity of glutathione peroxidase (GPx) (Wendel, 1981). NADPH, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and GSH reagents were purchased from Sigma (St. Louis, MO, USA). Other reagents used in this study were of analytical grades and obtained from standard commercial suppliers.

2.2. Exercise protocol

Physical exercise was performed during 8 weeks in a motorized treadmill built for rodents (Insight Ltda, SP/Brazil). Running exercise was performed at intensity of 60–70% maximal oxygen uptake (VO_2) (treadmill belt velocity between 9 m/min and 13 m/min), in sessions lasting 30 min, 5 times a week, always in the same period of day, in light time period (Malek, Huttemann, Lee, & Coburn, 2013). In the week before the start of intervention, rats performed a daily treadmill running for ten minutes to habituate before performing the first VO_2 test. An indirect VO_2 running test was performed to determine the individual intensity of exercise (starting with low velocity and increasing it in 5 m/min every 3 min until the rat was unable to keep running). Time to fatigue (min) and the work volume (m/min) were considered as an indirect measure of VO_2 maximum (Brooks & White, 1978; Cechetti et al., 2012). In the middle of exercise intervention (week 4), an additional indirect VO_2 running test was conducted to adjust the exercise intensity for each rat.

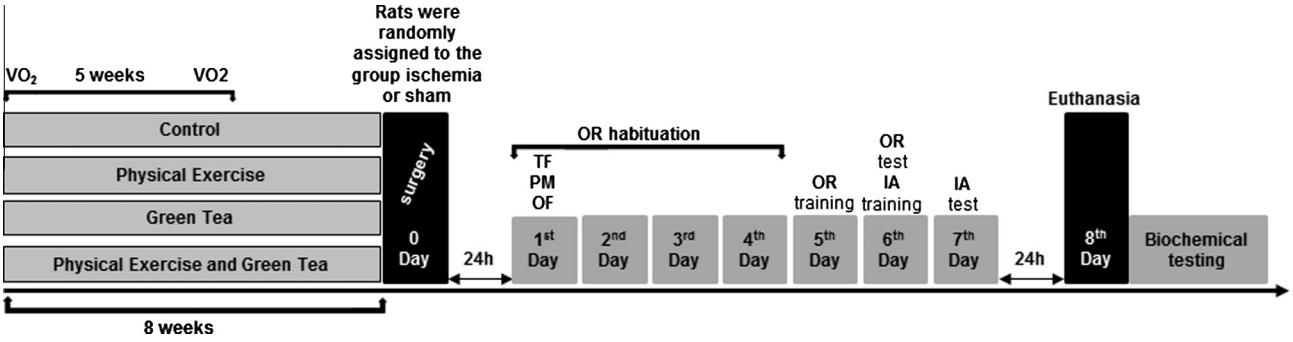

Fig. 1. Experimental design. OR – Object recognition memory test; TF – tail flick; OF – open field; PM – plus maze; IA – inhibitory avoidance memory test. Before the ischemia–reperfusion surgery, rats in the 4 initial proposed groups were randomly divided into 8 different groups, according sham or ischemia–reperfusion surgery. Each group had undergone different interventions during 8 weeks. Behavioral testing started 24 h after sham or ischemia–reperfusion surgery. Biochemical testing was the last step of the study.

2.3. Green tea supplementation

Rats received green tea mixed with drinking water (13.33 g/L), as described elsewhere (Mustata et al., 2005). Green tea was prepared daily in the early morning and administrated at ambient temperature. The liquid volume intake for each day was monitored. Green tea samples, Madrugada Co., used in this study were purchased from standard markets and analyzed by spectrophotometry using the Folin–Ciocalteu modified method (Chandra & De Mejia Gonzalez, 2004), which ensured the total polyphenols content (concentration 819.5 μg GAE/mL), and by high-performance liquid chromatography, which ensured presences of epicatechin (EGC) (concentration of 83.35 μg/mL), EGCG (299.56 μg/mL) and epicatechin gallate (ECG) (86.05 μg/mL).

2.4. Ischemia–reperfusion surgery

After 8 weeks of interventions, the rats were subjected to the ischemia–reperfusion or sham surgery. The surgery was performed always in the morning, under ketamine and xylazine anesthesia, 75 mg/kg and 10 mg/kg i.p., respectively. The rats were placed on a heating pad and shaved in the neck where a median incision was performed. The muscles' planes and trachea were deviated and common carotid arteries were freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained for occlusion (Collino et al., 2006). The temporary occlusion of the carotid arteries was performed using a vascular clip removed after 30 min. Restoration of blood flow in the carotid arteries was confirmed by careful observation by an experienced researcher; neck skin incision was then closed and sutured. During the surgical procedure, heating pad temperature was maintained at 37 °C to 38 °C until the rat wake up. Afterwards, the rat was transferred back to the cage house. Sham-operated rats underwent identical surgical procedures except for the no application of the vascular clip.

2.5. Behavioral testing

2.5.1. Object recognition memory test

Training and test in the object recognition task (OR) were performed in an open-field arena (50 × 50 × 50 cm) built with polyvinyl chloride plastic, plywood and transparent acrylic (Ennaceur & Delacour, 1988; Mello-Carpes & Izquierdo, 2013). Rats were first habituated to the apparatus during 20 min of free exploration in 4 consecutive days. For training, two different objects (A and B) were placed in the apparatus and rats were allowed to freely explore them during 5 min. The objects were made of metal, glass, or glazed ceramic. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects were not considered exploratory behaviors. A video camera was positioned over the OR arena, and the behavior was recorded using a video tracking system for offline analyses. After 24 h, in the test phase, one of the objects was randomly exchanged for a novel one (C), and the rats were reintroduced into the apparatus to freely explore the objects (familiar and new one) during 5 min. To avoid confounds by lingering olfactory stimuli and preferences, the objects and the arena were cleaned with 70% ethanol after each animal was tested.

2.5.2. Inhibitory avoidance memory test

Rats were trained in a one-trial step-down inhibitory avoidance task (IA) using a 50 × 25 × 25 cm plexiglass box with a 5 cm-high, 8 cm-wide, and 25 cm-long platform on the left end of a series of bronze bars which made up the floor of the box. For training, rats were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, a 2 s 0.5 mA scrambled foot shock was delivered. Memory retention was evaluated in a no reinforced test session carried out 24 h after training by quantifying the step-down latency (Barros et al., 2001; Fiorenza, Rosa, Izquierdo, & Myskiw, 2012; Mello, Benetti, Cammarota, & Izquierdo, 2009).

2.5.3. Control behavioral tasks

To analyze exploratory and locomotor activities and ensure that any procedure impaired such behaviors, each rat was placed on the left quadrant of a 50 × 50 × 39 cm open field arena made with wooden pained white, and with a frontal glass wall. Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossing and rearing, as measures for locomotor and exploratory activity, respectively, were measured over 5 min (Bonini et al., 2006).

To evaluate anxiety state, rats were exposed to an elevated plus maze. The time spent and the total number of entries into the open arms was recorded over a 5 min session (Pellow, Chopin, File, & Briley, 1985).

To ensure the IA testing efficacy, nociception was measured using the tail flick test (Tjolsen, Lund, Berge, & Hole, 1989), with pain induced by infrared light acting on the tail of the rat 5 cm away from the tip of the tail. Reaction time (tail-flick latency) was measured by the interval between placing the tail on the infrared light source and the voluntary withdrawal of the tail.

Data from these tests were compared between the groups to verify any impairment that could influence the behavioral results.

2.6. Biochemical testing

2.6.1. Tissue preparation

For the preparation of brain tissues, the rats were euthanized 24 h after the behavioral experiments were finished. The brain was removed and bilateral hippocampus and prefrontal cortex were quickly dissected out and homogenized in 50 mM Tris HCl, pH 7.4, (1/10, w/v). Afterwards, samples were centrifuged at 2400*g* for 20 min, and supernatants (S1) were used for assay.

2.6.2. Glutathione (GSH)

GSH levels were fluorometrically determined (Hissin & Hilf, 1976). An aliquot of homogenized was mixed (1:1) with perchloric acid ($HClO_4$) and centrifuged at 3000*g* for 10 min. After centrifugation, the protein pellet was discarded and free-SH groups were determined in the clear supernatant. An aliquot of supernatant was incubated with orto-phthaladehyde, and fluorescence was measured at excitation of 350 nm and emission of 420 nm. Results were expressed as nmol g^{-1} of tissue.

2.6.3. Glutathione peroxidase

Glutathione peroxidase (GPx) activity was measured spectrophotometrically (Wendel, 1981) in a system containing GSH/NADPH/GR by dismutation of H_2O_2 at 340 nm. S1 was added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity is indirectly measured by means of NADPH decay. H_2O_2 is decomposed generating GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase presents in the assay media at the expenses of NADPH. The enzymatic activity was expressed by the consumption of NADPH in nmol/min/mg of protein.

2.6.4. Catalase

Catalase activity was determined spectrophotometrically (Aebi, 1984) involving the monitoring of the consumption of H_2O_2 in the presence of the sample (20 μL) at 240 nm. Enzyme activity was expressed in units (1U 1 μmol H2O2/min decomposed at pH 7 and 25 °C).

2.6.5. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) content was assessed by a spectrofluorimetric method using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe (Loetchutinat et al., 2005). The sample (S1) was incubated in darkness with 5 μL DCFH-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular ROS. The formation of the oxidized fluorescent derivative (DCF), measured by DCF fluorescence intensity, was recorded at 520 nm (480 nm excitation), 30 min after the addition of DCFH-DA to the medium. Results were expressed as AU (arbitrary units).

2.6.6. Detection of TBARS level

Lipoperoxidation was evaluated by the thiobarbituric acid reactive substance (TBARS) test (Ohkawa et al., 1979). One aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95 °C for 2 h, and the color reaction was measured at 532 nm. Results were expressed as nmol of malondialdehyde (MDA) per mg protein.

2.7. Statistical analysis

Data were checked for normality of distribution using Shapiro–Wilk. Daily intake of water and rats' weight were compared between the experiment days using Anova one-way. Mean weight was compared between the start and at the end of interventions using *t*-test. Oxygen uptake values in the beginning and end of interventions were compared using *t*-test. Object exploration time in OR task was converted to percent of total exploration time and therefore a two-way analysis of variance considering groups and interventions was conducted. Exploration time for the familiar and novel object were further compared in each group using paired *t* test. For IA results, differences in the step down latencies between training and test were calculated. These differences were compared between the groups using Kruskall–Wallis test. Mann–Whitney test was used for specific comparisons between the groups (sham control *vs* ischemia control; ischemia control *vs* ischemia intervention groups). Wilcoxon test was used for specific intra-groups comparisons (training *vs* test). In OF, PM and TF tests the data were analyzed using ANOVA with Duncan post hoc if necessary. Biochemical results were compared using ANOVA for a mixed linear model (2 groups – sham and ischemia × 4 interventions – control, exercise, green tea, exercise and green tea) using a Bonferroni correction for multiple comparison. Where significant main effects or interaction were observed, *t*-test or ANOVA with Duncan post hoc were conducted to compare groups and interventions, respectively. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Animal weight and fluid intake

Individual weight gain of rats from all groups in daily measurements was similar between the groups and between the start and end of the interventions. The mean of water or green tea daily intake were similar between the groups (47.01 ± 3.14 ml for groups drinking green tea mixed with water, and 45.99 ± 1.35 ml for groups drinking water).

3.2. Indirect oxygen uptake (VO_2)

VO_2 measured for each rat was higher in the second VO_2 test than the beginning of the physical training (data not shown).

3.3. Behavioral results

3.3.1. Object recognition memory test

Long term recognition memory was evaluated using the OR paradigm. Rats were trained in the OR task when all of them, regardless of the group, explored two new objects (A and B) for about 50% of total exploration time each one (mean of all groups for object A = 44.1 ± 13.8%; and for object B = 55.9 ± 13.8%). An effect of the object tested was observed in the testing session, performed 24 h after training [$F_{(1,5)} = 76.48$, $P < 0.01$]. Further analysis indicated that the percent of time exploring the novel object (C) was higher than the time exploring the familiar object (A) in sham groups ($P < 0.05$; Fig. 2, sham groups). In this case rats explored longer the new object.

Rats from ischemia–reperfusion (group 5) spent similar exploration time for each one of the objects (Fig. 2, ischemia–reperfusion 5). An effect of the object tested was observed in the testing session, performed 24 h after training [$F_{(1,5)} = 8.67$, $P < 0.05$]. Further analysis indicated that rats from ischemia–reperfusion and physical exercise (group 6), and ischemia–reperfusion and green tea supplementation (group 7) spent significantly more time exploring the new object (C) compared to the familiar one (A) ($P < 0.01$; Fig. 2, ischemia–reperfusion 6 and 7). However, the rats from group 8, in which the exercise and the green tea supplementation were conducted simultaneously before the ischemia–reperfusion, spent similar exploration time for each one of the objects (Fig. 2, ischemia–reperfusion 8).

The total exploration time (s) in each training and testing session was similar for all groups (Table 1 – exploration time in OR).

3.3.2. Inhibitory avoidance memory test

In the evaluation of aversive long term memory consolidation using IA task, rats from all groups presented similar step down latency in the training session (mean of all groups: 2.9 ± 0.8 s).

In the test session, all groups significantly increased their step down latencies, as observed in intra-groups comparisons between training and testing latencies ($P < 0.05$ for groups 5 and 8, and

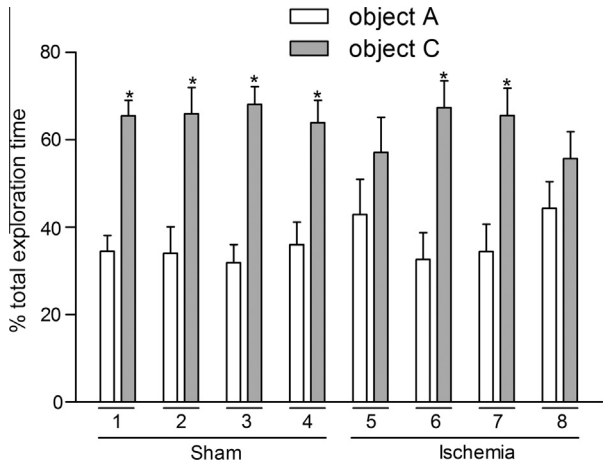

Fig. 2. Ischemia–reperfusion impairs memory in the object recognition task. Physical exercise and green tea showed a neuroprotetor effect when administrated isolated. Long term memory was tested in the object recognition task. 24 h after training animals were exposed to a familiar object (A) and a new one (C) during 5 min. Data of testing (mean ± standard-deviation) are presented for percent of the total exploration time, in seconds, in the experimental groups: 1: sham; 2: sham and exercise; 3: sham and green tea supplementation; 4: sham and exercise with green tea supplementation; 5: ischemia–reperfusion; 6: ischemia–reperfusion and exercise: 7: ischemia–reperfusion and green tea supplementation; 8: ischemia–reperfusion and exercise with green tea supplementation. *$P < 0.05$; *t* test comparing exploration time for objects A and C; $n = 8$–10 per group.

Table 1
Result of behavioral control tasks. Green tea supplementation and physical exercise along 8 weeks and ischemia–reperfusion surgery had no effect in the total time of exploration in OR task, locomotor and exploratory activities, anxiety and pain thresholds. Data are expressed as mean ± standard-deviation of the total time of exploration for object recognition task (OR), number of crossings and rearings (open field), total number of entries and the time spent in the open arms (plus maze), and time latency (tail flick). There were no differences between the groups ($P > 0.05$, one-way ANOVA). Experimental groups: 1: sham; 2: sham and exercise: 3: sham and green tea supplementation; 4: sham and exercise with green tea supplementation; 5: ischemia–reperfusion; 6: ischemia–reperfusion and exercise: 7: ischemia–reperfusion and green tea supplementation; 8: ischemia–reperfusion and exercise with green tea supplementation ($n = 8$–10 per group).

Behavioral tasks		Sham				Ischemia–reperfusion			
		1	2	3	4	5	6	7	8
Exploration time in OR	Total exploration time in training (s)	40.4 ± 19.1	30.9 ± 13.4	31.5 ± 13.0	36.3 ± 17.4	18.2 ± 12.6	33.7 ± 29.0	34.5 ± 21.4	27.6(13.1)
	Total exploration time in test (s)	39.3 ± 25.2	28.7 ± 20.6	38.2 ± 21.1	26.5 ± 18,4	23.2 ± 15.7	35.2 ± 23.5	25.6 ± 16.3	28.3(15.1)
Open field	Crossings (n°)	34.2 ± 22.7	35.8 ± 12.2	18.1 ± 5.8	22.1 ± 10.9	20.8 ± 16.5	25.3 ± 9.2	18.0 ± 12.7	21.0(14.2)
	Rearing (n°)	16.3 ± 10.8	14.9 ± 9.0	10.9 ± 5.2	11.8 ± 6.8	9.3 ± 7.9	11.0 ± 3.2	7.7 ± 5.8	8.2(7.9)
Plus maze	Total entries (n°)	12.8 ± 11.2	12.6 ± 14.2	11.9 ± 12.7	8.2 ± 7.0	11.1 ± 18.5	13.4 ± 14.2	8.7 ± 13.0	11.4(16.6)
	Time in open arms (s)	9.2 ± 11.0	19.9 ± 25.2	9.3 ± 11.9	16.0 ± 30.4	10.6 ± 27.8	13.7 ± 19.8	9.7 ± 14.8	12.2(18.6)
Tail flick	Times (s)	5.8 ± 1.8	5.1 ± 1.5	5.5 ± 1.4	4.6 ± 1.1	5.4 ± 1.7	5.2 ± 1.6	5.6 ± 1.9	5.9(1.8)

$P < 0.01$ for groups 1–4, 6 and 7 in Wilcoxon test – data not shown). However, the training-test step-down latencies differences were significant shorter in the group submitted to ischemia–reperfusion than in sham groups ($P < 0.01$, Fig. 3). Rats submitted to physical exercise or green tea supplementation previously to ischemia–reperfusion presented higher latencies differences than ischemia group ($P < 0.01$, Fig. 3).

3.3.3. Control behavioral tasks

Rats were exposed to an open-field arena, elevated plus maze and tail flick test after the surgery to verify exploratory and locomotor activity, anxiety, and pain threshold, respectively. No differences in the number of crossing and rearings in the open field task were observed after interventions (Table 1 – open field). The total number of entries and the time spent in the open arms (Table 1 – plus maze), as well as latencies in the tail flick test, were not altered in the different groups (Table 1 – tail flick).

3.4. Biochemical results: antioxidant markers

3.4.1. Glutathione (GSH)

A main effect for group [$F_{(1,5)} = 17.675$; $P = 0.008$] was observed. GSH concentration in the prefrontal cortex of ischemia–reperfusion group was lower than found in the sham group ($P < 0.01$ Fig. 4A, ischemia–reperfusion 5). Ischemia–reperfusion groups submitted to physical exercise and green tea supplementation, either in combination or not, avoided this reduction (Fig. 4A, $P < 0.01$, ischemia–reperfusion 6, 7 and 8). Among ischemia–reperfusion groups, exercise and the green tea isolated promoted increase of GSH levels when compared with paired sham animals ($P < 0.05$; Fig. 4A).

In hippocampus, a main effect for intervention [$F_{(3,15)} = 22.830$; $P < 0{,}001$] and an interaction group-intervention [$F_{(3,15)} = 24.678$; $P = 0.013$] were observed. GSH concentration in the hippocampus was reduced in the ischemia–reperfusion group compared to the sham group ($P < 0.05$; Fig. 4B, ischemia–reperfusion 5). Supplementation with green tea did not maintain GSH concentrations in the hippocampus of rats submitted to ischemia–reperfusion when compared to the sham green tea (Fig. 4B, ischemia–reperfusion 7). On the other hand, exercise, either isolated or associated to the green tea supplementation maintained GSH concentrations in the hippocampus (Fig. 4B, ischemia–reperfusion 6 and 8). Among sham groups, physical exercise combined with green tea supplementation increased GSH concentration in the hippocampus ($P < 0.01$; Fig. 4B, sham 4).

3.4.2. Glutathione peroxidase (GPx)

In the prefrontal cortex, an interaction group-intervention was observed [$F_{(3,15)} = 3.789$; $P = 0.033$]. GPx activity in the prefrontal cortex increased in the ischemia–reperfusion group compared to the sham animals ($P < 0.05$, Fig. 4C, ischemia–reperfusion 5). Physical exercise combined with green tea supplementation increased prefrontal cortex GPx activity in the ischemia–reperfusion animals compared to the sham physical exercise and green tea ($P < 0.01$, Fig. 4C, ischemia–reperfusion 8). In the sham animals, green tea supplementation increased GPx activity ($P < 0.01$, Fig. 4C, sham 3).

A main effect for intervention was observed in the hippocampus [$F_{(3,15)} = 5.553$; $P = 0.009$]. Activity of GPx in hippocampus of ischemia–reperfusion groups was similar to the sham (Fig. 4D). Comparisons between ischemia–reperfusion groups showed a lower activity of GPx in the hippocampus in the group ischemia–reperfusion and green tea supplementation.

3.4.3. Catalase

In the prefrontal cortex, a main effect for group [$F_{(1,5)} = 15.685$; $P = 0.011$]. Catalase activity in the prefrontal cortex was reduced in

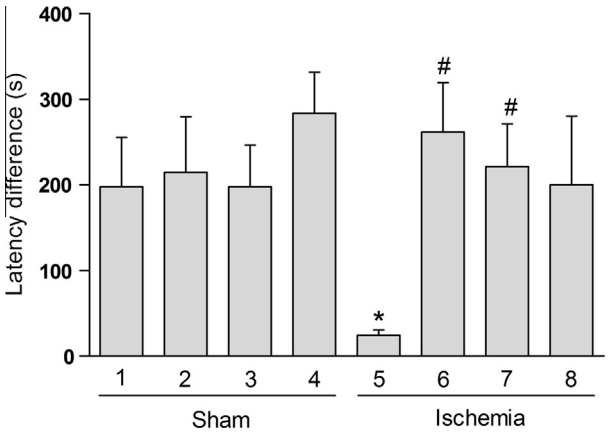

Fig. 3. Ischemia–reperfusion impairs aversive memory. Physical exercise and green tea have a neuroprotetor effect when administrated isolated. Difference between test and training step down latencies are presented in seconds considering mean and standard error data. Experimental groups: 1: sham; 2: sham and exercise: 3: sham and green tea supplementation; 4: sham and exercise with green tea supplementation; 5: ischemia–reperfusion; 6: ischemia–reperfusion and exercise: 7: ischemia–reperfusion and green tea supplementation; 8: ischemia–reperfusion and exercise with green tea supplementation. $^{*}P < 0.01$ in Mann–Whitney test (5 – ischemia – differed from all sham); $^{\#}P < 0.01$ in Mann–Whitney test (different of ischemia 5); $n = 8$–10 per group.

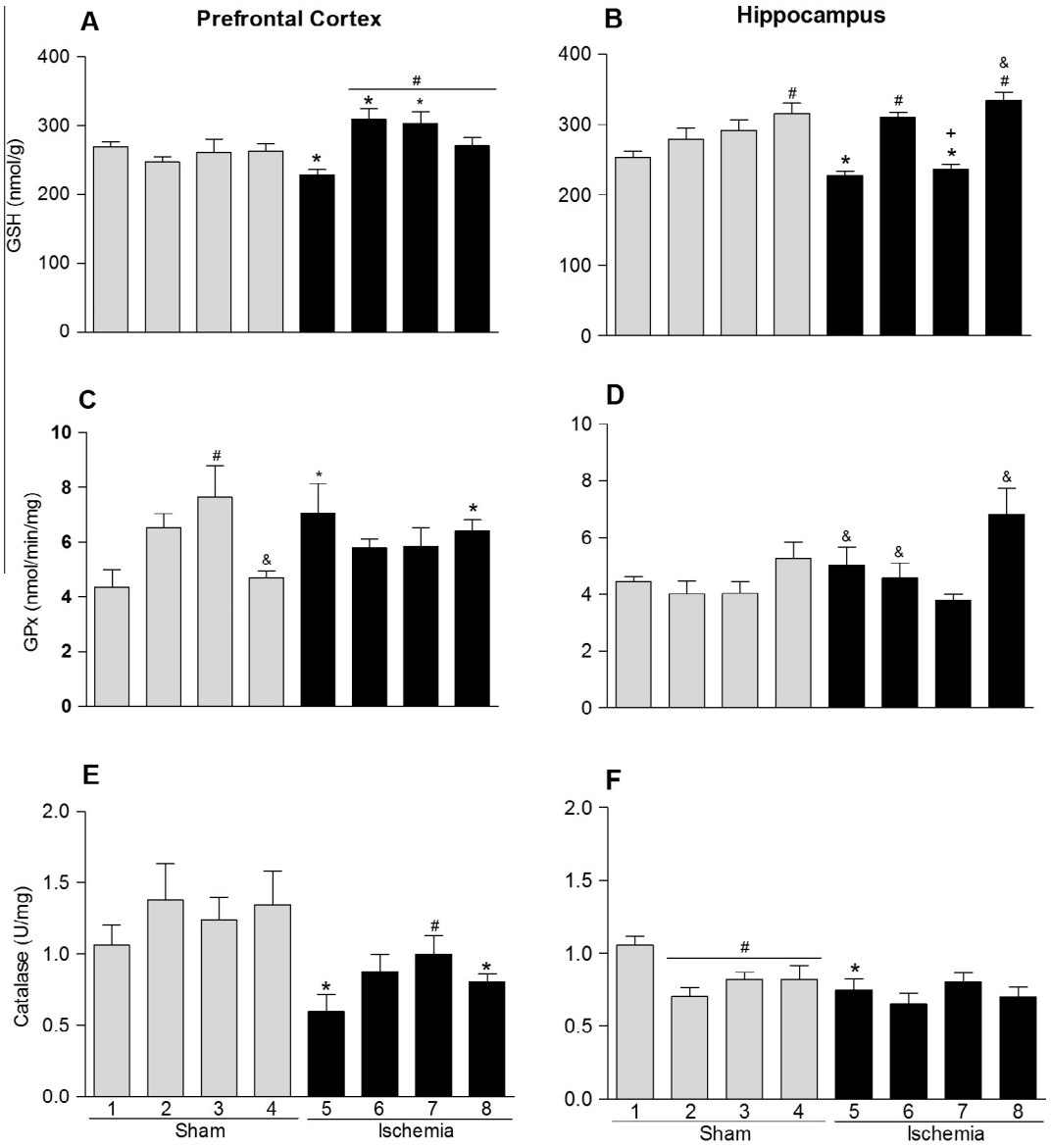

Fig. 4. Effect of ischemia–reperfusion, physical exercise and green tea supplementation on antioxidant markers in the prefrontal cortex (right column) and the hippocampus (left column). The signs indicate differences between/within groups: ∗ comparison between groups (*t* test; ischemia–reperfusion group *vs* its respectively sham); #, & or + evidence comparisons within groups (ANOVA and Duncan post hoc within sham or ischemia–reperfusion groups): where # means different of control; & means different of green tea supplementation, and + indicates different of exercise with green tea supplementation. Experimental groups: 1: sham; 2: sham and exercise: 3: sham and green tea supplementation; 4: sham and exercise with green tea supplementation; 5: ischemia–reperfusion; 6: ischemia–reperfusion and exercise: 7: ischemia–reperfusion and green tea supplementation; 8: ischemia–reperfusion and exercise with green tea supplementation.

the ischemia–reperfusion group ($P < 0.05$; Fig. 4E, ischemia–reperfusion 5), and in the ischemia–reperfusion group submitted to physical exercise and supplemented with green tea ($P < 0.05$; Fig. 4E, ischemia–reperfusion 8) when compared to their respective sham group. When the catalase activity was compared between ischemia–reperfusion groups, rats in the group supplemented with green tea presented higher catalase activity in the prefrontal cortex compared to the ischemia–reperfusion group ($P < 0.05$; Fig. 4E, ischemia–reperfusion 7).

In the hippocampus, a main effect for group [$F_{(1,5)} = 8.322$; $P = 0.034$] and a main effect for intervention [$F_{(3,15)} = 3.542$; $P = 0.041$] were observed. Rats from ischemia–reperfusion group presented lower activity of catalase in the hippocampus compared to the sham animals ($P < 0.01$, Fig. 4F, ischemia–reperfusion 5). Among the sham groups, physical exercise ($P < 0.01$; Fig. 4F, sham and exercise 2), green tea ($P < 0.05$; Fig. 4F, sham and green tea 3) and physical exercise associated with green tea supplementation reduced the catalase activity in the hippocampus ($P < 0.05$; Fig. 4F, sham and exercise with green tea supplementation 4).

3.5. Biochemical results: oxidative stress markers

3.5.1. Reactive oxygen species (ROS)

A main effect for group was observed in the hippocampus [$F_{(1,5)} = 8.043$; $P = 0.036$]. ROS levels in the prefrontal cortex were higher in the ischemia–reperfusion group than sham ($P < 0.05$; Fig. 5A, ischemia 5). In the ischemia–reperfusion groups, exercise increased ROS in comparison to the sham submitted to exercise ($P < 0.01$; Fig. 5A, ischemia–reperfusion 6).

An interaction group-intervention was observed in hippocampus [$F_{(3,15)} = 3.819$; $P = 0.032$]. Hippocampus presented higher ROS levels in the ischemia–reperfusion compared to the sham

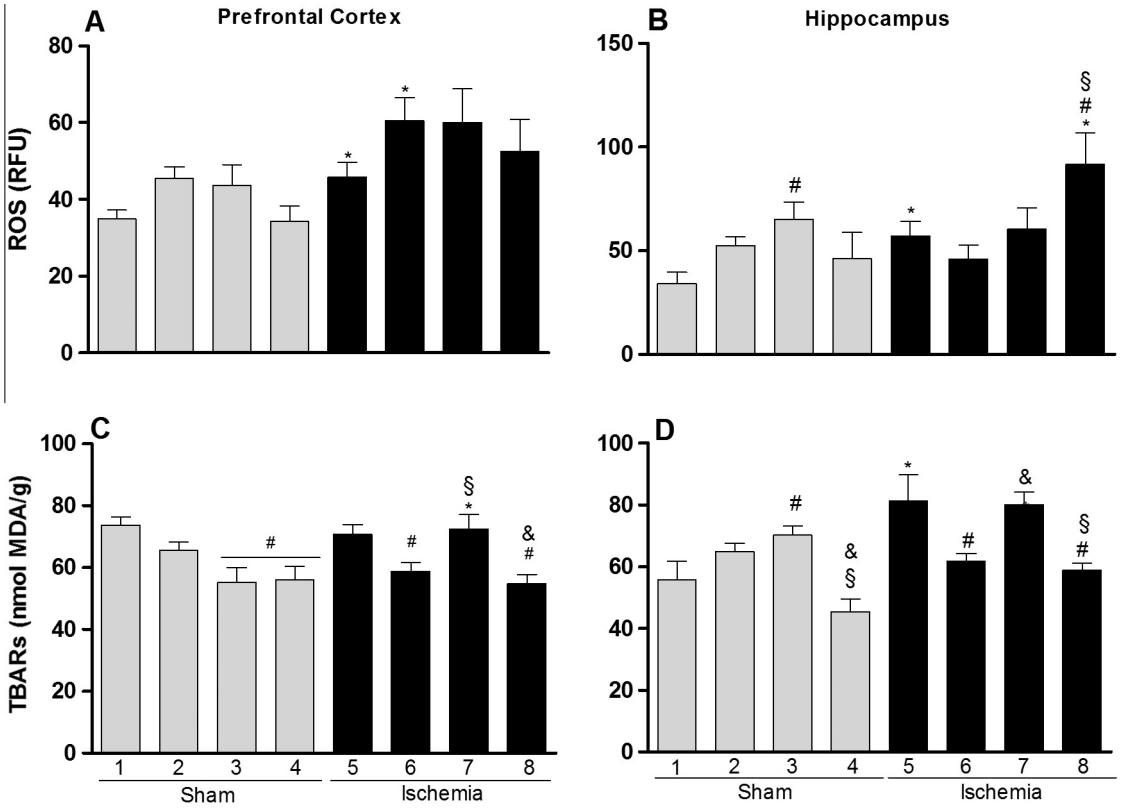

Fig. 5. Effects of ischemia–reperfusion, physical exercise, and green tea on biochemical markers in the prefrontal cortex (right) and hippocampus (left). The signs indicate differences between/within groups: ∗ between groups comparison (*t* test; ischemia–reperfusion group *vs* its respectively sham); #, & or + evidence within groups comparisons (ANOVA and Duncan post hoc within sham or ischemia–reperfusion groups): where # means different of control; & means different of green tea supplementation, and + indicates different of exercise with green tea supplementation. Experimental groups: 1: sham; 2: sham and exercise: 3: sham and green tea supplementation; 4: sham and exercise with green tea supplementation; 5: ischemia–reperfusion; 6: ischemia–reperfusion and exercise: 7: ischemia–reperfusion and green tea supplementation; 8: ischemia–reperfusion and exercise with green tea supplementation.

($P < 0.05$; Fig. 5B, ischemia–reperfusion 5). Sham rats supplemented with green tea presented higher hippocampus ROS levels than sham controls ($P < 0.01$; Fig. 5B, sham 3). Rats of the ischemia–reperfusion group submitted to physical exercise and green tea supplementation presented higher ROS levels in the hippocampus compared to the sham ($P < 0.05$; Fig. 5B, ischemia–reperfusion 8), ischemia–reperfusion control ($P < 0.05$; Fig. 5B, ischemia reperfusion 8), and ischemia–reperfusion submitted to physical exercise ($P < 0.01$; Fig. 5B, ischemia reperfusion 8).

3.5.2. Thiobarbituric acid reactive substances (TBARS)

A main effect for intervention [$F_{(3,15)} = 4.756$; $P = 0.016$] and an interaction group-intervention [$F_{(3,15)} = 3.564$; $P = 0.040$] were observed. The concentration of TBARS in the prefrontal cortex of ischemia–reperfusion group supplemented with green tea was higher than the control ischemia–reperfusion ($P < 0.05$; Fig. 5C, ischemia–reperfusion 7). In the sham groups, green tea supplementation, either isolated or combined with exercise, reduced TBARS concentration ($P < 0.01$; Fig. 5C, sham 3). Additionally, physical exercise, either isolated or associated with green tea, prevented the increase of TBARS concentration in ischemia–reperfusion rats (Fig. 5C, ischemia–reperfusion 6 ($P < 0.05$) and 8 ($P < 0.01$)).

In the hippocampus, a main effect for intervention [$F_{(3,15)} = 4.756$; $P = 0.016$] and an interaction group-intervention [$F_{(3,15)} = 3.564$; $P = 0.040$] were observed. Ischemia–reperfusion rats had increased TBARS concentration compared to the sham ($P < 0.05$; Fig. 5D, ischemia–reperfusion 5). Among the ischemia–reperfusion groups, physical exercise, either per se or associated to green tea supplementation, prevented increase of TBARS levels in the hippocampus when compared to the ischemia–reperfusion control ($P < 0.05$; Fig. 5D, ischemia–reperfusion 6 and 8). Within groups comparisons revealed that TBARS levels in hippocampus were increased in the sham group supplemented with green tea compared to the sham control ($P < 0.05$; Fig. 5D, sham 3). However, sham rats submitted to physical exercise combined with green tea supplementation presented lower TBARS levels than sham supplemented with green tea ($P < 0.01$; Fig. 5D, sham 4), and sham submitted to physical exercise ($P < 0.05$; Fig. 5D, sham 4).

4. Discussion

Here we investigated the neuroprotective potential of long term physical exercise and green tea supplementation, either in association or by separate, to prevent or reduce learning and memory deficits resulting from ischemia–reperfusion in a rat model. Our model showed to be valid as it resulted in cognitive deficits without impairment in locomotor and exploratory activities, as well as anxiety and pain thresholds.

Our results support the interpretation that the cognitive deficits were caused by ischemia–reperfusion surgery. Oxidative damages were observed in prefrontal cortex and hippocampus brain regions. A main finding was that combining of physical exercise and green tea in general did not elicit better results than when interventions were conducted separately.

Learning and memory deficits after ischemia–reperfusion may result from increased oxidative stress (ROS and TBARS) and decreased antioxidant status (GSH and GPx) in the hippocampus

and prefrontal cortex (Collino et al., 2006). Hippocampus is one of the brain areas more sensitive to ischemia–reperfusion (Zamani, Hassanshahi, Soleimani, & Zamani, 2013). The oxidative damages observed in hippocampus, evidenced by increased TBARS and ROS, can contribute to the impairment of learning function (da Silveira, Furini, Benetti, Monteiro Sda, & Izquierdo, 2013) and the aversive learning (Lima, de Bruin, Rios, & de Bruin, 2014).

We observed that physical exercise or green tea supplementation avoided memory deficits in the OR and IA task performed after ischemia–reperfusion. Additionally, physical exercise had positive effects on hippocampus and prefrontal antioxidant status by avoiding decreases of GSH, and on hippocampal pro-oxidants markers by avoiding increases in TBARS. Although with more discrete effects, our results suggest that green tea promotes GSH increases in prefrontal cortex and avoids catalase decreases in the same brain region. Such observations help to explain the effects of physical exercise and green tea on learning and memory. The neuroprotective effects of exercise on behavioral functions after a transient cerebral ischemia are consistent with previous researches (Li, Luan, Clark, Rafols, & Ding, 2004; Shih, Yang, & Wang, 2013; Tahamtan et al., 2013).

Ischemia–reperfusion also decreased concentration of catalase in the hippocampus and prefrontal cortex. In addition, ischemia–reperfusion increased the concentration of ROS in the hippocampus and prefrontal cortex, while TBARS increased in the hippocampus. Although these results were expected due the injury by ischemia reperfusion, they cannot be compared with any previous result, since our work seems to be the first to consider a long time after reperfusion.

Physical exercise improved the performance in long term recognition and aversive memory tasks in the ischemia–reperfusion rats. The physical exercise has been shown to potentiate hippocampal neurogenesis and cognitive functions (Castilla-Ortega et al., 2013; Cechetti et al., 2012; Kobilo et al., 2011; Marlatt et al., 2012; Vivar et al., 2013). It results from different mechanisms, including the capability of modulating oxidative stress (Hamakawa et al., 2013).

Despite of physical exercise, long term green tea supplementation also was related with improved learning and memory among ischemia–reperfusion groups, despite of association or not with exercise. It most likely resulted from the antioxidant activity of green tea catechines present in green tea (Park et al., 2009). Our initial hypothesis was that when these antioxidant strategies are combined, their positive effects could be potentiated (van Praag, 2009). However, interventions in association not prevent against the damages caused by ischemia–reperfusion; in these animals, behavioral performance was similar to those animals underwent the occlusion and received no prior intervention.

In addition, physical exercise associated with green tea supplementation significantly increased levels of ROS in the hippocampus after ischemia–reperfusion. While the concomitant regular physical exercise and intake of green tea has been a valid strategy to improve neuromuscular recovery after exercise (Haramizu, Ota, Hase, & Murase, 2011; Haramizu, Ota, Hase, & Murase, 2013; Jowko et al., 2012), it may has negative impact in the case of an ischemia–reperfusion event. The combined interventions increased ROS levels in hippocampus, which can be related to the fact that in this group/structure GSH and GPx were also higher than in the others, leading to an oxidative imbalance. It is interesting to observe that increase in ROS was associated with worst memory performance in this group.

In summary, our results suggest that mnemonic deficits associated to cerebral ischemic-reperfusion can be prevented by physical exercise and green tea when used isolated, but not when both interventions were associated. In addition, association of physical exercise and green tea increased ROS in the hippocampus. In general, regular physical exercise promoted better neuroprotection to an ischemic event.

References

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology, 105*, 121–126.

Barros, D. M., Mello e Souza, T., De David, T., Choi, H., Aguzzoli, A., Madche, C., et al. (2001). Simultaneous modulation of retrieval by dopaminergic D(1), beta-noradrenergic, serotonergic-1A and cholinergic muscarinic receptors in cortical structures of the rat. *Behavioural Brain Research, 124*, 1–7.

Berube-Parent, S., Pelletier, C., Dore, J., & Tremblay, A. (2005). Effects of encapsulated green tea and guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. *British Journal of Nutrition, 94*, 432–436.

Bonini, J. S., Bevilaqua, L. R., Zinn, C. G., Kerr, D. S., Medina, J. H., Izquierdo, I., et al. (2006). Angiotensin II disrupts inhibitory avoidance memory retrieval. *Hormones and Behavior, 50*, 308–313.

Brooks, G. A., & White, T. P. (1978). Determination of metabolic and heart rate responses of rats to treadmill exercise. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology, 45*, 1009–1015.

Castilla-Ortega, E., Rosell-Valle, C., Pedraza, C., Rodriguez de Fonseca, F., Estivill-Torrus, G., & Santin, L. J. (2013). Voluntary exercise followed by chronic stress strikingly increases mature adult-born hippocampal neurons and prevents stress-induced deficits in 'what-when-where' memory. *Neurobiology of Learning and Memory, 109C*, 62–73.

Cechetti, F., Worm, P. V., Elsner, V. R., Bertoldi, K., Sanches, E., Ben, J., et al. (2012). Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat. *Neurobiology of Learning and Memory, 97*, 90–96.

Chandra, S., & De Mejia Gonzalez, E. (2004). Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. *Journal of Agriculture and Food Chemistry, 52*, 3583–3589.

Collino, M., Aragno, M., Mastrocola, R., Gallicchio, M., Rosa, A. C., Dianzani, C., et al. (2006). Modulation of the oxidative stress and inflammatory response by PPAR-gamma agonists in the hippocampus of rats exposed to cerebral ischemia/reperfusion. *European Journal of Pharmacology, 530*, 70–80.

da Silveira, C. K., Furini, C. R., Benetti, F., Monteiro Sda, C., & Izquierdo, I. (2013). The role of histamine receptors in the consolidation of object recognition memory. *Neurobiology of Learning and Memory, 103*, 64–71.

Dal-Pizzol, F., Klamt, F., Frota, M. L., Jr., Andrades, M. E., Caregnato, F. F., Vianna, M. M., et al. (2001). Neonatal iron exposure induces oxidative stress in adult Wistar rat. *Brain Research. Developmental Brain Research, 130*, 109–114.

Dal-Pizzol, F., Klamt, F., Vianna, M. M., Schroder, N., Quevedo, J., Benfato, M. S., et al. (2000). Lipid peroxidation in hippocampus early and late after status epilepticus induced by pilocarpine or kainic acid in Wistar rats. *Neuroscience Letters, 291*, 179–182.

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*, 47–59.

Fiorenza, N. G., Rosa, J., Izquierdo, I., & Myskiw, J. C. (2012). Modulation of the extinction of two different fear-motivated tasks in three distinct brain areas. *Behavioural Brain Research, 232*, 210–216.

Hamakawa, M., Ishida, A., Tamakoshi, K., Shimada, H., Nakashima, H., Noguchi, T., et al. (2013). Repeated short-term daily exercise ameliorates oxidative cerebral damage and the resultant motor dysfunction after transient ischemia in rats. *Journal of Clinical Biochemistry and Nutrition, 53*, 8–14.

Haramizu, S., Ota, N., Hase, T., & Murase, T. (2011). Catechins attenuate eccentric exercise-induced inflammation and loss of force production in muscle in senescence-accelerated mice. *Journal of Applied Physiology, 111*, 1654–1663.

Haramizu, S., Ota, N., Hase, T., & Murase, T. (2013). Catechins suppress muscle inflammation and hasten performance recovery after exercise. *Medicine and Science in Sports and Exercise, 45*, 1694–1702.

Hissin, P. J., & Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry, 74*, 214–226.

Jowko, E., Sacharuk, J., Balasinska, B., Wilczak, J., Charmas, M., Ostaszewski, P., et al. (2012). Effect of a single dose of green tea polyphenols on the blood markers of exercise-induced oxidative stress in soccer players. *International Journal of Sport Nutrition and Exercise Metabolism, 22*, 486–496.

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y., & van Praag, H. (2011). Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learning & Memory, 18*, 605–609.

Li, J., Luan, X., Clark, J. C., Rafols, J. A., & Ding, Y. (2004). Neuroprotection against transient cerebral ischemia by exercise pre-conditioning in rats. *Neurological Research, 26*, 404–408.

Lima, A. M., de Bruin, V. M., Rios, E. R., & de Bruin, P. F. (2014). Differential effects of paradoxical sleep deprivation on memory and oxidative stress. *Naunyn-Schmiedeberg's Archives of Pharmacology*.

Loetchutinat, C., Kothan, S., Dechsupa, S., Meesungnoen, J., Jay-Gerin, J., & Mankhetkorn, S. (2005). Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. *Radiation Physics and Chemistry, 72*, 323–331.

Malek, M. H., Huttemann, M., Lee, I., & Coburn, J. W. (2013). Similar skeletal muscle angiogenic and mitochondrial signalling following 8 weeks of endurance

exercise in mice: Discontinuous versus continuous training. *Experimental Physiology, 98*, 807–818.
Marlatt, M. W., Potter, M. C., Lucassen, P. J., & van Praag, H. (2012). Running throughout middle-age improves memory function, hippocampal neurogenesis, and BDNF levels in female C57BL/6J mice. *Developmental Neurobiology, 72*, 943–952.
Mello, P. B., Benetti, F., Cammarota, M., & Izquierdo, I. (2009). Physical exercise can reverse the deficit in fear memory induced by maternal deprivation. *Neurobiology of Learning and Memory, 92*, 364–369.
Mello-Carpes, P. B., & Izquierdo, I. (2013). The nucleus of the solitary tract → nucleus paragigantocellularis → locus coeruleus → CA1 region of dorsal hippocampus pathway is important for consolidation of object recognition memory. *Neurobiology of Learning and Memory, 100*, 56–63.
Mustata, G. T., Rosca, M., Biemel, K. M., Reihl, O., Smith, M. A., Viswanathan, A., et al. (2005). Paradoxical effects of green tea (*Camellia sinensis*) and antioxidant vitamins in diabetic rats: Improved retinopathy and renal mitochondrial defects but deterioration of collagen matrix glycoxidation and cross-linking. *Diabetes, 54*, 517–526.
Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry, 95*, 351–358.
Park, J. W., Jang, Y. H., Kim, J. M., Lee, H., Park, W. K., Lim, M. B., et al. (2009). Green tea polyphenol epigallocatechin gallate reduces neuronal cell damage and up-regulation of MMP-9 activity in hippocampal CA1 and CA2 areas following transient global cerebral ischemia. *Journal of Neuroscience Research, 87*, 567–575.
Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open: Closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods, 14*, 149–167.
Sarkaki, A., Rafieirad, M., Hossini, S. E., Farbood, Y., Motamedi, F., Mansouri, S. M., et al. (2013). Improvement in memory and brain long-term potentiation deficits due to permanent hypoperfusion/ischemia by Grape seed extract in rats. *Iranian Journal of Basic Medical Sciences, 16*, 1004–1010.
Shih, P. C., Yang, Y. R., & Wang, R. Y. (2013). Effects of exercise intensity on spatial memory performance and hippocampal synaptic plasticity in transient brain ischemic rats. *PLoS ONE, 8*, e78163.
Tahamtan, M., Allahtavakoli, M., Abbasnejad, M., Roohbakhsh, A., Taghipour, Z., Taghavi, M., et al. (2013). Exercise preconditioning improves behavioral functions following transient cerebral ischemia induced by 4-vessel occlusion (4-VO) in rats. *Archives of Iranian Medicine, 16*, 697–704.
Tian, S., Zhang, Y., Yang, X., Yu, K., Shen, X., Zhang, L., et al. (2013). Early exercise training improves ischemic outcome in rats by cerebral hemodynamics. *Brain Research, 1533*, 114–121.
Tjolsen, A., Lund, A., Berge, O. G., & Hole, K. (1989). An improved method for tail-flick testing with adjustment for tail-skin temperature. *Journal of Neuroscience Methods, 26*, 259–265.
van Praag, H. (2008). Neurogenesis and exercise: Past and future directions. *Neuromolecular Medicine, 10*, 128–140.
van Praag, H. (2009). Exercise and the brain: Something to chew on. *Trends in Neurosciences, 32*, 283–290.
Vivar, C., Potter, M. C., & van Praag, H. (2013). All about running: Synaptic plasticity, growth factors and adult hippocampal neurogenesis. *Current Topics in Behavioural Neuroscience, 15*, 189–210.
Voss, M. W., Vivar, C., Kramer, A. F., & van Praag, H. (2013). Bridging animal and human models of exercise-induced brain plasticity. *Trends in Cognitive Sciences, 17*, 525–544.
Wendel, A. (1981). Glutathione peroxidase. *Methods in Enzymology, 77*, 325–333.
Wu, K. J., Hsieh, M. T., Wu, C. R., Wood, W. G., & Chen, Y. F. (2012). Green tea extract ameliorates learning and memory deficits in ischemic rats via its active component polyphenol epigallocatechin-3-gallate by modulation of oxidative stress and neuroinflammation. *Evidence-Based Complementary and Alternative Medicine, 2012*, 163106.
Xu, Y., Zhang, J. J., Xiong, L., Zhang, L., Sun, D., & Liu, H. (2010). Green tea polyphenols inhibit cognitive impairment induced by chronic cerebral hypoperfusion via modulating oxidative stress. *Journal of Nutritional Biochemistry, 21*, 741–748.
Yang, X., He, Z., Zhang, Q., Wu, Y., Hu, Y., Wang, X., et al. (2012). Pre-Ischemic treadmill training for prevention of ischemic brain injury via regulation of glutamate and its transporter GLT-1. *International Journal of Molecular Sciences, 13*, 9447–9459.
Zamani, M., Hassanshahi, J., Soleimani, M., & Zamani, F. (2013). Neuroprotective effect of olive oil in the hippocampus CA1 neurons following ischemia: Reperfusion in mice. *Journal of Neurosciences in Rural Practice, 4*, 164–170.