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The effect of NADPH-oxidase inhibitor apocynin on cognitive impairment induced by moderate lateral fluid percussion injury: Role of inflammatory and oxidative brain damage

Ana Paula Oliveira Ferreira [a,b], Fernanda Silva Rodrigues [a,b], Iuri Domingues Della-Pace [a,b], Bibiana Castagna Mota [a,b], Sara Marchesan Oliveira [b], Camila de Campos Velho Gewehr [b], Franciane Bobinski [c], Clarissa Vasconcelos de Oliveira [d], Juliana Sperotto Brum [e], Mauro Schneider Oliveira [d], Ana Flavia Furian [f], Claudio Severo Lombardo de Barros [e], Juliano Ferreira [b], Adair Roberto Soares dos Santos [c], Michele Rechia Fighera [a,b,g], Luiz Fernando Freire Royes [a,b,*]

[a] Laboratório de Bioquímica do Exercício, Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Programa de Pós – Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianólpolis, SC, Brazil
[d] Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[e] Departamento de Patologia, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[f] Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[g] Departamento de Neuropsiquiatria, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

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ABSTRACT

Traumatic brain injury (TBI) is a devastating disease that commonly causes persistent mental disturbances and cognitive deficits. Although studies have indicated that overproduction of free radicals, especially superoxide (O_2^-) derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a common underlying mechanism of pathophysiology of TBI, little information is available regarding the role of apocynin, an NADPH oxidase inhibitor, in neurological consequences of TBI. Therefore, the present study evaluated the therapeutic potential of apocynin for treatment of inflammatory and oxidative damage, in addition to determining its action on neuromotor and memory impairments caused by moderate fluid percussion injury in mice (mLFPI). Statistical analysis revealed that apocynin (5 mg/kg), when injected subcutaneously (s.c.) 30 min and 24 h after injury, had no effect on neuromotor deficit and brain edema, however it provided protection against mLFPI-induced object recognition memory impairment 7 days after neuronal injury. The same treatment protected against mLFPI-induced IL-1β, TNF-α, nitric oxide metabolite content (NO_x) 3 and 24 h after neuronal injury. Moreover, apocynin treatment reduced oxidative damage (protein carbonyl, lipoperoxidation) and was effective against mLFPI-induced Na^+, K^+-ATPase activity inhibition. The present results were accompanied by effective reduction in lesion volume when analyzed 7 days after neuronal injury. These data suggest that superoxide (O_2^-) derived from NADPH oxidase can contribute significantly to cognitive impairment, and that the post injury treatment with specific NADPH oxidase inhibitors, such as apocynin, may provide a new therapeutic approach to the control of neurological disabilities induced by TBI.

1. Introduction

Traumatic brain injury (TBI) is characterized as an insult to the brain caused by an outside force, resulting in skull fractures, intracranial injuries, loss of consciousness and motor functional deficits. The brain damage induced by TBI results from both the primary mechanical impact and secondary degenerative response (Davis, 2000). Secondary mechanisms are potentially amenable to post-injury therapeutic intervention because of their delayed onset and include neuroinflammation, oxidative stress, brain edema and delayed cell death (Lotocki et al., 2009; Toklu et al., 2009; Zhang et al., 2012). It is important to note that the survival rate from TBI has been improved with modern clinical

* Corresponding author. Address: Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, Universidade Federal de Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 3220 9378; fax: +55 55 3220 8241.
E-mail address: nandoroyes@yahoo.com.br (L.F.F. Royes).

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management practices. However many survivors of TBI still display neuronal death and suffer from permanent cognitive impairment (Singh, 2003).

In line with this view, a considerable body of evidence has indicated that oxidative stress and functional deficits occurring after TBI are interrelated events and studies of the mechanisms involved in this interface can open new avenues to better understanding of TBI (Lima et al., 2008; Marklund et al., 2001). Furthermore, it has been demonstrated that ROS production elicited by TBI induces cellular death (Clausen et al., 2004), blood brain barrier breakdown (BBB) (Pun et al., 2009) and failure of energy metabolism after brain injury (Arun et al., 2013; Prins, 2008). The mechanisms of ROS production include mitochondrial breakdown, intracellular calcium overload and increase in activity of pro-oxidant enzymes (Lambert and Brand, 2009; Lewen et al., 2000; Solaroglu et al., 2005; Zhang et al., 2012).

The nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is a pro-oxidant enzyme that catalyzes the reduction of molecular oxygen and oxidation of NADPH to generate superoxide radicals (O_2^-) (Bedard and Krause, 2007; Infanger et al., 2006; Kahles and Brandes, 2012). NADPH oxidase is a multiunit enzyme composed of several subunits that include several isoforms of NOX (NOX 1–5) (Bedard and Krause, 2007). Although few studies have evaluated the role of $gp91^{phox}$ (catalytic subunit of the enzyme, NOX_2) in TBI, experimental evidences suggest that use of specific NADPH oxidase inhibitors may have significant efficacy in the treatment of TBI (Dohi et al., 2010; Lo et al., 2007). A candidate for this is Apocynin, a natural organic compound isolated from the Himalayan medicinal herb *Picrohiza kurroa* used as an efficient inhibitor of the NADPH–oxidase complex (Hayashi et al., 2005; Stolk et al., 1994; Van den Worm et al., 2001). Recently, it was demonstrated that treatment with high doses of apocynin protected against ROS production, microglial activation, BBB disruption and neuronal death after weight drop brain injury in rats (Choi et al., 2012). Moreover, pre- or post-treatment with apocynin protects against early microglial activation, reduces lipoperoxidation and deposition of b-amyloid protein after controlled cortical impact in mice (Zhang et al., 2012). It is important to note that treatment with apocynin prior to diffuse brain injury also attenuates the expression and activation of NOX_2 protein, in addition to reducing brain edema and spatial learning deficit after neuronal injury (Song et al., 2012).

Although there is increasing evidence supporting the role of NADPH oxidase in the pathology of TBI, more detailed studies are required to assess the antioxidant potential of apocynin administration in diseases that present cognitive impairment, such as TBI. For this purpose, we evaluated the effect of apocynin treatment on inflammatory and oxidative damage, and determining its actions on neuromotor and memory impairments caused by moderate fluid percussion injury in mice (mLFPI).

2. Materials and methods

2.1. Materials

Apocynin, bovine serum albumin, ethylenediaminetetraacetic acid disodium salt (EDTA), ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), trichloroacetic acid (TCA), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide, thiobarbituric acid (TBA) 2,4-dinitrophenylhydrazine (DNPH) sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$) and ouabain adenosine triphosphate (ATP) and sodium molybdate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lidocaine hydrochloride and ketamine hydrochloride were obtained from Cristália Produtos Quimicos e Farmacêuticos Ltda (São Paulo, SP, Brazil). Xylazine hydrochloride was obtained from Vallée S.A. – Produtos Veterinários (São Paulo, SP, Brazil) and ceftriaxone sodium was obtained from SEM Sigma Pharma (S. Bernardo do Campo, SP, Brazil).

2.2. Subjects and maintenance

Male Swiss mice (28–32 g) provided by the Animal House of the Federal University of Santa Maria, were maintained in a room with a controlled temperature (24 ± 1 °C),12 h light/dark cycle (lights on at 6:00), and standard laboratory chow and tap water *ad libitum*. We attest that all the experiments were conducted in compliance with the current Brazilian Laws (Law No. 6638 of 1979 – Standards for the Teaching–Scientific Practice of Animal use) and that experiments were previously approved by the Committee on the Use and Care of Laboratory Animals of the Federal University of Santa Maria (process number: 113/2010).

2.3. Experimental design

To determine the best dose of apocynin for the treatment of neuromotor and cognitive deficits induced by mLFPI, the animals were injected subcutaneously (s.c.) with vehicle (5% ethanol in saline solution) or apocynin (at doses of 0.05, 0.5 or 5 mg/kg) 30 min and 24 h after induction of TBI. The neuromotor evaluation was performed at time intervals of 3 h, 6 h, and 1 day after injury. The memory test and histological analyses were performed 7 days after injury, as described in Fig. 1A.

In order to determine whether the treatment effective against memory deficit also protects against biochemical alterations induced by mLFPI, the animals were injected s.c. with vehicle (5% ethanol in saline solution) or apocynin (5 mg/kg) 30 min and 24 h after injury, and biochemical analysis were performed 3 h and 1 day after neuronal injury, as described in Fig. 1B.

2.4. Traumatic brain injury

The moderate lateral fluid percussion injury (mLFPI) was induced according to the method of Carbonell et al. (1998). Briefly, mice were anesthetized with a mixture of xylazine (10 mg/kg)/ketamine (100 mg/kg) and placed in a stereotaxic head holder. The scalp was reflected and the skull exposed through a midline incision. A topical anesthetic 2% lidocaine hydrochloride was applied to the skull and the fascia was scraped from the skull. A burr hole of 3 mm in diameter was drilled in the right parietal bone between bregma and lambda and between the sagittal suture and lateral ridge over the right hemisphere, taking care to keep the dura mater intact. A plastic cannula was placed over the craniotomy with dental cement. When the dental cement hardened the cannula was filled with 4% chloramphenicol and closed with a proper plastic cap. At 24 h after the surgical procedure, the animals were anesthetized with Isoflurane, the injury cannula was attached to the fluid percussion device, and the animals were placed on a heat pad maintained at 37 ± 0.2 °C. TBI was produced by a fluid-percussion device developed in our laboratory. A brief (10–15 ms) transient pressure fluid pulse (1.8 ± 0.41 atm) impact was applied against the exposed dura. Pressure pulses were measured extracranially by a transducer (hydraulic fluid control, Belo Horizonte, MG, Brazil) and recorded on a storage oscilloscope (Tektronix TDS 210). Sham-operated animals underwent an identical procedure, with the exception of mLFPI. Immediately after these procedures, the cannula was topped with dental cement. The time elapsed until the animal spontaneously righted itself was recorded as an acute

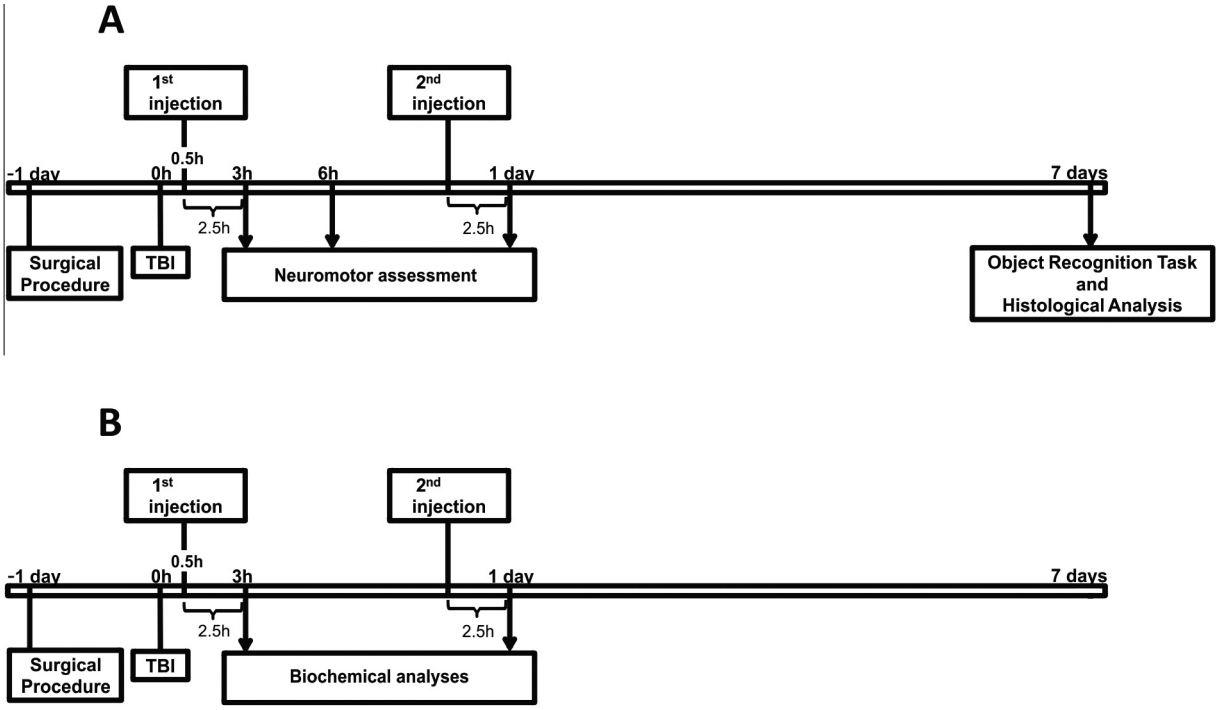

Fig. 1. (A) Representation of experimental design with the effect of apocynin treatment (0.05, 0.5 and 5 mg/kg) on neuromotor assessment 3, 6 and 1 day after mLFPI (B). The effect of apocynin (5 mg/kg, s.c.) on biochemical alterations 3 h and 1 day after neuronal injury. The neuromotor and biochemical analysis were realized 2.5 h after the drug administration. The objected recognition task and histologic analysis were determined 7 days after neuronal injury.

neurological assessment, and defined as the righting reflex time (or unconsciousness). The TBI caused immediate unconsciousness that lasted for 219.3 ± 7.0 s, demonstrating that injury induced by LFPI was within the range of moderate injury (Hosseini and Lifshitz, 2009; Morehead et al., 1994). All animals received ceftriaxone (200 mg/kg, i.p.) immediately after surgery and after trauma. To reduce the unspecific effect of antibiotic treatment all sham and traumatized animals tested in this study received the same antibiotic therapy. Although, there is no available literature indicating pharmacokinetic interactions between apocynin and ceftriaxone, it is important to note that ceftriaxone has anti-inflammatory and anti-apoptotic properties (Romano et al., 2004).

2.5. Assessment of neuromotor function

The neuromotor function was assessed at time intervals of 3, 6 h and 1 day after injury. These time points were selected from a time course performed in another study, not yet published, in which the TCE caused neuromotor impairment only at the mentioned times. The neuromotor function was determined by means of a neuroscore test, as described by Raghupathi et al. (1998). Briefly, animals were subjected to a grid-walk test for 1 min in order to asses of the number of foot-faults. Subsequently, forelimb and hindlimb functions were evaluated by suspending the animals by the tail and observing how they grasped the top of the cage when they were lowered toward it (to test the forelimbs) and the same procedure was used for hindlimb spreading and extension during suspension (to test the hindlimbs). Finally, animals were tested for both right and left resistance to lateral pulsion. Animals were scored from 0 (severely impaired) to 4 (normal) for each of the following indices: fore-limb function, hind-limb function and resistance to lateral pulsion. The maximum score for each animal was 12. Evaluation of neurological motor function was conducted by an experienced investigator who was blinded to all groups.

2.6. Evaluation of object recognition task (ORT) and elevated plus maze

The objects to be discriminated were figures of similar size and texture (8–10 cm high). The objects were selected on the basis of previous observations, which demonstrated a lack of preferential exploration for one object (A) over the other (B) [exploration of *A* in seconds was 16.6 ± 3.44 and exploration of *B* was 16.8 ± 4.09; $t(10) = 0.159$; $p > 0.05$]. To avoid olfactory stimuli, the objects to be discriminated were cleaned carefully initially and washed with an ethanol solution (30% in water) after each individual session. The testing arena was a plastic box (width: 20 cm; length: 30 cm; height: 15 cm, with the floor divided into 24 squares measuring 5 × 5 cm each) that was dimly illuminated so that the environment was visually uniform. Twenty-four hours before testing, the mice were allowed to explore the testing box for 10 min to reduce neophobic responses and habituate to the stimuli present in the empty arena. During this habituation, the open-field test was performed. This test was carried out to identify motor disabilities and lasted for 10 min. During this time, an observer, who was not aware of pharmacological treatments, manually recorded the number of crossing and rearing responses. In the first trial two identical objects were placed in the box. Mice were placed in the box for 5 min and exploratory activity was manually recorded. After a delay of 4 h, mice were re-introduced into the same cage in which one of the objects was replaced by a new one, for 5 min. The cumulative time spent by the mouse at each of the objects was manually recorded by an observer, who was not aware of pharmacological treatments. Exploration of an object was defined as follows: directing the nose to the object or touching it with the nose; turning around or sitting on the object was not considered as exploratory behavior. The percentage of the total exploration time that the animal spent investigating the novel object was the measure of recognition memory, defined by the discrimination index (DI). This was calculated using the following formula: (time spent in investigating the new object) – (time spent in

investigating the known object)/time spent in investigating both objects × 100.

After ORT, the animals were tested on the elevated plus-maze. The apparatus comprises two open arms (25 × 5 cm) across from each other and perpendicular to two closed arms (25 × 5 × 15 cm) with a center platform (5 × 5 cm). The apparatus is made of wood, the platform is white and the walls are transparent acrylic. The entire apparatus is raised 50 cm above the floor. Initially, subjects were placed on the center platform of the maze facing an enclosed arm. During the test the mice are allowed to move freely about the maze for 10 min. The percentage of entries into each arm, the percentage of time spent in each arm, and the percentage of time spent in central platform are recorded and these measurements serve as an index of anxiety-like behavior as described by (Komada et al., 2008). After each trial, all arms and the center area were cleaned with 30% ethanol solution, to prevent a bias based on olfactory cues.

2.7. Cytokine immunoassay

The interleukin (L-1β) and tumor necrosis factor-α (TNF-α) content were determined in ipsilateral cortex homogenate containing bovine serum albumin (BSA 10 mg/ml), 2 mM EGTA, 2 mM EDTA and 0.2 mM PMSF in phosphate buffered saline (PBS 0.1 M, pH7.4). Cytokine levels were measured using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA), in accordance with the manufacturer's protocol. The concentration of cytokines was normalized to the protein concentration contained in the samples. The results were expressed in pg/mg of protein.

2.8. Assay of NO_x (NO_2 plus NO_3) as a marker of NO synthesis

For NO_x determination, an aliquot of 100 μL of ipsilateral homogenates containing 2 mM EGTA, 2 mM EDTA and 0.2 mM PMSF in phosphate buffered saline was added to acetonitrile (96%, HPLC grade). Afterwards, the homogenate was centrifuged at 3000*g* for 30 min at 4 °C and the supernatant was separated for the analysis of the NO_x content as described by Miranda et al. (2001). The resulting pellet was suspended in NaOH (6 M) for protein determination by the Bradford method and the concentration of NO_x was normalized to the protein concentration contained in the samples. The results were expressed in nmol of NO_x/mg of protein.

2.9. Evaluation of brain edema by water content

To determine the ipsilateral water content a subset of animals was sacrificed 3 h and 1 day after trauma. The ipsilateral hemispheres were immediately removed. After obtained wet weight (WW) of fresh brain, tissue samples were dried in a desiccating oven at 105 °C for 24 h and weighed to obtain the dry weight (DW). Tissue water content (%) was calculated as (WW−WD)/WW × 100 according to Zweckberger et al. (2006).

2.10. Measurement of thiobarbituric acid reactive species (TBARS)

The TBARS content was estimated by the method of Ohkawa et al. (1979). Briefly, the ipsi lateral cortex homogenates containing BHT (80 μM) were incubated in a medium containing 30 μL cortex homogenate, 20 μL of 8.1% sodium dodecyl sulfate (SDS), 80 μL buffered acetic acid (500 mM, pH 3.4), and 150 μL of 0.8% thiobarbituric acid (TBA). The mixture was made up to 400 μL with type I ultrapure water and heated to 95 °C for 60 min in water bath using a glass ball as condenser. The absorbance of each sample was measured in the supernatant at 532 nm in a Hitachi U-2001 spectrophotometer (Hitachi Instruments Incorporation, Schaumburg, IL, USA).

2.11. Measurement of protein carbonyl content

The total protein carbonyl content was determined by the method described by Yan et al. (1995) and adapted for brain tissue by Schneider Oliveira et al. (2004). Briefly, ipsilateral cortex homogenates were adjusted to 759–800 μg of protein/ml in each sample and 500 μL aliquots were mixed with 0.1 ml 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.1 ml HCl (2 M). After incubation at room temperature for 1 h in a dark environment, 250 μL denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1 ml heptane (99.5%) and 1 ml ethanol (99.8%) were added sequentially and mixed under vortex agitation for 40 s and centrifuged for 15 min. Afterwards, the protein isolated from the interface was twice washed with 1 ml ethyl acetate/ethanol 1:1 (v/v) and suspended in 500 μL ml denaturing buffer. Each DNPH sample was read at 370 nm in a Hitachi U-2001 spectrophotometer against the corresponding HCl sample (blank) and total carbonylation was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}, as described by (Levine et al., 1990).

2.12. Na^+, K^+-ATPase activity measurement

Na^+, K^+-ATPase activity was measured according to Silva et al. (2013). Briefly, the reaction medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl2, and 50 μg protein in the presence or absence of ouabain (1 mM) to a final volume of 350 μL. The reaction was started by adding adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37 °C, reaction was stopped by adding 70 μL trichloroacetic acid (TCA, 50%). Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow and the Na^+, K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain).

2.13. Evaluation of the injured cortical volume

After the elevated plus maze test, the mice were killed for histologic analysis. Under deep anesthesia (ketamine hydrochloride, 200 mg/kg, i.p.) they were transcardially perfused with 100 mL of heparinized saline (1000 UI/ml) followed by 100 mL of formaldehyde (4%) in PBS (0, 1 M), then the brains were carefully removed from the skull. The measurement of lesion volume was performed according to Ziebell et al. (2011) with some modifications. Briefly, six sections from bregma levels −0.5 to −3 mm, spaced 500 μm apart, were stained with hematoxylin and eosin, and digitally photographed using a stereomicroscope (Olympus BX51) with a digital camera (Olympus DP25). The peripheries of the lesion were traced on each image according Fig. 8B–E by a pathologist with help of a microscope at 4× magnification. The area of cortical lesion was calculated using calibrated image analysis software program Image J (NIH, Bethesda, MD, USA). Volumes were calculated using the formula $\Sigma(An + An + 1) \times d/2$ where A is the lesioned area in each section and, d is the distance between sections.

2.14. Data analysis

Data were expressed as means ± S.E.M. or median ± interquartile range. Data were analyzed by one, two or three way analysis of variance (ANOVA) or by non-parametric tests such as Kruskal–Wallis or Friedman tests depending on the experimental design.

Post-hoc analyses were performed with the Newman–Keuls test, or Dunn test when appropriate. $p < 0.05$ was considered significant.

3. Results

3.1. Apocynin has no statistically significant effect on neuromotor deficit induced by mLFPI in mice

To assess whether inhibition of NOX_2 by apocynin alters the neurological disabilities resulting from mLFPI, we injected the mice with vehicle or apocynin (0.05, 0.5 and 5 mg/kg; s.c.) 30 min and 24 h after mLFPI induction and their neuromotor function were tested at time intervals of 3 and 6 h and 1 day (test performed 2 h and 30 min after last drug injection) after trauma induction. The result of this experiment is shown in Fig. 2A. The Friedman test revealed that mLFPI induced neuromotor damage over time [$F(5) = 13.86$; $p < 0.001$]. A trend, although without statistical significance, was noted towards attenuation of the neuroscore deficit in animals treated with TBI/apocynin 0.5 mg/kg and in TBI/apocynin 5 mg/kg in comparison with TBI/vehicle-treated mice. The post hoc analysis performed at each time point is shown in Fig. 2B–D.

3.2. Apocynin post-treatment is effective in reducing the object recognition deficit induced by mLFPI in mice

The effect of different doses of apocynin in ORT is demonstrated in Fig. 3. Our result showed that apocynin 0.05 mg/kg had no effect on memory impairment caused by mLFPI. On the other hand, the dose of 0.5 mg/kg demonstrated only a trend towards reversal of memory deficit, although without statistical significance. However, the statistical analysis also revealed that DI reduction caused by trauma was significantly reversed by Apocynin at the dosage of 5 mg/kg [$F(5, 31) = 4.708$; $p < 0.01$; Fig. 3]. To exclude the effect of locomotor and exploratory disabilities on ORT we performed the open-field test as shown in Table 1. No statistically significant difference was found between the tested groups in crossing [$F(5,31) = 1.271$; $p = 0.314$] or rearing responses [$F(5,31) = 1.708$;

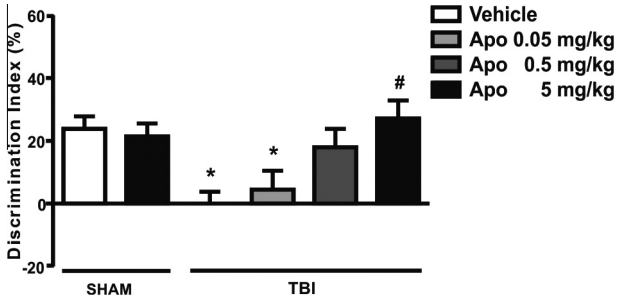

Fig. 3. Effect of apocynin (0.05, 0.5 and 5 mg/kg; s.c.) on recognition memory impairment induced by mLFPI 7 days after injury. Values represent mean ± S.E.M. for $n = 6–7$ per group. $^{*}P < 0.05$ compared with Sham/vehicle group. $^{\#}P < 0.05$ compared with TBI/vehicle group (Newman–Keuls multiple comparison test).

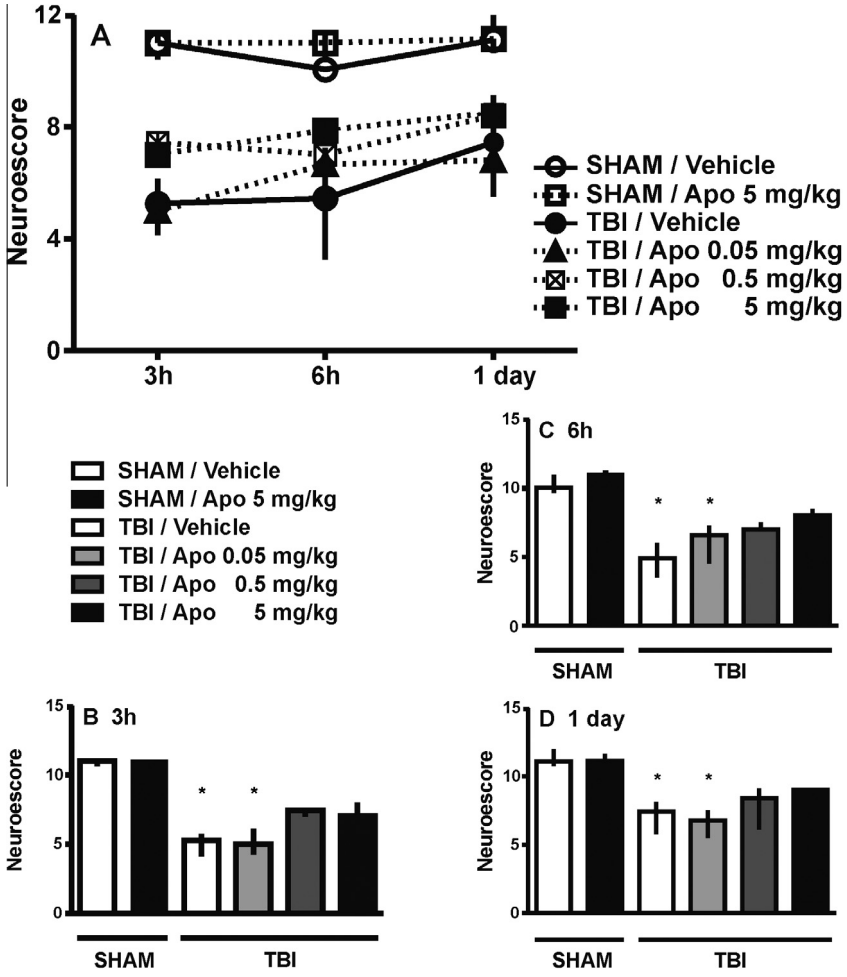

Fig. 2. Effect of apocynin (0.05, 0.5 and 5 mg/kg; s.c.) on neuromotor impairment induced by mLFPI performed 3, 6 h and 1 day after injury. The post hoc analysis performed at each time point is shown in Fig. 2B–D. Values represent median and interquartile for $n = 6–7$ per group. $^{*}P < 0.05$ compared with sham group (Dunn's multiple comparison test).

Table 1
Locomotor and exploratory activity 7 days after mLFPI.

Groups	Crossing	Rearing	N
Sham/vehicle	158.0 ± 20.1	59.4 ± 2.1	6
Sham/Apo 5 mg/kg	150.3 ± 19.5	57.0 ± 5.5	6
TBI/vehicle	182.4 ± 6.1	72.2 ± 6.1	6
TBI/Apo 0.05 mg/kg	194.4 ± 12.9	78.8 ± 4.3	6
TBI/Apo 0.5 mg/kg	146.8 ± 17.6	50.8 ± 12.7	6
TBI/Apo 5 mg/kg	162.6 ± 16.1	60.2 ± 6.4	7

$p = 0.162$]. To exclude the effects of anxiety on memory, we analyzed whether apocynin treatments alter anxiogenic like behavioral as shown in Table 2. No significant difference was found between the tested groups in percentage of time spent in open arms [$F(5,31) = 0.3043$; $p = 0.906$], in percentage of number of entries in open arms $F(5,31) = 0.7371$; $p = 0.601$], in percentage of time spent in enclosed arms $F(5,31) = 0.8171$; $p = 0.547$], in percentage of number of entries in enclosed arms $F(5,31) = 0.7371$; $p = 0.601$], in percentage of time spent in the central area $F(5,31) = 1.489$; $p = 0.2219$].

3.3. Apocynin attenuates the acute inflammatory response induced by mLFPI

To evaluate whether the dose of apocynin 5 mg/kg, would also be effective in reducing the mLFPI-induced inflammation in the ipsi lateral cortex, we subjected the mice to the same protocol treatment and investigated the brain edema and IL-1β, TNF-α and NO_x content in the ipsilateral cortex at time points of 3 h and 1 day after injury. The results shown in Fig. 4, revealed that mLFPI induced an increase in ipsi lateral levels of IL-1β [$F(1,44) = 28.41$; $p < 0.001$; Fig. 4A], TNF-α [$F(1,44) = 9.62$; $p < 0.01$; Fig. 4B], NO_x [$F(1,44) = 33.66$; $p < 0.001$; Fig. 5C] and water content [$F(1,32) = 62.56$; $p < 0.001$; Fig. 4D]. Notably, the levels of IL-1β [$F(1,44) = 4.19$; $p < 0.05$] and NO_x [$F(1,44) = 11.67$; $p < 0.01$] obtained at 1 day were higher than the values obtained at 3 h, but no effect of time on TNF-α levels was found. On the other hand, contrary to that which we expected, the water content found at 3 h was greater than that found at 1 day after injury [$F(1,32) = 7.28$; $p < 0.05$]. The apocynin (5 mg/kg; s.c.) treatment also significantly attenuated mLFPI-induced increase in NO_x content [$F(1, 44) = 8.11$; $p < 0,01$; Fig. 4C], reduced the IL-1β [$F(1,44) = 4.23$; $p < 0.05$; Fig. 4A] and TNF-α content

Table 2
Anxiolitic or anxiogenic like behavior 7 days after mLFPI.

Group	% T.O.	% No. E.O.	% T.E.	% No. E.E.	% T.M.	N
Sham/vehicle	10.3 ± 3.2	21.3 ± 3.1	76.3 ± 4.0	78.7 ± 3.1	13.4 ± 2.1	6
Sham/Apo 5 mg/kg	13.1 ± 2.4	28.0 ± 2.8	66.1 ± 2.8	72.0 ± 2.8	20.9 ± 3.6	6
TBI/vehicle	14.0 ± 3.0	28.4 ± 4.8	71.0 ± 4.4	71.6 ± 4.8	15.2 ± 3.1	6
TBI/Apo 0.05 mg/kg	11.3 ± 1.8	17.9 ± 4.0	70.8 ± 2.5	82.1 ± 4.0	17.9 ± 3.2	6
TBI/Apo 0.5 mg/kg	12.4 ± 4.4	26.2 ± 6.7	67.8 ± 4.6	73.8 ± 6.7	19.9 ± 3.9	6
TBI/Apo 5 mg/kg	15.2 ± 4.1	24.9 ± 5.3	72.7 ± 4.5	75.1 ± 5.3	12.1 ± 1.4	7

% T.O. percent of time spent in open arms; % No. E.O. percent of number of entries in open arms; % T.E. percent of time spent in enclosed arms; % No. E.E. percent of number of entries in enclosed arms. % T.M. percent of time spent in the central area.

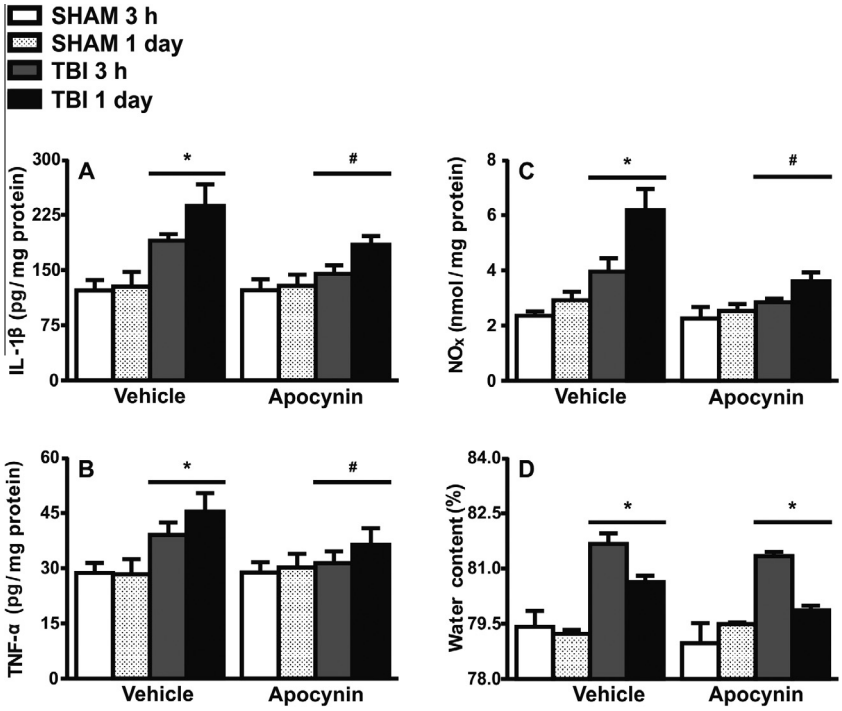

Fig. 4. Apocynin (5 mg/kg, s.c.) attenuates the mLFPI-induced increase in IL-1β (A), TNF-α (B) and NO_x (C), but have no effects on water content (D) 3 h and 1 day after brain lesion. Values represent mean ± S.E.M. for $n = 5–7$ per group. $^{*}P < 0.05$ compared with Sham/vehicle groups. $^{\#}P < 0.05$ compared with TBI/vehicle groups (three way Anova).

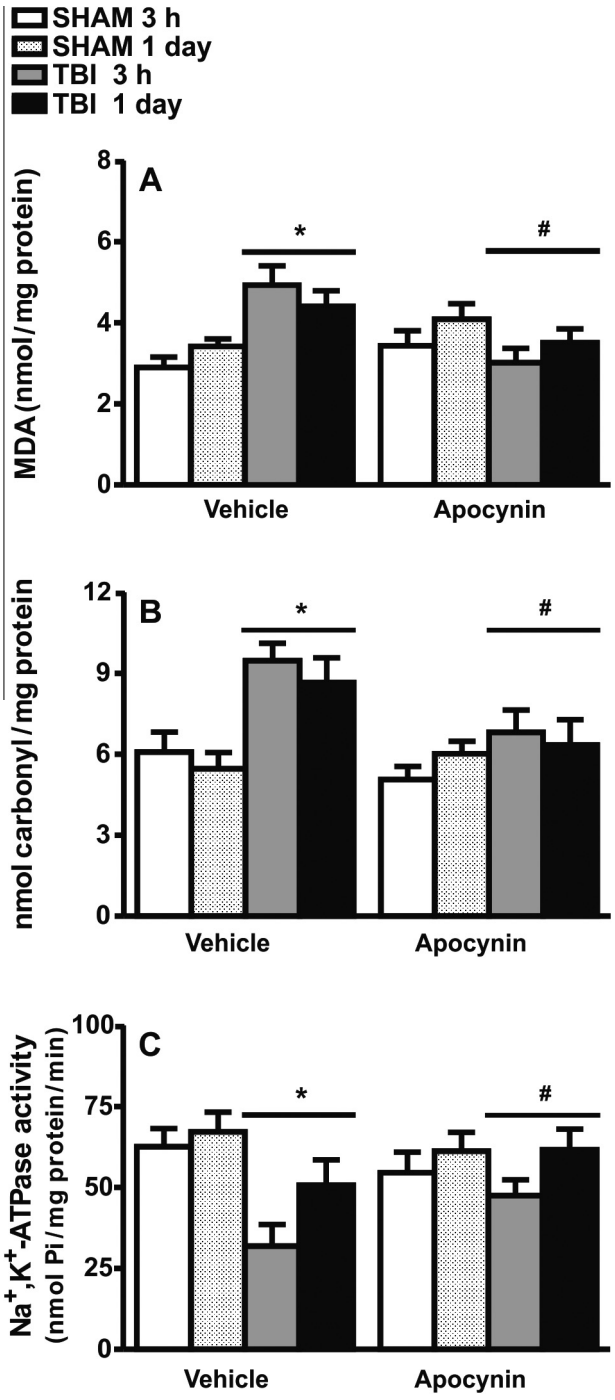

Fig. 5. Apocynin (5 mg/kg, s.c.) attenuates the mLFPI-induced increase in TBARS (A); protein carbonyl content (B) and inhibition of Na^+, K^+, ATPase activity (C) 3 and 24 h after neuronal injury. Values represent mean ± S.E.M. for $n = 7$ per group. $^*P < 0.05$ compared with Sham/vehicle groups. $^\#P < 0.05$ compared with TBI/vehicle groups (three way Anova).

[$F(1,44) = 4.50$; $p < 0.05$; Fig. 4B]. It was interesting to note that, apocynin did not significantly decrease the brain edema resulting from mLFPI [$F(1,32) = 1.32$; $p > 0.05$; Fig 4D].

3.4. Effect of apocynin on oxidative damage

It is well known that NADPH oxidase is a pro-oxidant enzyme, and this is why we decided to evaluate the effects of inhibiting it with apocynin, on the oxidative stress caused by trauma, as is demonstrated in Fig. 5. The statistical analyses showed that mLFPI increased the content of protein carbonyl [$F(1,48) = 17.99$; $p < 0.001$; Fig. 5B), TBARS [$F(1,48) = 3.58$; $p < 0.05$; Fig. 5A], in addition to decreasing Na^+, K^+ ATPase activity [$F(1,48) = 9.13$; $p < 0.01$, Fig. 5C], an enzyme sensitive to oxidative stress (Lima et al., 2008; Mota et al., 2012; Souza et al., 2009). In contrast with the results of the inflammatory parameters, we did not find any effect of time on TBARS [$F(1,48) = 1,31$; $p > 0.05$] or on protein carbonyl content [$F(1,48) = 0.21$; $p > 0.05$). However, the inhibition of Na^+ K^+ ATPase activity at 3 h was higher than it was at 1 day after trauma [$F(1,48) = 6,18$; $p < 0.05$]. Our data also demonstrated that apocynin (5 mg/kg; s.c.) attenuated the increase in TBARS [$F(1,48) = 16.36$; $p < 0.001$; Fig. 5A] and in protein carbonyl [$F(1,48) = 4.91$; $p < 0.05$; Fig. 5B] induced by mLFPI. This treatment was also able to attenuate Na/K ATPase inhibition, an enzyme sensitive to oxidative stress, induced by mLFPI [$F(1,48) = 5.24$; $p < 0.05$; Fig. 5C].

3.5. Apocynin reduces the cortical lesion volume

It is well known that acute inflammatory and oxidative brain damage contribute to cell death after TBI. Thus, we decided to evaluate whether the Apocynin treatment also reduces the brain lesion volume 7 days after injury. Statistical analyses demonstrated that mLFPI induced a large cortical injury [$F(1,20) = 54.12$; $p < 0.0001$; Fig. 6A], as shown by the representative figures Fig. 6D (TBI/vehicle; 15.2 ± 1.8) vs. Fig. 6B (SHAM/vehicle; 2.8 ± 0.6 mm^3). The treatment with Apocynin (5 mg/kg) significantly reduced the lesion volume induced by trauma [$F(1,20) = 4.711$; $p < 0.05$; Fig. 6A] as demonstrated by representative Fig. 6E (TBI/Apocynin; 8.2 ± 1.8 mm^3). The treatment with apocynin also decreased the small manipulation lesion present in sham/vehicle mice [$F(1,20) = 10.54$; $p < 0.005$]. The stereological analysis of lesions also demonstrated that mLFPI induced a marked loss of gray matter and a perilesional area with several necrotic neurons. In some cases, there was loss of white matter and moderate inflammatory infiltrate composed primarily of neutrophils and hemorrhage. On the other hand, in the sham animals we observed a focal and slight loss of gray matter characterized by a moderate dimple in the neural parenchyma. Differently from groups TBI/vehicle, the traumatized animals treated with apocynin showed a smaller level of hemorrhage, inflammatory infiltrate and necrosis of neural tissue. Fig. 7E–G shows representative images of other animals that also corroborate the above mentioned stereological analysis. Image 7E demonstrated the hemorrhagic focus in a TBI/vehicle mouse. Image 7F shows a TBI/vehicle mouse that contains neutrophil infiltration. Image 7G corresponds to a TBI/Apocynin animal and demonstrates the protective effects of apocynin, characterized by a minor hemorrhage, inflammatory infiltrate and necrosis of neural tissue (G). These results corroborate the deleterious role of NADPH oxidase in the inflammation and neurodegeneration induced by mLFPI.

4. Discussion

The results presented in this report revealed that treatment with Apocynin, an efficient inhibitor of the NADPH–oxidase complex, promoted protection against mLFPI-induced cognition impairment but had no effect on neuromotor dysfunction. It is important to note that all treatments used in this study did not alter locomotor and/or anxiety-like behavior when analyzed 7 days after mLFPI, suggesting that the effects elicited by apocynin cannot be attributed to locomotor and/or anxiogenic-like behavior. Neurochemical analysis also demonstrated that apocynin administration reduced the cytokine release (IL-1β and TNF-α) and oxidative damage induced by mLFPI, characterized here by the increase in NO_x, TBARS and protein carbonyl content. Moreover, the protection

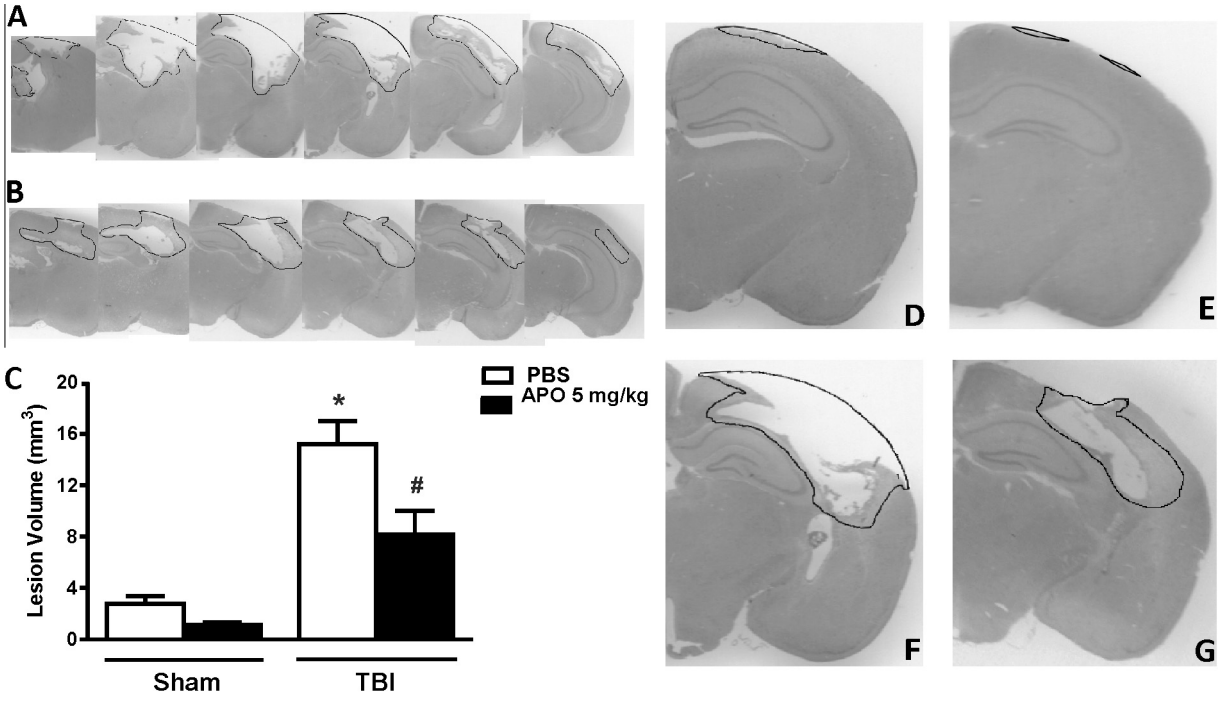

Fig. 6. Apocynin (5 mg/kg, s.c.) attenuates the mLFPI-induced brain lesion. The various analyzed bregma levels to the rostral/caudal lesion distribution of TBI/vehicle (A) and of TBI/Apocynin (B). Effects of apocynin lesion volume (mm^3) induced by mLFPI (C). Representative images of groups Sham/vehicle (D) Sham/Apocynin (E) TBI/vehicle (F) and TBI/Apocynin (G). Values represent mean ± S.E.M. for $n = 6$ per group. $^{*}P < 0.05$ compared with Sham/vehicle group. $^{\#}P < 0.05$ compared with TBI/vehicle group (two way Anova).

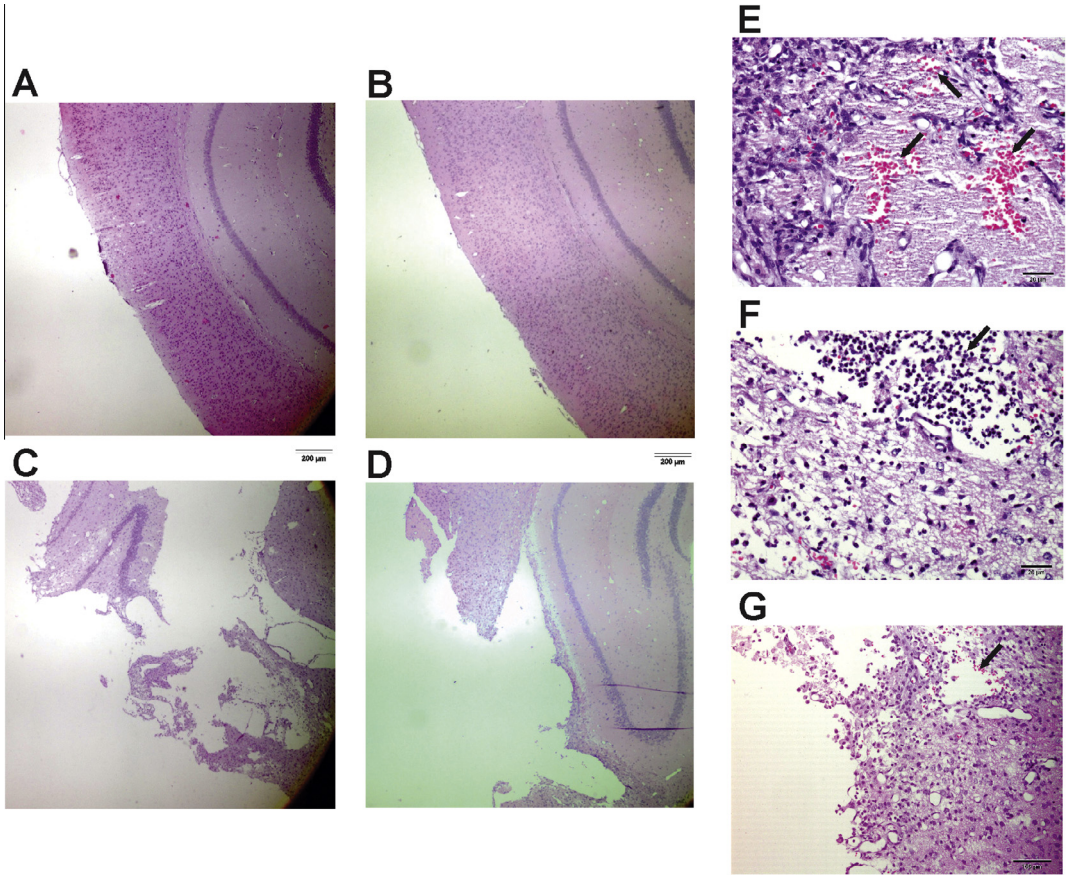

Fig. 7. Effect of apocynin on TBI-induced damage in the cerebral cortex. H&E staining demonstrates the anatomical and cellular differences between groups. Sham/vehicle (A); Sham/Apocynin (B); TBI/vehicle (C); TBI/Apocynin (D); image of a TBI/vehicle mouse showing hemorrhagic focus (E); image of a TBI/vehicle mouse showing neutrophil infiltration in TBI/vehicle mice (F); image of a TBI/Apocynin mouse showing minor hemorrhage, inflammatory infiltrate and necrosis of neural tissue (G).

observed against mLFPI-induced Na^+, K^+-ATPase activity inhibition induced by acute apocynin treatment reinforces the idea that the failure of some selected targets, caused by free radical attack, may increase cellular damage in this model of TBI. Thus, our data revealed that mLFPI induced a large cortical injury and that the treatment with apocynin significantly reduced the lesion volume induced by trauma. Although the antibiotic therapy used in this study had anti-inflammatory and anti-apoptotic properties (Romano et al., 2004), the positive results obtained with apocynin cannot be attributed to the antibiotic, since control animals received de same treatment.

In CNS, the NADPH oxidase plays an important role in the normal cellular processes of neurons, such as long-term potentiation (LTP) and hippocampus-dependent memory (Infanger et al., 2006). On the other hand, under pathological conditions, an overactivation of NADPH oxidase contributes significantly to the pathology of TBI via a complex cascade of biochemical events that lead to neuroinflammation, oxidative stress and delayed cell death (Harting et al., 2008; Lotocki et al., 2004). There are experimental evidences that corroborate this sentence, which indicate the effective involvement of NADPH oxidase-dependent ROS generation in the activation of transcriptors, (nuclear factor-κB (NF-κB), pro-inflammatory cytokine and prostaglandin E2 production in microglial cells (Yang et al., 2007) as well as the microglial proliferation induced by pro-inflammatory cytokines (Mander et al., 2006). Furthermore, it has been shown that the inhibition of NADPH oxidase-dependent redox signaling by apocynin significantly decreases the iNOS expression in microvascular endothelial cells (Wu et al., 2008), and that $gp91^{phox}$ knockout mice have a reduced up regulation of iNOS after ischemic stroke (Chen et al., 2011). Likewise, our results showed that acute treatment with apocynin was effective against the increase in IL-1β, TNF-α and NO_x when analyzed at time intervals of 3 and 24 h after brain injury. These data suggest that the reduction in ROS content resulting from NADPH oxidase inhibition affected the intracellular signaling that triggers the production of inflammatory mediators.

In the present study we revealed for the first time that an alteration in the profile of cerebral inflammatory status induced by apocynin limits long-term secondary degeneration, here characterized by cognitive impairment. The apocynin treatment also protected against mLFPI-induced protein carbonylation, increase in TBARS and Na^+, K^+-ATPase inhibition. Considering that the Na^+, K^+-ATPase enzyme is especially sensitive to oxidative stress (Lima et al., 2009, 2008; Souza et al., 2009) and its inhibition induces spatial learning deficits (Zhan et al., 2004) it is possible that NADPH oxidase ROS production is involved in the decrease in Na^+, K^+-ATPase activity and thereby contributes to later cognitive deficit elicited by mLFPI.

It has long been known that the Na^+, K^+-ATPase activity is particularly sensitive to reactive species, which alter plasma membrane lipid composition (Jamme et al., 1995) and the redox state of regulatory sulfhydryl groups (Morel et al., 1998). In this context, both oxidative and nitrosative stress induce protein carbonylation (Dean et al., 1997) which is related to increased susceptibility to proteolysis and marked impairment of protein functionality (Dalle-Donne et al., 2006). From the biochemical point of view, the most important consequence of oxidative modification is that Na^+, K^+-ATPase molecules have lost the ability to interact with one another, decreasing oligomeric structure formation and leading to subsequent suppression of the rate of hydrolysis (Dobrota et al., 1999). Thus, the effective protection exerted by apocynin against mLFPI-induced Na^+, K^+-ATPase inhibition provides a possible mechanism by which the early blockade of NADPH oxidase prevents the progression of secondary brain damage. Corroborating this hypothesis, we performed a histological analysis 7 days post trauma (the time in which memory deficits were developed). Our experimental findings showed that mLFPI induced neural damage here characterized by an increase in lesion volume, and that the treatment with apocynin was effective in reducing lesion volume when analyzed 7 days after injury. The beneficial effects of this drug were also observed on the small manipulation lesion present in sham animals, reinforcing the protective action of this drug on general brain injuries. These results corroborate those of previous studies that have shown the significant role of NADPH oxidase in ROS generation, oxidative stress damage and neuronal cell death following TBI (Choi et al., 2012). Furthermore, the ability of apocynin to reduce neuronal cell death, neurological impairment and mortality in the stroke studies reinforces the assumption that the beneficial effects of Apocynin are specially due to the inhibition of NADPH oxidase (Jackman et al., 2009).

It is interesting to note that we did not verify any protective effect of apocynin on brain water content or against neuromotor disability induced by mLFPI. The absence of beneficial results with treatment with apocynin in brain edema could be indicative that NADPH oxidase activity is not involved in the development of brain edema induced by TBI. Although, there are experimental findings demonstrating that apocynin (5 mg/kg; i.p.) and gp91ds-tat treatment (another NADPH oxidase inhibitor) reduces brain edema induced by cold brain injury and controlled cortical impact, respectively (Jinnouchi et al., 2007; Zhang et al., 2012) our results are in agreement with other previous studies that did not show any reduction in brain water content with pre- or post-injury treatments with apocynin in different brain injury models (Jackman et al., 2009; Lo et al., 2007; Tang et al., 2008). One possible reason for these discrepant results is the low doses used in present study and the different methodologies used. Therefore, further studies are necessary to elucidate the involvement of NADPH oxidase in the development of brain edema after TBI. The results presented in this report also revealed that Apocynin treatment did not alter transient motor dysfunction induced by mLFPI suggesting that in this trauma model, the NADPH oxidase system is pathologically relevant especially at more advanced stages when degenerative processes prevail. It is important to note that role of the enzyme NADPH oxidase in the development of neuromotor deficit induced by brain injury continues to be controversial. While some authors have suggested that apocynin shows beneficial effects when administered before ischemia (Jackman et al., 2009; Tang et al., 2008) other studies obtained with gp91phox knockout mice have demonstrated a harmful role (Tang et al., 2005) or no involvement of this enzyme in neuromotor damage (Liu et al., 2007). In a model of surgically-induced brain injury, the $gp91^{phox}$ knockout animals, but not the apocynin-treated mice demonstrated improved neurological scores (Lo et al., 2007). Conversely, in another model of traumatic brain injury (controlled cortical injury) the authors obtained improvements with apocynin (5 mg/kg; i.p) treatment in neuromotor deficit and only a trend toward improvements in spatial learning (Loane et al., 2013). Although the reason for these disagreements cannot be established, the differences between experimental models, as well as diversity of treatment protocols (low doses, different time intervals of evaluation and different routes of administration) may determine these contradictions.

5. Conclusions

In summary, in the present study we showed that a single episode of mLFPI induced neurological dysfunction characterized by early neuromotor dysfunction, neuroinflammation, oxidative stress generation and Na^+, K^+-ATPase activity inhibition. These modifications induced by mLFPI were followed by cognitive impairment and brain lesion when analyzed 7 days after injury. Our data also revealed that early treatment with Apocynin reduced

secondary brain damage and neurological deficits, here characterized by a reduction in recognition memory. Thus, the present study indicates that post injury treatment with specific NADPH oxidase inhibitors, such as apocynin, may provide a new therapeutic approach to the control of neurological disabilities induced by TBI.

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