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Previous physical exercise alters the hepatic profile of oxidative-inflammatory status and limits the secondary brain damage induced by severe traumatic brain injury in rats

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Key points

- An early inflammatory response and oxidative stress are implicated in the signal transduction that alters both hepatic redox status and mitochondrial function after traumatic brain injury (TBI).
- Peripheral oxidative/inflammatory responses contribute to neuronal dysfunction after TBI
- Exercise training alters the profile of oxidative-inflammatory status in liver and protects against acute hyperglycaemia and a cerebral inflammatory response after TBI.
- Approaches such as exercise training, which attenuates neuronal damage after TBI, may have therapeutic potential through modulation of responses by metabolic organs.
- The vulnerability of the body to oxidative/inflammatory in TBI is significantly enhanced in sedentary compared to physically active counterparts.

Abstract Although systemic responses have been described after traumatic brain injury (TBI), little is known regarding potential interactions between brain and peripheral organs after neuronal injury. Accordingly, we aimed to investigate whether a peripheral oxidative/inflammatory response contributes to neuronal dysfunction after TBI, as well as the prophylactic role of exercise training. Animals were submitted to fluid percussion injury after 6 weeks of swimming training. Previous exercise training increased mRNA expression of X receptor alpha and ATP-binding cassette transporter, and decreased inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF)-α and interleukin (IL)-6 expression *per se* in liver. Interestingly, exercise training protected against hepatic inflammation (COX-2, iNOS, TNF-α and IL-6), oxidative stress (decreases in non-protein sulfhydryl and glutathione, as well as increases in 2′,7′-dichlorofluorescein diacetate oxidation and protein carbonyl), which altered hepatic redox status (increases in myeloperoxidase and superoxide dismutase activity, as well as inhibition of catalase activity) mitochondrial function (decreases in methyl-tetrazolium and $\Delta\psi$ as well as inhibition of citrate synthase activity) and ion gradient homeostasis (inhibition of Na^+,K^+-ATPase activity inhibition) when analysed 24 h after TBI. Previous exercise training also protected against dysglycaemia, impaired hepatic signalling (increase in phosphorylated c-Jun

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NH2-terminal kinase, phosphorylated decreases in insulin receptor substrate and phosphorylated AKT expression), high levels of circulating and neuronal cytokines, the opening of the blood–brain barrier, neutrophil infiltration and Na^+,K^+-ATPase activity inhibition in the ipsilateral cortex after TBI. Moreover, the impairment of protein function, neurobehavioural (neuromotor dysfunction and spatial learning) disability and hippocampal cell damage in sedentary rats suggests that exercise training also modulates peripheral oxidative/inflammatory pathways in TBI, which corroborates the ever increasing evidence regarding health-related outcomes with respect to a physically active lifestyle.

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Abbreviations ABCA1, ATP-binding cassette transporter; ALT, aminotransferase; AST, aspartate aminotransferase; BBB, blood–brain barrier; CAT, catalase; COX-2, cyclooxygenase-2; CT, synthase; DCFH-DA, 2′,7′-dichlorfluorescein diacetate; ELISA, enzyme-linked immunosorbent assay; FPI, fluid percussion injury; GSH, reduced glutathione; H&E, haematoxylin and eosin; HOMA2%S, homeostasis model assessment; IL, interleukin; iNOS, inducible nitric oxide synthase; IRS, insulin receptor substrate; JNK, c-Jun NH2-terminal kinase; LT, lactate threshold; LXR-α, liver X receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TBI, traumatic brain injury; TNF, tumor necrosis factor.

Introduction

Traumatic brain injury (TBI) is a public health concern highly related to morbidity and mortality. This neurological injury is characterized by a combination of immediate mechanical dysfunction of the brain tissue, and secondary damage developed over a period of hours to days following the injury. Furthermore, TBI-related injuries are not limited to the CNS because protein metabolism alterations are linked to nitrogen transfer from muscles to the liver after a TBI, which participates in the synthesis of inflammatory proteins (Mansoor *et al.* 1996; Mansoor *et al.* 1997) and leads to local dysfunctions in different tissues (Mirzayan *et al.* 2008).

From a metabolic perspective, injuries caused by TBI are not limited to the CNS. In this context, TBI-induced hepatic inflammation reinforces the idea that neuronal injury signals may impact on the function of organs distant from the injury site (Moinard *et al.* 2008; Anthony & Couch, 2014). Indeed, the production of acute phase proteins after CNS injury leads to systemic inflammatory response syndrome, characterized by local damage as a result of the accumulation of blood inflammatory cells in different organs, such as the liver (Bao *et al.* 2011). Recent studies also have demonstrated that fast hepatic chemokine production after TBI amplifies focal injury (Anthony *et al.* 2012; Villapol *et al.* 2015*a*). However, the role of these hepatic inflammatory mediators on the neuronal dysfunction after TBI remains largely unknown.

Liver is involved in endogenous glucose production, which is regulated by insulin through insulin binding and subsequent activation of the insulin receptor substrate (IRS) (Wang *et al.* 2014). In this context, the unsuppressed hepatic glucose output significantly contributes to fasting hyperglycaemia as observed in patients with diabetes and/or glucose intolerance (Rizza, 2010). Furthermore, injuries (including TBI) commonly induce high glucose reactions (emergency insulin resistance) similar to high glucose and insulin levels in type-2 diabetes (Ljungqvist *et al.* 2000; Tsatsoulis *et al.* 2013), although the mechanism of the disease is not completely understood.

The experimental model of TBI induced by fluid percussion injury (FPI) has demonstrated major decreases in liver weight and protein content, which were associated with homeostasis impairment (Moinard *et al.* 2005; Moinard *et al.* 2008). Although not as energy-dependent as the skeletal muscle, the liver is also a highly demanding metabolic organ. In this sense, the relationships between energy metabolism, redox biochemistry and reactive oxygen species (ROS) generation at mitochondrial level are highly relevant, considering this organelle is also a primary target of uncontrolled ROS production (Ray, 2012). ROS overproduction has long been known to damage cell components in the liver, such as nucleic acids, protein and lipids (Apel & Hirt, 2004; Bergamini *et al.* 2004; Dong *et al.* 2014). Furthermore, the combination of abnormal oxygen metabolism and ATP depletion in organs such as the liver results in the collapse of the mitochondrial machinery, with the cell being destined for necrotic death (O'Connell *et al.* 2012).

Over past several decades, experimental and clinical evidence has validated the contribution of ROS and inflammation to TBI-induced multidimensional secondary injury responses (Bains & Hall, 2012). However, only a few studies have addressed the role of the liver in TBI-induced toxicity (Kamm *et al.* 2006; Wang & Yang, 2010; Yang *et al.* 2013). Furthermore, the understanding of post-injury neuroendocrine dysregulation in TBI is

essential for establishing scientific-based rehabilitative strategies. One of these strategies may be to support exercise training from a pre-clinical, prophylactic, point of view, considering its role in the reduction of damage onset after a TBI, which limits the secondary neuronal death outcomes and promotes neural repair and behavioural rehabilitation (Lima *et al.* 2009; Kim *et al.* 2010; Mota *et al.* 2012).

Regarding systemic adaptations elicited by exercise, cross-sectional studies have suggested that regular training protects against diseases associated with chronic low-grade systemic inflammation (Petersen & Pedersen, 2005). Aerobic exercise has been effective in reducing food intake in animals fed a fructose-rich diet by reducing inflammatory pathways triggered through c-Jun NH2-terminal kinase (JNK) phosphorylation and nuclear factor kappa B activation in both the liver and skeletal muscle (Botezelli *et al.* 2016). Aerobic exercise training also increased liver X receptor (LXR)-α, a member of the ligand-activated transcription factor that regulates lipid metabolism, plasma high-density cholesterol and inflammatory responses (Kazeminasab *et al.* 2012; Fu *et al.* 2014). This hepatic modulation of pro- and anti-inflammatory responses (Abd El-Kader *et al.* 2014; Motta *et al.* 2015), with intermittent states of rest and the exercise-related anti-oxidant effect, reinforces the rationale that metabolic adaptations to training are not restricted to the exercising muscles (Lima *et al.* 2013).

Liver plays essential roles during exercise, particularly in the energy metabolism, as well as inflammatory and oxidative responses (Barcelos *et al.* 2017). Although the immune modulation induced by exercise training provides a unique non-pharmacological therapeutic approach for controlling TBI-related secondary damage (Ang & Gomez-Pinilla, 2007; Motta & Dutton, 2013), little information regarding the prophylactic role of exercise training in the liver is currently available. In this sense, we aimed to investigate whether: (i) acute TBI induces insulin resistance in the liver; (ii) induced resistance is associated with hepatic inflammatory responses; (iii) inflammation-related hepatic chemokine release amplifies the neuronal injury response, as well as how these mediators contribute to cognitive dysfunction after TBI; and (iv) interference in the secondary injury development elicited by previous physical exercise breaks the progression of neuronal damage and cognitive dysfunction after TBI.

Materials and methods

Experimental design

The present study consists of two independent experiments, in which animals were randomly assigned to sedentary and exercise groups. Twenty-four hours after the last training session, animals underwent surgery for the FPI protocol, which resulted in four groups: sedentary/sham, sedentary/TBI, exercise/sham and exercise/TBI. Then, animals were subjected to a neuromotor evaluation 24 h after FPI. Immediately afterward, animals were killed and ipsilateral cortex and liver samples were obtained for biochemical analysis (Experiment 1). Another subset of animals performed memory tests 48 h after FPI. Immediately afterward, animals were killed by decapitation and ipsilateral hippocampus samples were obtained for biochemical and histological assays (Experiment 2) (Fig. 1).

Animal and reagents

Male Wistar rats (250–350 g) were maintained under 12:12 h light/dark photocycle at 24 ± 1ºC and 55% relative humidity, with free access to food and water. Experimental procedures were conducted in accordance with national and international legislations (Brazilian College of Animal Experimentation and US Public Health Service Policy on Humane Care and Use of Laboratory Animals guidelines) and previously approved by the Ethics Committee on Animal Research of the Universidade Federal de Santa Maria (Protocol number 081/2014). The experiments were carried out in accordance with the principles described by Grundy (2015).

Exercise training protocol and lactate threshold assay

Exercise training was carried out as previously described by Souza *et al.* (2009). Briefly, animals were placed in a cylindrical pool (diameter 1.05 m, height 60 cm). The swimming training protocol lasted 6 weeks with 60 min sessions five times per week. The water temperature was 32°C and swimming sessions were performed between 09.00 h and 11.00 h. Animals underwent a swimming adaptation period without weights during the first week of training. After adaptation, rats were submitted to swimming training with a workload of 5% of body weight to improve endurance. This training intensity is based on 80–90% of lactate threshold (LT) (Gobatto *et al.* 2001) and no animal drowned or struggled to keep the head above water. Sedentary groups were placed in a different tank with shallow water (5 cm) at 32°C for 30 min, 5 days per week, without workload. Twenty-four hours after the last training session, LT were evaluated in sedentary ($n = 6$) and trained ($n = 6$) rats. The LT test was conducted in accordance with the protocol described previously (Marquezi *et al.* 2003). The test consisted on swimming sessions of 3 min (1 min of resting) with progressive workload corresponding to 4%, 5%, 6%, 7% and 8% of the body weight of each animal. During resting periods, blood samples (25 μl) were collected from the tail vein into heparinized capillary tubes to

determine lactate concentration. The LT for each animal was calculated based on the point of inflection of the graph when the lactate concentration was plotted against the corresponding exercise workload.

Traumatic brain injury (TBI)

Twenty-four hours after the LT (Experiment 1), sedentary and trained rats were subjected to lateral FPI as described by D'Ambrosio *et al.* (2004). Briefly, animals were anaesthetized with a single injection of xylene (10 mg kg^{-1})/ketamine (100 mg kg^{-1}) mixture and placed in a rodent stereotaxic apparatus. Heart rate, temperature, tale and eyelid reflexes were monitored to verify anaesthetic depth and duration. A burr hole of 3 mm in diameter was drilled on the right convexity, 2 mm posterior to the bregma and 3 mm lateral to the midline, avoiding dura mater injury. A plastic injury cannula was placed over the craniotomy with acrylic cement. When the cement hardened, the cannula was filled with chloramphenicol and closed with a proper plastic cap. The animal was then removed from the stereotaxic device and returned to the cage. After 24 h, animals were anaesthetized with isofluorane, had the injury cannula attached to the FPI device, and were placed in a heat pad maintained at 37 ± 0.2°C. Frontoparietal TBI (2 mm posterior to the bregma and 3 mm lateral to the midline) was produced by a FPI device developed in our laboratory. A brief (10–15 ms) transient pressure fluid pulse (4.05 ± 0.17 atm) impact was applied against the exposed dura mater. Pressure pulses were measured extracranially by a transducer (Fluid Control, AmScien Instruments, Brazil) and recorded on a storage oscilloscope (Gould Ltd, Hainault, UK). During the recovery period from anaesthesia and injury, neurological function was assessed by determining the presence or absence of reflexes, including paw-pinch withdrawal, corneal, pinna, respiratory drive and righting reflex. During the surgery and subsequent recovery, body temperature was maintained with a circulating water-heating pad. Immediately upon responding to a paw pinch, anaesthesia was restored and the skull was sutured. Neomycin was applied on the suture and the rats were placed in a heated recovery chamber before being returned to their cages. The sham group underwent the same procedures and was coupled to the injury device, although no fluid pulse was delivered.

Assessment of neuromotor function

Twenty-four hours after neuronal injury, the neuromotor function was tested via the neuroescore test as described by Raghupathi *et al.* (1998). Briefly, animals were subjected to a grid-walk test for 1 min to allow assessment of the number of foot-faults. Subsequently, forelimb and hindlimb functions were evaluated by suspending animals by the tail and observing how they grasped the top of the cage when lowered towards it (for the forelimbs) and with the same pattern with respect to spread and hindlimb extension during the suspension (for hindlimbs). Finally,

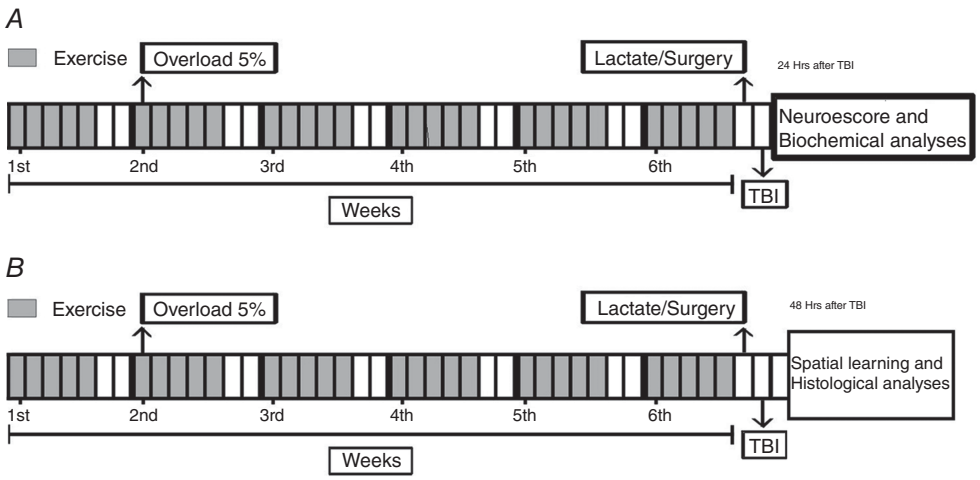

Figure 1. Schematic representation of the experimental design with the exercise training protocol
In the experiment 1 (*A*), animals underwent a swimming adaptation period without weights during the first week of training. After the swimming adaptation, animals trained with an extra overload equivalent to 5%/body weight during 5 weeks. One day after the last exercise session, animals were submitted to LT. Twenty-four hours after LT, sedentary and the trained rats were subjected to lateral FPI. One day after this procedure, motor function was assessed by the neuroescore test (Experiment 1). Immediately after behavioural analysis, animals were killed for biochemical and histological analysis in ipsilateral cortex and liver. In the second experiment (*B*), exercise training and FPI procedures applied were the same as described in Experiment 1, except that object recognition memory test was carried out 48 h after FPI. Immediately after this behavioural test, animals were killed for histological analysis in ipsilateral hippocampus.

Table 1. Sequences and size of the primers used for quantitative PCR amplifications

Gene	Forward primer (5′- to 3′)	Reverse primer (5′- to 3′)	Size bp
LXRs	CCTGATGTTTCTCCTGACTC	TGACTCCAACCCTATCCTTA	147
β-actin	GGAGAAGATTTGGCACCACAC	GGATGGCTACGTACATGGCTG	164
ABCA1	CTTGCTTCCGTTATCCAACTCCAG	GCTGTAATGTTCTCAGGACCTTGTG	162
iNOS	CTTGCAAGTCCAAGTCTTGC	GTATGTGTCTGCAGATGTGCTG	369
TNF-α	TTCGAGTGACAAGCCTGTAGC	AGATTGACCTCAGCGCTGAGT	390
IL-6	CATATGAGCTGAAAGCTCTCCA	GACACAGATTCCATGGTGAAGTC	435
COX-2	CCCCCACAGTCAAAGACACT	AGGCAATGCGGTTCTGATAC	348
GAPDH	GTATGACTCCACTCACGGCAA	GGTCTCGCTCCTGGAAGATG	132

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: forelimb function, hindlimb function and resistance to lateral pulsion. The maximum score for each animal was 12. Evaluation of neurological motor function was conducted by an experienced researcher who was blinded to all groups.

Blood, liver and ipsilateral cortex tissue preparation

After the neuroescore test, rats were re-anaesthetized with isofluorane and decapitated. Livers and ipsilateral cortex were rapidly dissected and stored at −80°C for further biochemical assays. Trunk blood also was collected and allowed to clot for 10 min at room temperature, centrifuged 1500 *g* for 15 min and stored at −80°C for further biochemical assays.

Determination of glucose and insulin resistance calculation

An Aviva Accu-check monitor (Roche Diagnostic Corp., Indianapolis, IN, USA) was used to analyse blood glucose levels. For the insulin test, blood concentration in each group was determined using an enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instruction (Linco Research, St Charles, MO, USA). The Homeostasis Model Assessment (HOMA2-%S) was calculated with a virtual platform released by the Diabetes Trials Unit, University of Oxford: HOMA2 Calculator (http://www.dtu.ox.ac.uk/homacalculator/index.php).

Cytokine, aspartate and alanine aminotransferase levels in blood

Serum interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels were measured using a commercially available ELISA kit, in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with a Hitachi 7020 automatic analyser (Hitachi, Tokyo, Japan) using commercially available reagents (Shino-Test Corporation, Tokyo, Japan).

RT-PCR in liver samples

Hepatic total RNA was obtained by using a Trizol reagent (Life Technologies, Carlsbad, CA, USA) and quantified by spectrophotometry (NanoDrop 1000; Thermo Scientific, Waltham, MA, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA). First-standard cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). The negative control (no transcriptase control) was performed in parallel. First-standard cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems, Paisley, UK) and then amplified using TaqMan® Universal PCR Master Mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) (Filippin *et al.* 2011). The Taqman® probes for inducible nitric oxide synthase (iNOS) (Rn00561640 m1) and cyclooxygenase-2 (COX-2) (Rn01483828 m1) were acquired from Applied Biosystems. The primer sequences for LXR-α, ATP-binding cassette transporter (ABCA)1, iNOS, COX-2 and β-actin are presented in Table 1. Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method, as described previously (García-Mediavilla *et al.* 2005; Tuñón *et al.* 2011). The cycle number at which the transcripts were detectable (C_t) was normalized to the cycle number, referred to as ΔCT.

Cytokine immunoassay

The contents of IL-6 and TNF-α were determined in liver homogenates. Cytokine levels were measured using a commercially available ELISA kit from R&D Systems, in accordance with the manufacturer's instructions. The

concentration of cytokines was normalized to protein concentration of samples the and results are expressed as pg mg^{-1} protein.

Western blot analysis

Liver samples were homogenized at 4°C in 300 μl of 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The homogenate was centrifuged at 4°C for 30 min at 13 000 *g*. The supernatant fraction was collected and stored at −80°C in aliquots until use. Samples containing 40 μg of protein were fractionated by SDS-PAGE and then, transferred to a polyvinylidene fluoride membrane by a Trans-Blot TurboTM Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight at 4°C with the corresponding antibodies (Gobatto *et al.* 2001). Antibodies against iNOS (130 kDa), pIRS (130 kDa), pJNK (48 kDa) and pAkt (60 kDa) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibody against COX-2 (70 kDa) was purchased from Abcam (Cambridge, UK). After washing with TBST, the membranes were incubated for 1 h at room temperature with secondary HRP conjugated antibody (dilution 1:5000; Dako, Glostrup, Denmark), and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). The blots were stripped and probed again for anti-β-actin (42 kDa) antibody (Sigma-Aldrich) to confirm equal protein loading (Gobatto *et al.* 2001). The density of the specific bands was quantified with an imaging densitometer (Image J, version 1.46a; NIH, Bethesda, MD, USA).

Estimation of ROS production, non-protein sulfhydryl, protein carbonyl and reduced glutathione (GSH) content

Production of ROS was estimated in liver mitochondria with the fluorescence probe 2′,7′-dichlorfluorescein diacetate (DCFH-DA) as described by Myhre *et al.* (2003). Free -SH groups were determined as described by Ellman & Lysko (1967). Total protein carbonyl content was determined using the method described by Yan *et al.* (1995) and Levine *et al.* (1990). For measurement of GSH levels, the method previously described by Hissin & Hilf (1976) was used.

Superoxide dismutase (SOD) and catalase (CAT) enzyme activities

The hepatic SOD activity was measured as described by Mirsra and Fridovich (1972). CAT activity was determined by following the decomposition of hydrogen peroxide in accordance with the method proposed by Aebi (1984).

Mitochondrial 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction

The hepatic MTT assays were carried out with a modification (Cohen *et al.* 1997) of the method described by Berridge and Tan (1993) the respiration buffer was used as stock buffer.

Mitochondrial membrane potential ($\Delta\psi$) and citrate synthase (CS) activity

The hepatic mitochondrial $\Delta\Psi m$ determination was estimated by fluorescence changes in safranine-O, assayed as described previously (Akerman & Wikstrom, 1976). CS activity was determined spectrophotometrically using the method of Srere & Brooks (1969), which measures the appearance of free CoA.

Na^+,K^+-ATPase activity

Hepatic Na^+,K^+-ATPase activity was measured as described by Silva *et al.* (2013*a*). The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske & Subbarow (1927). The Michaelis–Menten constant (K_m) for ATP was then calculated using the non-linear fit function of Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Evaluation of MPO activity as a marker of neutrophil inflitration

MPO activity in the ipsilateral cortex was assayed in accordance with the method of Suzuki *et al.* (1983), with some modifications. Briefly, perilesional cortex samples were homogenized with 10 volumes of 50 mM sodium acetate buffer (pH 5.4) plus 0.5% hexadecyltrimethylammonium bromide, centrifuged (11 200 *g* at 4°C for 20 min) and the supernatants were collected. Next, 10 μl of supernatant and 230 μl of 50 mM sodium acetate buffer (pH 5.4) containing 15% of 0.3 mM H_2O_2 were added in triplicate to a 96-well plate. The reaction was initiated by the addition of 20 μl of 18.4 mM tetramethylbenzidine. The mixture was incubated for 50 min at 37 °C follwed by immersion in an ice bath. The reaction was stopped by adding 30 μl of acetic acid and absorbance was monitored at 630 nm.

Cerebral cytokine immunoassay and Na^+,K^+-ATPase activity

The contents of IL-6 and TNF-α were determined in ipsilateral cortex homogenized in solution containing BSA (10 mg ml^{-1}), 2 mM EGTA, 2 mM EDTA and 0.2 mM phenylmethylsulphonyl fluoride in PBS (pH 7.4). Cytokine levels were measured using a commercially

available ELISA kit from R&D Systems, in accordance with the manufacturer's instructions. The concentration of cytokines was normalized to the protein concentration contained in the samples. The results are expressed as pg/mg^{-1} protein. The activity of Na^+,K^+-ATPase was determined in the ipsilateral cortex homogenized using the method of Silva *et al.* (2013*b*).

Evaluation of blood–brain barrier (BBB) permeability

For evaluation of BBB permeability to small molecular mass compounds, 24 h after neuronal injury, a subset of animals was injected with sodium fluorescein (10 mg in 0.1 ml of sterile saline I.P.) as described by Olsen *et al.* (2007). In brief, animals were anaesthetized with ketamine HCl (200 mg kg^{-1}) I.P., 45 min after sodium fluorescein injection for blood sampling. The brain was removed, weighed, homogenized (dilution 1:10) in trichloroacetic acid, centrifuged at 1250 *g* for 5 min and then the supernatant was stored at −70°C until biochemical analysis. Samples were analysed on fluorometer (emission 538 nm; extinction 480 nm). BBB permeability degree was measured as the percentage of sodium fluorescein in the brain per amount of sodium fluorescein in a milliliter of serum.

Barnes maze assay

To determine the effect of previous physical exercise and TBI on spatial learning memory, a subset of animals was trained to solve the Barnes maze 48 h after neuronal injury. The Barnes maze is a validated test often used for the assessment of spatial learning and memory in rodents (Barnes, 1979). The Barnes maze paradigm exploits the natural inclination of small rodents to seek escape to a darkly lit, sheltered environment when placed in an open arena under bright, aversive illumination. Our maze consists of a circular wooden table (diameter 120 cm, thickness 3.5 cm) elevated 90 cm above the floor.

Twenty holes (diameter 6 cm) were equidistantly located around the perimeter and centred 5 cm from it. The apparatus was located in a 4 × 4 m test room where four visuospatial cues made of rigid black paper (rectangle, circle, cross, triangle) were affixed to the walls but not directly over any maze hole; this increases the spatial component of the Barnes maze during training. A black wooden escape tunnel (15 × 10 × 30 cm) was placed beneath one hole, selected randomly for each rat but remained constant throughout training sessions for a given rat. The remaining 19 holes led only to a false escape box (15 × 10 × 10 cm) which, from the platform, appeared indistinguishable from an escape box but was too small to be entered. False boxes removed visual cues that might be observed through an open hole. There was a bright illumination of 300 lux over the maze.

On the first day of the experiment, rats were moved to a testing room and left undisturbed for 60 min. Following this habituation period, rats were trained to find the escape hole; they were placed in the escape box for 1 min, then into a cylindrical opaque chamber (start box) in the centre of the maze. With light on, the start box was removed and rats were allowed to explore freely and find the escape box. A maximum of 180 s to find it was allowed. Each rat was given three trials per day, over four consecutive days. In each trial, we recorded the time to reach the escape tunnel and the number of wrong holes visited. A visited hole was defined when the animal put it head into the hole. The arena, as well as the boxes, was wiped clean using distillated water both between each training session for a given rat and between each rat.

Histological procedures

To determine the effect of TBI and exercise training on neuronal damage, immediately after behavioural (spatial learning) analysis, rats were deeply anaesthetized with thiopental (125 mg kg^{-1}; I.P.) and transcardially perfused with 600 ml of NaCl 0.9% that contained heparin (5 IU ml^{-1}) followed by 600 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and serial coronal sections of 50 μm (between −1.5 and – 2.1 from bregma) were cut on a vibratome (model KD400; Zhejiang Jinhua Kedi Instrumental Equipment Co., Zhejiang, China). Sections were washed in phosphate buffer and subjected to the Giemsa staining processing (Iniguez *et al.*, 1985). The staining was performed in duplicate for each rat (i.e. two sections per animal) and a single slide contained sections for all four groups at 35 days after TBI. Sections were mounted on gelatinized glass slides and stained for 30 s in a commercially available Giemsa solution (New Prov, Pinhais, PR, Brazil), washed three times for 2 min in distilled water, dehydrated in three graded ethanol solutions (70%, 90% and 100%), cleared for 2 min in xylene and mounted with Cytoseal 60 (Thermo Scientific). The evaluation of the Giemsa-stained slides was conducted with 10× magnification under a light microscope (DFC290; Leica Microsystems, Wetzlar, Germany) and images of the slices were taken with the Leica Application Suite, version 3.8 (Leica Microsystems). The nucleus of neurons was used as the counting unit. The number of cells within the dentate hilus was assessed at each section, and counts were averaged, resulting in a single value for each rat.

For hepatic damage, tissue blocks were placed in 10% buffered formaldehyde solution for 48 h before being embedded in paraffin. Liver histology was assessed by light microscopy (BH2; Olympus, Tokyo, Japan) of haematoxylin and eosin (H&E)-stained 4 μm sections in a blinded fashion. Ten random fields on each slide were assessed for necrosis by standard morphological

criteria (loss of architecture, vacuolization, karyolysis, increased eosinophilia) and the extent of necrosis was semiquantitatively estimated by assigning a severity score on a scale of 0–4 as described previously by Sigala *et al.* (2004) (0, absent; 1, mild; 2, moderate; 3, severe; 4, total necrotic destruction of the liver).

Protein determination

The protein content was colorimetrically measured using BSA (1 mg ml^{-1}) as a standard (Bradford, 1976).

Statistical analysis

Data are expressed as the mean ± SEM or median ± inter-quartile range. Data analysis was conducted using one or two-way ANOVA or non-parametric tests, such as the Kruskal–Wallis test, depending on the experimental design. *Post hoc* analyses were performed using Tukey's test when appropriate. $P < 0.05$ was considered statistically significant.

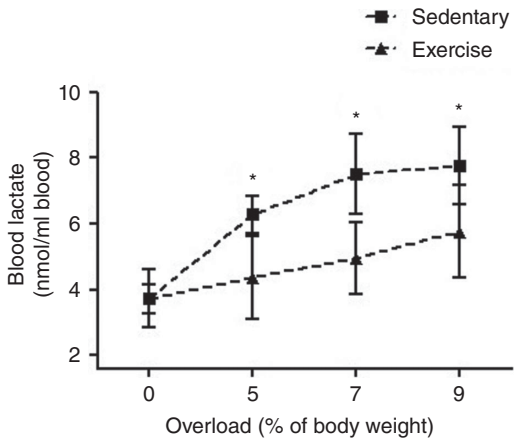

Figure 2. Effect of exercise training on the lactate threshold test
Data are expressed as the mean ± SEM for $n = 6$. $^{*}P < 0.05$ compared to trained group (*F* test for simple effect).

Results

The lactate threshold test (LT)

In the present study we showed a clear stabilization of blood lactate concentration in trained rats compared to the sedentary group ($F_{1,16} = 7.09$; $P < 0.05$) (Fig. 2). This finding indicates exercise training increased the aerobic resistance of the animals (Rambo *et al.* 2009; Souza *et al.* 2009; Lima *et al.* 2013).

Glycogen levels and LXR-α and ABCA1 receptors

Our experimental data revealed that FPI did not alter glycogen levels in the liver ($F_{1,20} = 0.65$; $P > 0.05$) (Fig. 3*A*), although exercise training was able to increase its content ($F_{1,20} = 16.96$; $P < 0.05$) (Fig. 3*A*). In the present study, we showed that mRNA expression of LXR-α ($F_{1,20} = 0.27$; $P > 0.05$) (Fig. 3*B*) and ABCA1 ($F_{1,20} = 0.11$; $P > 0.05$) (Fig. 3*C*) was not altered by the FPI injury. On the other hand, exercise training increased hepatic LXR-α ($F_{1,20} = 26.96$; $P < 0.05$) and ABCA1 ($F_{1,20} = 15.68$; $P < 0.05$) mRNA expression levels in sham and TBI groups 24 h after neuronal injury.

Inflammatory responses

To further examine the role of pro-inflammatory enzymes in liver 24 h after neuronal injury, iNOS and COX-2 protein expression were measured by real-time RT-PCR and western blotting. Previous exercise training decreased both iNOS ($F_{3,20} = 8.09$; $P < 0.05$) (Fig. 4*A*) and COX-2 ($F_{3,20} = 36.26$; $P < 0.05$) (Fig. 4*B*) mRNA expression and protected against FPI-induced mRNA increases of these proteins. In the same way, the immunoreactivity of iNOS ($F_{3,20} = 25.82$; $P < 0.05$) (Fig. 4*C*) and COX-2 ($F_{3,20} = 20.71$; $P < 0.05$) (Fig. 4*D*) was reduced in the TBI-exercised group. An immunoassay for cytokine levels also revealed that exercise training decreased hepatic levels of TNF-α ($F_{1,20} = 36.12$; $P < 0.05$) (Fig. 4*E*) and protected against FPI-induced IL-6 increases

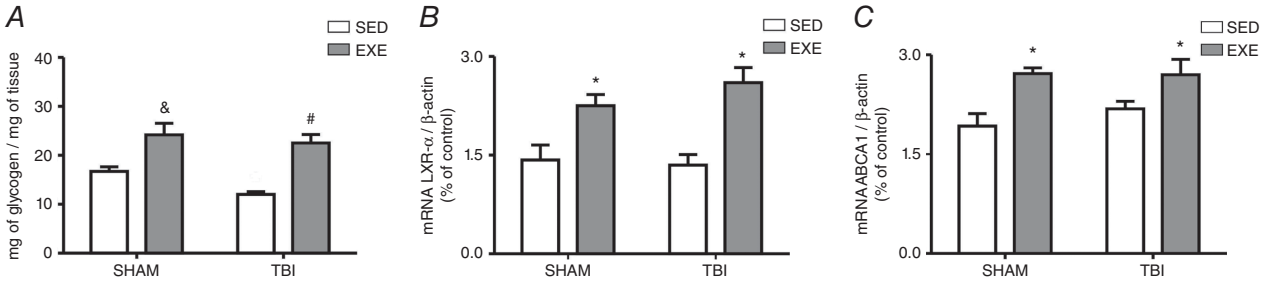

Figure 3. The effect of exercise training and FPI on Glycogen and LXR and ABCA1 receptors
Exercise training and FPI effects on hepatic glycogen content (*A*), as well as mRNA expression of LXR-α (*B*) and ABCA1 (*B*) 24 h after neuronal injury. Data are expressed as the mean ± SEM ($n = 6$). $^{*}P < 0.05$ compared to all other groups. $^{\&}P < 0.05$ compared to the sedentary/sham group. $^{\#}P < 0.05$ compared to the sedentary/TBI group.

($F_{3,20} = 20.02$; $P < 0.05$) (Fig. 4*F*) and TNF-α content ($F_{3,20} = 31.19$; $P < 0.05$) (Fig. 4*E*). In the present study, we revealed that pro-inflammatory responses induced by FPI were accompanied by increased immunoreactivity of pJNK ($F_{3,20} = 10.50$; $P < 0.05$) (Fig. 5*A*) and decreased pIRS ($F_{3,20} = 13.60$; $P < 0.05$) (Fig. 5*B*) and pAKT ($F_{3,20} = 10.13$; $P < 0.05$) (Fig. 5*C*) content, whereas previous exercise training protected against these deleterious effect.

Oxidative stress and redox status markers

The results obtained in the present study reveal that training attenuated DCFH-DA oxidation ($F_{1,22} = 26.40$; $P < 0.05$) (Fig. 6*A*) and decreased protein carbonyl content ($F_{1,18} = 8.67$; $P < 0.05$) (Fig. 6*B*). In the same line, increases in free –SH groups ($F_{1,20} = 19.13$; $P < 0.05$) (Fig. 6*C*) and GSH levels ($F_{1,16} = 25.46$; $P < 0.05$) (Fig. 6*D*) were seen after exercise training. Following FPI-induced injury, exercise training prevented DCFH-DA oxidation

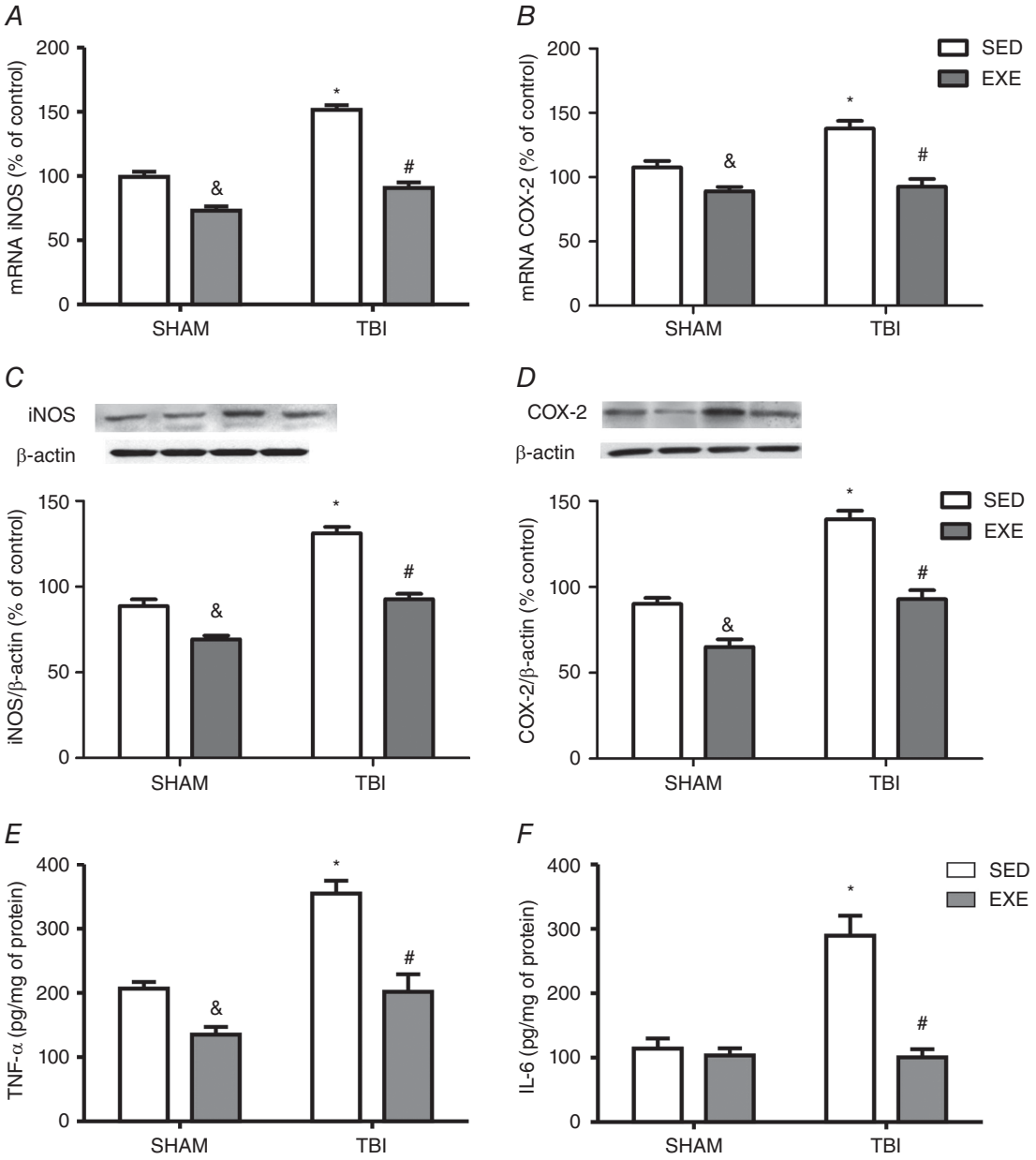

Figure 4. The effect of exercise training and FPI on inflammatory responses
Exercise training and FPI effects on liver mRNA expression of iNOS (*A*) and COX-2 (*B*), as well as protein content of iNOS (*C*) and COX-2 (*D*), and also TNF-α (*E*) and IL-6 (*F*) levels 24 h after neuronal injury. Data are expressed as the mean ± SEM ($n = 6$–7). $^{*}P < 0.05$ compared to all other groups. $^{\&}P < 0.05$ compared to the sedentary/sham group. $^{\#}P < 0.05$ compared to the sedentary/TBI group.

($F_{3,22}$ = 26.40; $P < 0.05$) (Fig. 6*A*) and protein carbonylation increases ($F_{3,18} = 26.17$; $P < 0.05$) (Fig. 6*B*), whereas free -SH groups ($F_{3,20}$ = 20.05) ($P < 0.05$) (Fig. 6*C*) and GSH levels ($F_{3,16} = 18.90$; $P < 0.05$) (Fig. 6*D*) were increased compared to TBI-sedentary rats. Statistical analysis also revealed that previous exercise training protected against FPI-induced SOD activity increases ($F_{3,20}$ = 9.26; $P < 0.05$) (Fig. 6*F*) and CAT inhibition

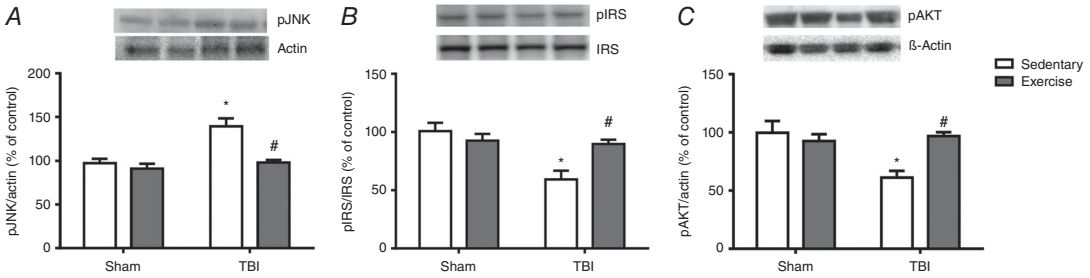

Figure 5. The effect of exercise training and FPI on hepatic pJNK, pIRS and pAKT immunoreactivity
Exercise training and FPI effects on liver protein content of pJNK (*A*), pIRS/IRS ratio (*B*) and pAKT (*C*) 24 h after neuronal injury. Data are expressed as the mean ± SEM (n = 6–7). $^{*}P < 0.05$ compared to all other groups. $^{\#}P < 0.05$ compared to the sedentary/TBI group.

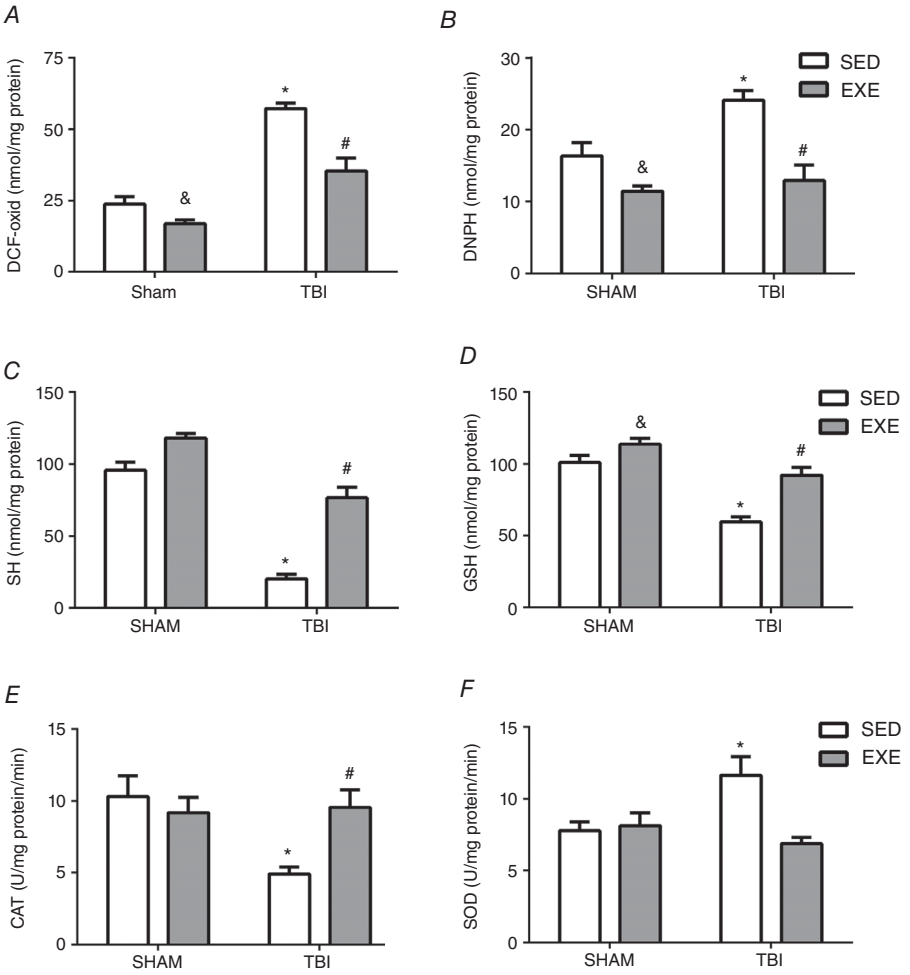

Figure 6. The effect of exercise training and FPI on hepatic oxidative and redox status
Exercise training and FPI effects on liver DCFH-DA oxidation (*A*), DNPH (*B*), free-SH (*C*) and GSH levels (*D*), as well as CAT (*E*) and SOD (*F*) activities 24 h after neuronal injury. Data are expressed as the mean ± SEM (n = 6–7). $^{*}P < 0.05$ compared to all other groups. $^{\&}P < 0.05$ compared to the sedentary/sham group. $^{\#}P < 0.05$ compared to the sedentary/TBI group.

($F_{3,20} = 6.62$; $P < 0.001$) (Fig. 6*E*) when analysed 24 h after neuronal injury.

Mitochondrial assays

Exercise training was effective against FPI-induced liver mitochondria dysfunction, as characterized by increases for both dehydrogenases, measured as MTT reduction ($F_{3,24} = 6.41$; $P < 0.05$) (Fig. 7*A*), and CS activity ($F_{3,20} = 5.01$; $P < 0.05$) (Fig. 7*B*) 24 h after neuronal injury. Moreover, $\Delta\psi$decreased after FPI ($F_{3,20} = 7.51$ $P < 0.05$) (Fig. 7*C*), whereas exercise training reverted this effect back to baseline values.

Na^+,K^+-ATPase and ATP K_m

Our experimental data showed that FPI decreased Na^+,K^+-ATPase activity ($F_{1,20} = 10.99$; $P < 0.05$) (Fig. 7*D*). Additionally, functional analysis of Na^+,K^+-ATPase dependence for ATP revealed that ATP K_m was also altered with FPI ($F_{1,20} = 8.19$; $P < 0.05$) (Fig. 7*E*). However, exercise training prevented both a FPI-induced Na^+,K^+-ATPase decrease ($F_{3,20} = 8.58$ $P < 0.05$) (Fig. 7*D*) and an ATP K_m increase ($F_{3,20} = 5.51$ $P < 0.05$) (Fig. 7*E*) 24 h after neuronal injury.

Change of metabolic characteristics and serum cytokine levels after TBI

Statistical analyses revealed that FPI induced blood insulin ($F_{1,20} = 17.32$; $P < 0.05$) (Fig. 9*A*) and glucose ($F_{1,20} = 8.90$; $P < 0.05$) (Fig. 9*B*) increases; previous exercise training protected against hyperglycaemia. Calculation of HOMA-2% indicated that insulin sensitivity was low in FPI-sedentary compared to the FPI-trained group 24 h after neuronal injury ($F_{1,20} = 7.38$; $P < 0.05$) (Fig. 9*C*).

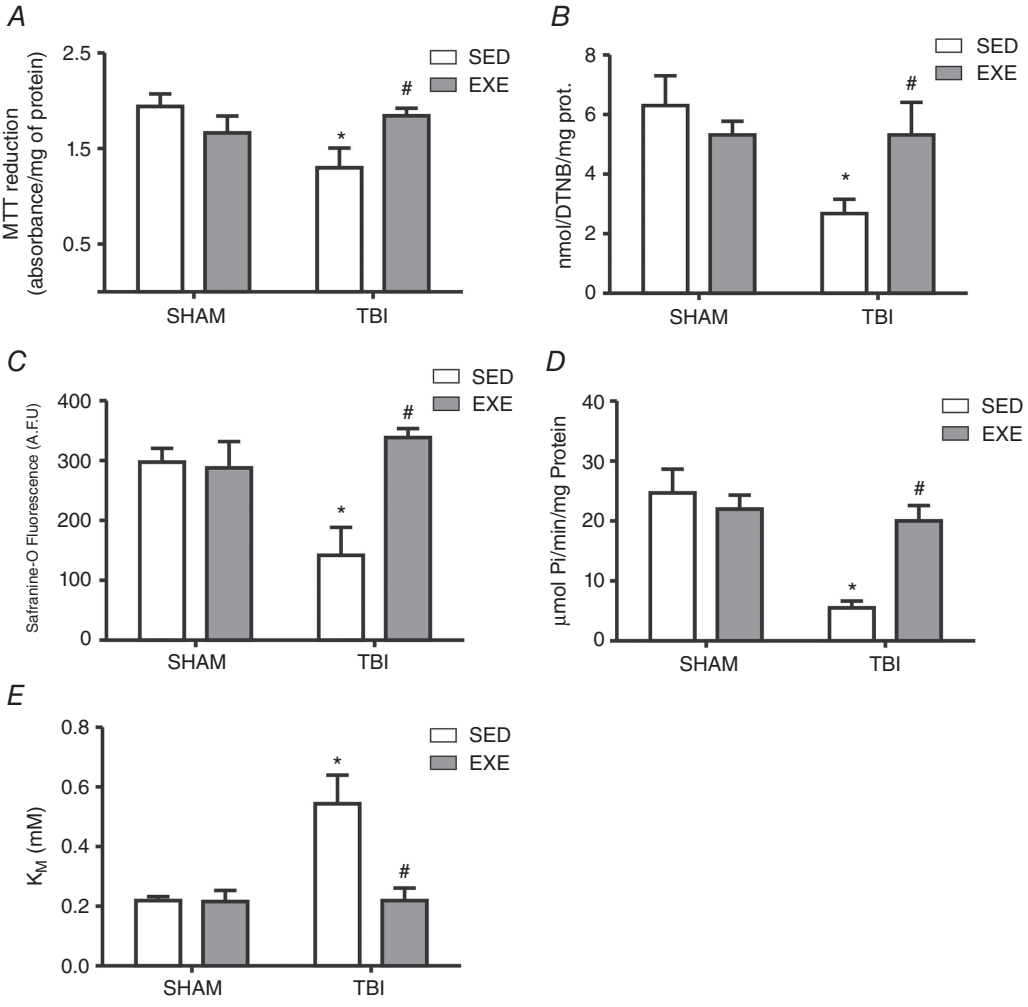

Figure 7. The effect of exercise training and FPI on hepatic mitochondrial function
Exercise training and FPI effects on liver mitochondrial MTT reduction (*A*), CS (*B*), $\Delta\psi$ (*C*), Na^+, K^+-ATPase activity (*D*) and K_m for ATP (*E*) 24 h after TBI. Data are expressed as the mean ± SEM ($n = 5$–8). $^*P < 0.05$ compared to all other groups. $^\#P < 0.05$ compared to the sedentary/TBI group.

Table 2. Metabolic profile of sham-operated and TBI-rats

		Day 1 post TBI	
Parameter	Baseline (n = 6–8)	Sedentary group (n = 6–8)	Trained group (n = 6–8)
Body weight (g)			
Sham	319.9 ± 2.7	316.9 ± 2.9	322.5 ± 3.0
TBI	314.7 ± 3.1	316.8 ± 3.6	320.6 ± 2.1
Liver weight (g)			
Sham		9.9 ± 0.5	10.1 ± 0.3
TBI		10.0 ± 1.6	9.7 ± 0.4
Serum IL-6 (pg ml^{-1})			
Sham		104.24 ± 31.0	98.09 ± 28.5
TBI		225.70 ± 69.10*	106.18 ± 31.4#
Serum TNF-α (pg ml^{-1})			
Sham		160.84 ± 42.1	159.39 ± 38.5
TBI		338.89 ± 89.99*	198.18 ± 59.4#
Serum ALT (IU mL^{-1})			
Sham		18.13 ± 4.1	24.19 ± 7.5
TBI		22.33 ± 6.1	23.13 ± 5.6
Serum AST (IU mL^{-1})			
Sham		102.4 ± 7.98	103.39 ± 8.50
TBI		106.3 ± 12.09	108.18 ± 11.10

Data are given as the mean ± SEM. *P < 0.05 sham/group. #P < 0.05 TBI/group.

As shown in Table 2, previous exercise training protected against serum IL-6 ($F_{3,20}$ = 14.15 P < 0.05) and TNF-α ($F_{3,20}$ = 16.89; P < 0.05) increases 24 h after FPI.

Liver damage markers

Statistical analyses revealed that neither FPI, nor exercise training altered liver and body weights, as well as serum ALT ($F_{1,20}$ = 1.09; P > 0.05) (Table 2) and AST ($F_{1,20}$ = 0.58; P > 0.05) (Table 2) concentrations, compared to the respective control groups. Equally, H&E staining did not show tissue damage in hepatic cells and histological assessment revealed that livers from the different groups displayed a well-preserved architecture 24 h after FPI injury (Fig. 8).

Opening of the blood-brain barrier (BBB) followed by neurtrophils infiltration and cerebral inflammation, contributes to failure of Na^+,K^+-ATPase activity

In the present study, we showed that previous exercise training significantly attenuated an FPI-induced increase of plasma fluorescein extravasation (indicator of BBB breakdown) ($F_{3,20}$ = 8,50; P < 0.05) (Fig. 10*A*) and protected against MPO activity increases ($F_{3,20}$ = 7,04; P < 0.05) (Fig. 10*B*) 24 h after neuronal injury. Exercise training was also effective against FPI-induced IL-6 ($F_{3,20}$ = 10.15) (P < 0.05) (Fig. 10*C*) and TNF-α content ($F_{3,20}$ = 18.06; P < 0.05) (Fig. 10*D*) increases, as well as Na^+,K^+-ATPase activity inhibition ($F_{3,20}$ = 14,74; P < 0.05;Fig. 10*E*).

Exercise training protects against TBI-induced motor impairment and spatial learning dysfunction

Motor impairment is a common TBI disability (Mota *et al.* 2012; da Silva Fiorin *et al.* 2016). Using the composite neuroscore, we demonstrated that FPI-induced motor impairment ($F_{1,20}$ = 10.58; P < 0.05) (Fig. 10*F*) and previous exercise training protected against neurological impairment 24 h after neuronal injury. Results showed that 6 weeks of swimming training enhanced spatial learning *per se* characterized by escape latency ($F_{1,42}$ = 5.10 P < 0.05) (Fig. 11*B* and *C*) and decreased number of errors (F(1,42 = 20.21 P < 0.05) (Fig. 12*B*–*D*) in the naive group compared to the sedentary animals. Exercise training also protected against FPI-induced increases on escape latency ($F_{2,42}$ = 7.03 P < 0.05) (Fig. 11*B* and *C*) and number of errors ($F_{2,42}$ = 6.23 P < 0.05) (Fig. 12*B*–*D*). The open field test revealed that neither swimming training, nor TBI altered crossing numbers ($F_{2,42}$ = 0.10; P > 0.05) or rearing responses ($F_{2,42}$ = 0.35; P > 0.05), indicating that FPI-related impaired performance in the Barnes maze was unrelated to motor disabilities (supplementary data).

Exercise training protects against hippocampal cell loss after FPI

Considering secondary injury processes lead to necrotic, apoptotic and autophagic neuronal cell death and synaptic loss (Bigford *et al.* 2009), we performed histological analyses in the hippocampus. H&E staining demonstrated previous exercise training protected against cell loss

in the dentate gyrus hilus after the Barnes maze test ($F_{3,21} = 13.25$; $P < 0.05$) (Fig. 13*A*).

Discussion

The results reported in the present study confirm and extend previous findings indicating that a single FPI episode in the rat parietal cortex induces a major peripheral inflammatory response (Moinard *et al.* 2008). From a metabolic point of view, our experimental data suggest that an early inflammatory response and oxidative stress are implicated in the signal transduction that alters redox status, mitochondrial function and insulin signalling in the liver. By contrast, the stress response in this TBI model did not induce early hepatocellular damage, although

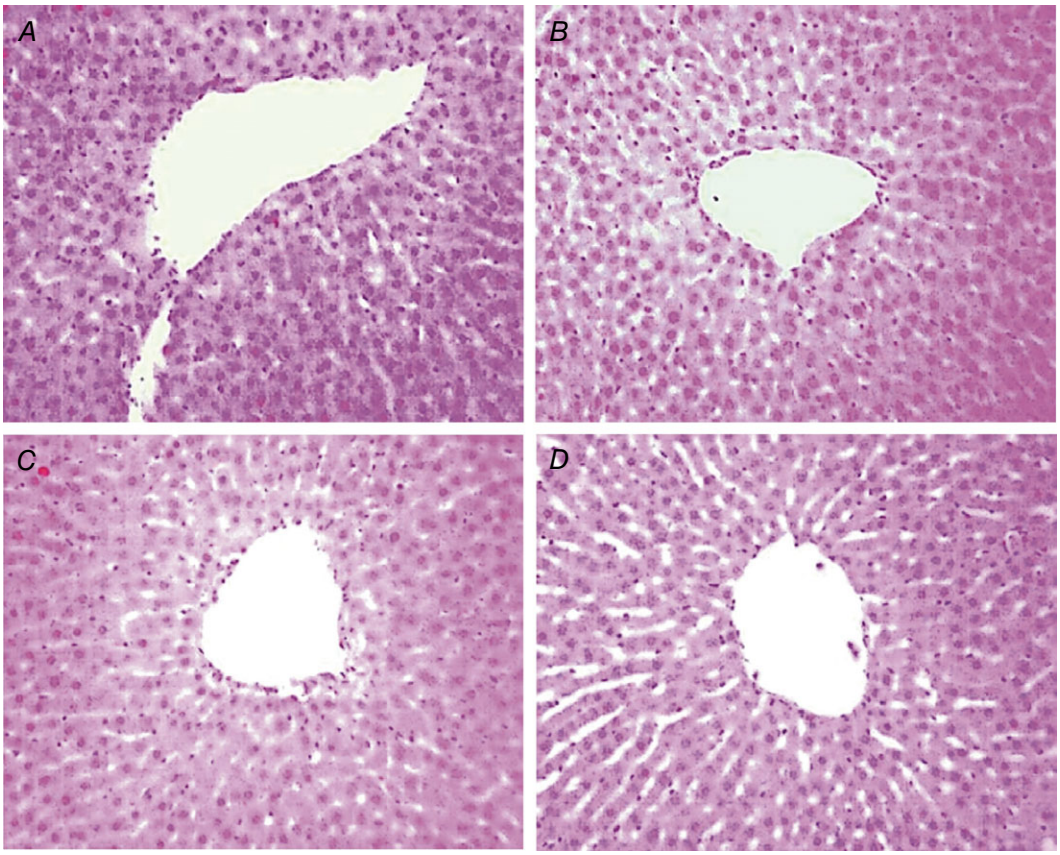

Figure 8. Exercise training and FPI effects on hepatocellular morphology 24 h after TBI
Histological analysis in the liver displays as: sham/sedentary group (*A*), TBI/sedentary (*B*), sham/trained (*C*) and TBI/trained (*D*) animals demonstrated by H&E staining. [Colour figure can be viewed at wileyonlinelibrary.com]

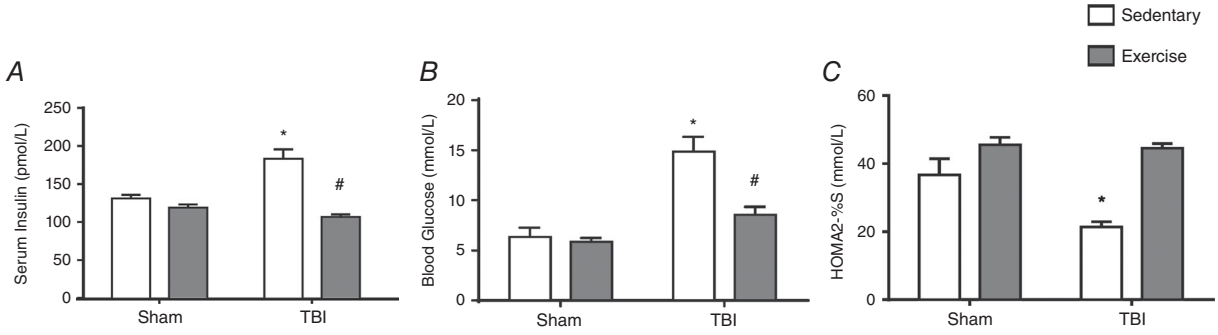

Figure 9. The effect of exercise training and FPI on metabolic profile
Exercise training and FPI effects on plasma insulin (*A*), glucose (*B*) and HOMA2 %S (*C*) 24 h after TBI. Data are expressed as the mean ± SEM ($n = 6$–7). $^{*}P < 0.05$ compared to all other groups. $^{\#}P < 0.05$ compared to the sedentary/TBI group.

the ion gradient collapse provides an explanation for the crucial role that the liver may play in secondary damage after a neuronal injury (Anthony *et al.* 2012).

Recently, considerable evidence has demonstrated that hyperglycaemia (both peak glucose and persistent hyperglycaemia) is one of the most common secondary complications of severe TBI (Shi *et al.* 2016). In line with this view, the present study shows that blood glucose, insulin and HOMA-2%S were significantly elevated following acute neuronal injury in sedentary rats. The development of hyperglycaemia after neuronal injury was accompanied by hepatic immunoreactivity of a

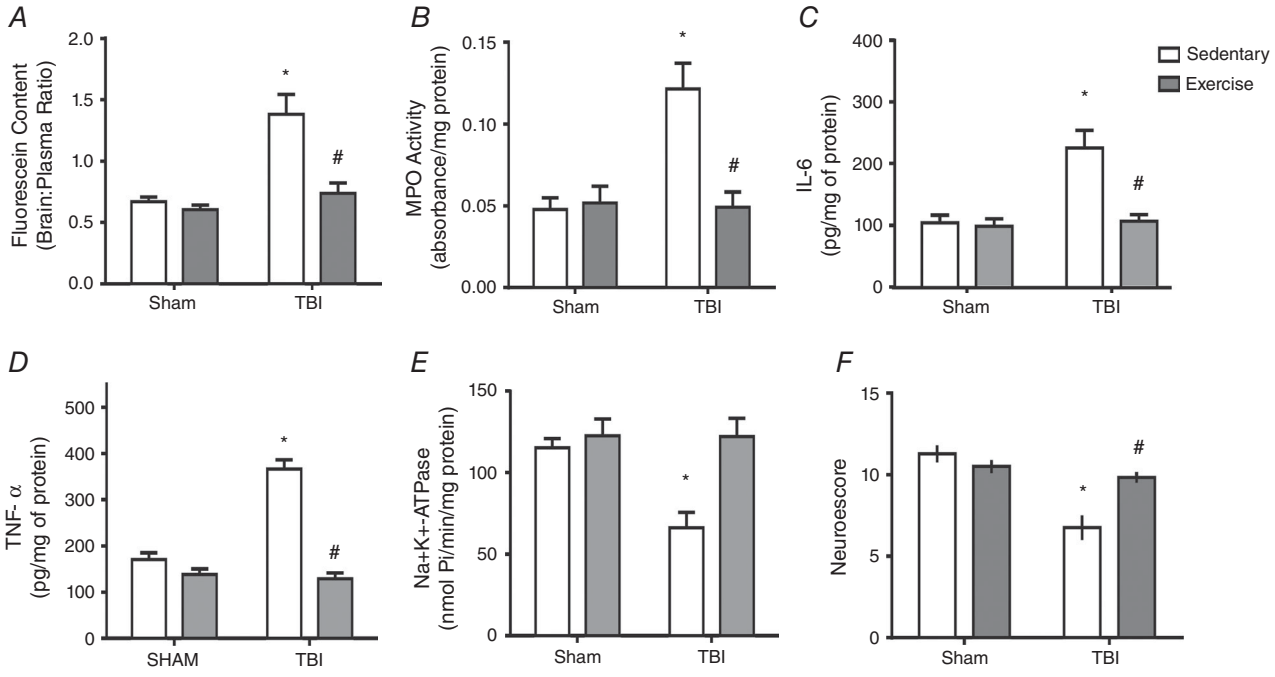

Figure 10. The effect of exercise training on neuronal oxidative inflammatory-related cascades after FPI
Exercise training and FPI effects on plasma fluorescein extravasation (indicator of BBB breakdown) (*A*), neuronal MPO activity (*B*), IL-6 (*C*), TNF-α levels (*D*), Na^+,K^+-ATPase activity (*E*) and neuroscore (*F*) 24 h after TBI. Data are expressed as the mean ± SEM (n = 6–7). *P < 0.05 compared to all other groups. $^{\#}P$ < 0.05 compared to the sedentary/TBI group.

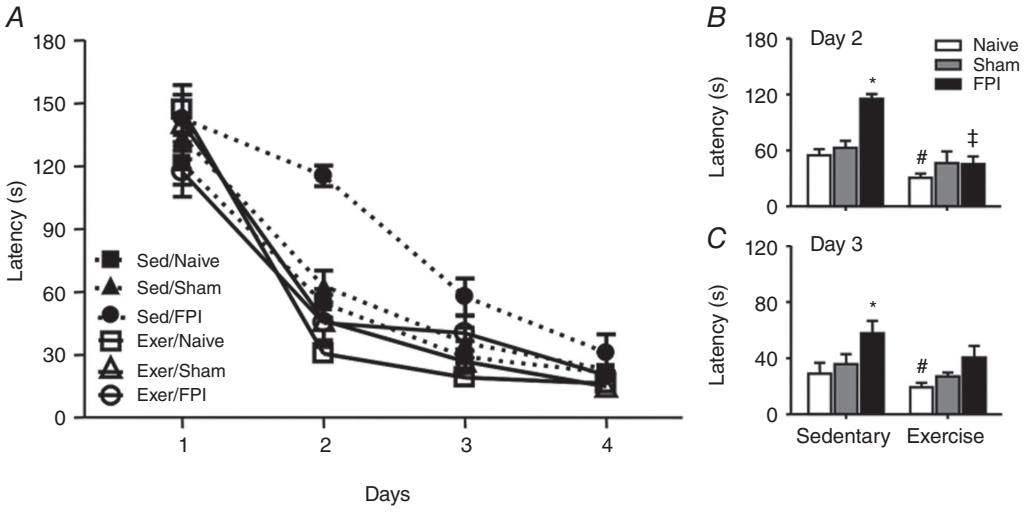

Figure 11. Previous physical training protects against FPI-induced spatial learning dysfunction
Exercise training and FPI effects on escape latency in Barnes maze test (*A*). *B* and *C*, previous swimming training decreased the latency for escape *per se* and protected against latency for escape increase induced by FPI. Data are the mean ± SEM (n = 7–9 in each group). *P < 0.05 compared to sham and naive sedentary group. $^{\#}$ P < 0.05 compared to the naïve-sedentary group.

pJNK increase and pIRS and pAKT decreases. Our data mimicked the findings of previous studies in experimental haemorrhage and burn models and further highlight the importance of local hepatic inflammation and ROS generation in the pathogenesis of glucose metabolic abnormalities in acute illnesses (Xu *et al.* 2008; Wang *et al.* 2011). Our experimental data also reinforce that the idea that TBI-related injuries are not limited to the CNS. In this sense, the hepatic acute-phase response leads to the mobilization of leukocytes, cytokines and chemokines (Villapol *et al.* 2015*b*). These signalling molecules enter the bloodstream and reach the cerebral circulation to affect the inflammatory status of the brain.

In the present study, we have demonstrated that high levels of circulating cytokines elicited by FPI affect the brain response to injury and contribute to BBB opening, neurtrophil infiltration and Na^+,K^+-ATPase activity inhibition. These results are in line with previous findings from our group suggesting that a single episode of FPI affects the inflammatory status of the brain (Mota *et al.* 2012), impairs performance in spatial learning and induces cell loss in the dentate gyrus hilus (Lima *et al.* 2008; da Silva Fiorin *et al.* 2016). However, the understanding of secondary damage post-injury is essential for establishing scientific-based rehabilitation following TBI. One of the possible strategies may be the inclusion of exercise training as a prophylactic means for TBI sequelae management. Evidence accumulated over the years has demonstrated that exercise training exerts a broad range of beneficial effects on the body, including improvements

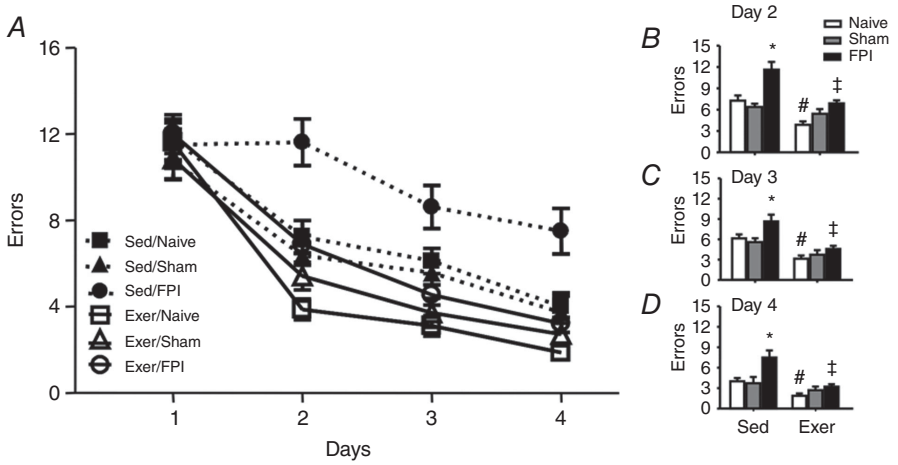

Figure 12. Previous physical training protects against the number of errors increase after FPI
Exercise training and FPI effects on the number of errors in Barnes maze test (*A*). *B–D*, previous swimming training decreased the number of errors *per se* and protected against the number of errors increase induced by FPI. Data are the mean ± SEM (n = 7–9 in each group). $^*P < 0.05$ compared to the sham and naive sedentary group. $^{\#}P <$ 0.05 compared with naïve-sedentary group.

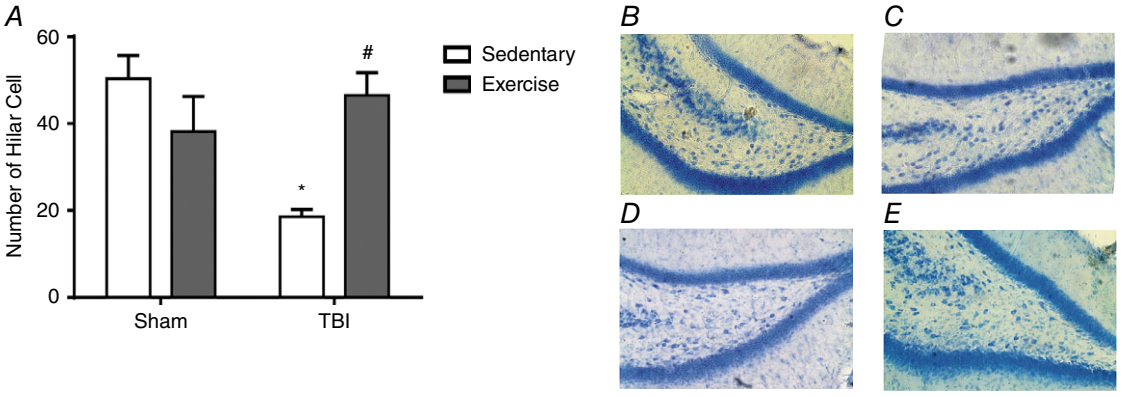

Figure 13. Previous physical training protects hippocampal cell loss after FPI
Histological analysis (H&E staining) demonstrated previous exercise training protected against cell loss in the dentate gyrus hilus induced by FPI (*A*). Histological analysis in the displays: sham/sedentary group (*B*), sham/trained (*C*), TBI/sedentary (*D*) and TBI/trained (*E*) animals. Data are expressed as the mean ± SEM (n = 6–7). $^*P < 0.05$ compared to all other groups. $^{\#}P < 0.05$ compared to the sedentary/TBI group. [Colour figure can be viewed at wileyonlinelibrary.com]

in cardiovascular function and a potential reduction in the incidence of several neurological diseases (Radak *et al.* 2008). In the present study, a clear stabilization of blood lactate concentration in the trained group during LT confirms the assumption that training increases aerobic resistance (Gobatto *et al.* 2001; Lima *et al.* 2009; Rambo *et al.* 2009; Souza *et al.* 2009; Lima *et al.* 2013). In addition, the results show that hepatic LXR-α and ABCA1 mRNA expression is higher in trained animals. LXRs are ligand-activated transcription factors that play an important role in the hepatic metabolism regulation of lipids and carbohydrates.

Apart from being glucose sensors (Mitro *et al.* 2007), LXRs also induce genes that inhibit the expression of inflammatory mediators, such as the ABCA1 (Spillmann *et al.* 2014). Because glucose is an endogenous LXR ligand and ABCA1 inhibits the expression of inflammatory mediators (Spillmann *et al.* 2014), it is plausible to propose that the anti-inflammatory effects elicited by exercise training involve hepatic glycogen metabolism (Hoene & Weigert, 2010) followed by LXR-α and ABCA1 mRNA increases (Ghanbari-Niaki *et al.* 2007; Kazeminasab *et al.* 2013). Noteworthy, inflammatory modulation may differ among TBI acute and delayed phases (Ziebell & Morganti-Kossmann, 2010). Although physiological nitric oxide (NO) production has beneficial anti-microbial and anti-tumor effects, excessive NO produced by iNOS mediates inflammatory signalling, causing ROS-mediated cell damage via peroxynitrite (Huang *et al.* 2012). Furthermore, lipid peroxidation initiated by peroxynitrite releases arachidonic acid from the cell membrane, which in turn activates COX-2 (Salvemini *et al.* 1995; Huang *et al.* 2012). Considering that prostaglandin E_2, a product of COX-2, is one of the strongest inflammatory mediators, it is plausible to propose the protection elicited by exercise training may be associated to prostaglandin E_2 blockade through COX-2 downregulation. Western blot assays have revealed that exercise training also protected against FPI-induced increases in IL-6 and TNF-α content in the liver. Because the hepatic pro-inflammatory cytokines TNF-α and IL-6 are relevant mediators for the stimulation of PGs synthesis by COX-2, our experimental data suggest that this exercise-mediated cytokine inhibition may account for COX-2 downregulation (Anthony *et al.* 2012). These data are consistent with the hypothesis that favourable changes in the hepatic anti-inflammatory status elicited by previous exercise training may exert a prophylactic effect on the early inflammatory response after severe TBI.

Liver is the main organ involved in endogenous glucose production, which is regulated by insulin though IRS and AKT pathways (Bugianesi *et al.* 2005). In this sense, the increase of blood glucose, insulin and HOMA-2%S after neuronal injury in the sedentary FPI group corroborates data from obese animal and human studies indicating that hepatic cytokine expression (TNF-α) activates intracellular JNK and phosphorylate insulin receptor substrate (IRS-1) at serine residues (Cai *et al.* 2005; Moschen & Tilg, 2008). Our experimental data also suggest that acute stress, such as TBI, may lead to hyperglycaemia, in the absence of diabetes, and also that strategies such as exercise training can enhance glucose control and reduce damage as a result of hyperglycaemia to several cells (Tsuzuki *et al.* 2015; Shi *et al.* 2016). Indeed, our experimental protocol of exercise training protected against blood glucose, insulin and HOMA-2%S increases, as well as hepatic immunoreactivity of pJNK increase, and also pIRS and pAKT decreases after neuronal injury.

Regarding the effects of TBI on liver function, it is important to consider that neutrophils produce superoxide anions ($O_2^{\cdot -}$) via NADPH oxidases: $O_2^{\cdot -}$ is further dismutated by SOD into hydrogen peroxide (H_2O_2) and molecular oxygen. In the presence of CAT, H_2O_2 is converted into H_2O and oxygen; otherwise, it can be used as a substrate by MPO to produce hypochlorous acid (HOCl), a highly reactive ROS that is associated with the production of protein carbonyls (Yan *et al.* 1996; Handelman *et al.* 1998). The increases in MPO and SOD activity combined with dampened CAT in the FPI sedentary group may indicate that higher levels of H_2O_2 formed by the SOD-mediated $O_2^{\cdot -}$ dismutation persist, increasing H_2O_2 production. The DCFH-oxidation data corroborate this rationale, considering that this is a H_2O_2-based assay (LeBel *et al.* 1992). Furthermore, the compromised CAT activity, a H_2O_2 decomposing enzyme, is usually a result of excessive H_2O_2, as well as 4-HNE, which forms aldehyde-protein adducts inactivating its active site (Kostyuk *et al.* 2010; Lubrano *et al.* 2015). Under these circumstances, the development of compensatory responses elicited by exercise training may be effective in attenuating FPI-induced redox status disruption, characterized in the present study by a decrease in GSH and -SH groups combined with increased protein carbonylation and DCFH-DA in the liver (Salo *et al.* 1991; Viguie *et al.* 1993; Leeuwenburgh & Heinecke, 2001). As such, our experimental data agree with the assumption that exercise training reinforces the liver anti-oxidant system (Boveris & Navarro, 2008; Sun *et al.* 2010).

In the present study, previous exercise training was effective against FPI-induced liver mitochondria dysfunction. It is worth noting that ROS generation is a necessary and unavoidable consequence of aerobic metabolism, and the rate of ROS generation in biological tissue is closely related to oxygen consumption (Toldy *et al.* 2005). Consequently, mitochondria are probably both a source and target of oxidant agents (Boveris & Navarro, 2008). Furthermore, the development of compensatory responses to oxidative stress elicited by exercise training (Salo *et al.* 1991; Viguie *et al.* 1993;

Leeuwenburgh & Heinecke, 2001) is a reflection of decreased markers of ROS generation, which translates into the maintenance of mitochondrial homeostasis in FPI-trained rats. This hypothesis has been upheld in animal studies, which indicate that a significant adaptive response to exercise training involves a greatly increased endurance capacity, facilitated by increases of O_2 consumption and mitochondrial biogenesis (Boveris & Navarro, 2008; Packer *et al.* 2008). In the present study, exercise training prevented the downregulation of relevant mitochondrial functioning parameters, such as the $\Delta\psi$ as well as CS and dehydrogenases activities.

Any cellular constituent may be a ROS target, although selected targets are more prone to induce toxicity (Lima *et al.* 2009). The Na^+,K^+-ATPase appears to be particularly sensitive to ROS-induced damage, considering that its inhibition has been associated with alterations in membrane lipid composition, -SH groups redox and amino acid residues induced by ROS (Jamme *et al.* 1995; Siems *et al.* 1996). Therefore, it is tempting to propose that the presently reported FPI-induced ROS generation and Na^+,K^+-ATPase activity decrease in liver are causally related. Although the stress response of liver did not induce early hepatocellular damage, the collapse of ion gradient homeostasis provides an explanation for the crucial role played by the liver in secondary damage after neuronal injury (Anthony *et al.* 2012). Indeed, the increase in the Michaelis–Menten constant (i.e. the K_m) for the main Na^+,K^+-ATPase substrate in the FPI-sedentary group suggests that ATP affinity is reduced in the liver of these animals. Importantly, the reduced affinity of hepatic Na^+,K^+-ATPase for ATP could be exacerbated by mitochondrial energy metabolism dysfunction. This finding is particularly important, considering the exercise training protocol induced compensatory responses to oxidative stress and protected against FPI-induced Na^+,K^+-ATPase activity diminution. Thus, the findings of the present study support the assumption that exercise training modulates oxidative-inflammatory functions in the liver (Barcelos *et al.* 2017) and thus delays or prevents secondary cascades that lead to several disabilities after a TBI (Griesbach *et al.* 2004; Gomez-Pinilla & Kostenkova, 2008).

Furthermore, favourable changes in the hepatic oxidative-inflammatory status elicited by previous exercise training may exert prophylactic effects on acute hyperglycaemia and the cerebral inflammatory response induced by severe TBI. It is important to note that the influx of leukocytes to an inflammatory site is orchestrated by a sequential upregulation of adhesion molecules on vascular endothelium, leading post-traumatic oedema and BBB breakdown (Baskaya *et al.* 1997; Unterberg *et al.* 1997). Moreover, an imbalance in the ratio of harmful ROS/RNS ratio and BBB dysfunction after TBI causes delayed neuronal dysfunction and death through secondary processes involving increased excitatory amino acids levels and ionic equilibrium loss (Lenzlinger *et al.* 2004; Shlosberg *et al.* 2010). Our experimental data agree with this assumption when TBI induced plasma fluorescein extravasation (an indicator of BBB breakdown) and cerebral MPO activity increase, whereas previous exercise training attenuated this neuronal dysfunction. Exercise training was also effective against an increase in IL-6 and TNF-α content, and Na^+,K^+-ATPase activity inhibition. Considering that alterations in the redox state of regulatory -SH groups in selected targets, such as Na^+, K^+-ATPase (Morel *et al.* 1998), increases cellular excitability after TBI (Rao *et al.* 2008), our data reinforce the idea that ROS-induced Na^+,K^+-ATPase inhibition may contribute to FPI-induced neurological dysfunction characterized by early neuromotor dysfunction and spatial learning impairment (Lima *et al.* 2008; Mota *et al.* 2012; da Silva Fiorin *et al.* 2016).

Recently, a considerable body of evidence has demonstrated that oxidative inflammatory-related cascades resulting from TBI have been implicated in altered signal transduction in peripheral organs and the CNS (Hall *et al.* 2004; Bayir *et al.* 2007; Lima *et al.* 2013). Although a pre-injury regimen for humans may not be the most effective treatment because the time of injury cannot be predicted (Vaynman & Gomez-Pinilla, 2005), the effective protection exerted by exercise training in this model of TBI is of particular interest because it supports the assumption that metabolic chances elicited by exercise delay or prevent secondary cascades which lead to long-term cell damage after TBI. Indeed, our experimental data revealed that previous exercise training protected from cell loss induced by TBI. Considering that a loss in number of interneurons after experimental TBI (Lowenstein *et al.* 1992; Santhakumar *et al.* 2000; Grady *et al.* 2003; Hall *et al.* 2005) can be associated with a reduction in granule cells synaptic inhibition (Hunt *et al.* 2011; Pavlov *et al.* 2011; Gupta *et al.* 2012), we suggest that exercise-induced reduction of the initial damage limits the long-term secondary degeneration and supports neural repair or behavioural compensation after neuronal injury (Wannamethee & Shaper, 1992; Wang *et al.* 2001; Klein *et al.* 2003). However, it is worth noting that simultaneous hippocampal cell loss and spatial memory dysfunction in this TBI model do not necessary imply a cause–effect relationship between these events. It is important to note that hippocampal cells are a mixed population of mostly inhibitory interneurons and the assay used does not allow the identification of these cells. Consequently, our experimental data are speculative and additional studies are necessary to clarify the involvement of these pathways in the prophylactic effect of exercise training after TBI.

In conclusion, the present study describes TBI induction of inflammation and oxidative stress of rats, which disrupt redox status and mitochondrial function in the liver. These responses originate not from early hepatocellular damage, but from a collapse of the ion gradient homeostasis followed by dysglycaemia and impaired hepatic signalling observed in sedentary rats after neuronal injury, which suggests that neuronal injury signals may impact on organs away from the injury site. High levels of circulating cytokines after TBI also affected brain function, characterized in the present study by BBB opening, neutrophil infiltration and oxidative-inflammatory increases. Neuronal inflammation and oxidative stress leads to a marked impairment of protein function, neurobehavioural disability and long-term cell damage after TBI. Although a pre-injury prophylactic strategy may not be the most effective treatment for humans because the occurrence of injury cannot be predicted, the effect of exercise on the expression/activity of specific endogenous proteins is particularly interesting. The findings reported in the present study support the idea that exercise training modulates oxidative-inflammatory functions (Barcelos *et al.* 2017) and thus delays or prevents secondary cascades that lead to several disabilities after a TBI (Griesbach *et al.* 2004; Gomez-Pinilla & Kostenkova, 2008). Therefore, approaches aiming to counteract or attenuate secondary damage developed over a period of hours to days after a TBI, such as exercise training, may have preventive and/or therapeutic potential through the modulation of responses by metabolic-related organs, such as the liver and brain.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author's contributions

LFFR, MRTC, GB and JGG conceived and designed the experiments. MRTC, APOF, GLB, LRHS, MEPJ, FF, RPB, MJC and GA performed the assays. LFFR, MRTC, MRF and MECL analysed the data. LFFR, MRTC, MECL, MRF, GB and JGG drafted the article. All authors approved the final version of the manuscript submitted for publication.

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