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Research Report

Adaptation to oxidative challenge induced by chronic physical exercise prevents Na^+,K^+-ATPase activity inhibition after traumatic brain injury

Frederico Diniz Lima[a], *Mauro Schneider Oliveira*[a,b], *Ana Flávia Furian*[a,b], *Mauren Assis Souza*[a], *Leonardo Magno Rambo*[a], *Leandro Rodrigo Ribeiro*[a], *Luiz Fernando Almeida Silva*[a,c], *Leandro Thies Retamoso*[a,c], *Maurício Scopel Hoffmann*[a], *Danieli Valnes Magni*[d], *Leticia Pereira*[a], *Michele Rechia Fighera*[a,b,e], *Carlos Fernando Mello*[a], *Luiz Fernando Freire Royes*[a,d,e,*]

[a]*Centro de Ciências da Saúde, Laboratório de Neurotoxicidade e Psicofarmacologia, Departamento de Fisiologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*

[b]*Programa de Pós-graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil*

[c]*Centro de Educação Física e Desportos, Departamento de Métodos e Técnicas Desportivas, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*

[d]*Centro de Ciências Naturais e Exatas, Laboratório de Neurotoxicidade, Departamento de Química, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*

[e]*Centro de Ciências da Saúde, Departamento de Pediatria, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*

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ABSTRACT

Physical exercise is likely to alter brain function and to afford neuroprotection in several neurological diseases. Although the favorable effects of physical exercise on traumatic brain injury (TBI) patients is well known, little information is available regarding the role of free radicals in the improvement induced by physical exercise in an experimental model of TBI induced by fluid percussion injury (FPI). Thus, we investigated whether 6 weeks of swimming training protects against oxidative damage (measured by protein carbonylation and thiobarbituric acid-reactive substances-TBARS) and neurochemical alterations represented by immunodetection of α subunit and activity of Na^+,K^+-ATPase after FPI in cerebral cortex of rats. Statistical analysis revealed that physical training protected against FPI-induced TBARS and protein carbonylation increase. In addition, physical training was effective against Na^+,K^+-ATPase enzyme activity inhibition and α_1 subunit level decrease after FPI. Pearson's correlation analysis revealed that the decrease in levels of catalytic α_1 subunit of Na^+,K^+-ATPase induced FPI correlated with TBARS and protein carbonylation content increase. Furthermore, the effective protection exerted by physical training against FPI-induced free radical correlated with the immunocontent of the catalytic α_1 subunit maintenance. These data suggest that TBI-induced reactive oxygen species (ROS) generation decreases Na^+,K^+-ATPase activity by decreasing the total number of enzyme molecules, and

* *Corresponding author*. Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. Fax: +55 55 3220 8031.
E-mail address: nandoroyes@yahoo.com.br (L.F.F. Royes).

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that physical exercise protects against this effect. Therefore, the effective protection of selected targets, such as Na^+,K^+-ATPase induced by physical training, supports the idea that physical training may exert prophylactic effects on neuronal cell dysfunction and damage associated with TBI.

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1. Introduction

Traumatic brain injury causes delayed neuronal dysfunction and death through secondary processes involving increased excitatory amino acids levels, loss of ionic equilibrium, decreased ATP production (Faden et al., 1989; Ross and Soltesz, 2000; Osteen et al., 2001; Sullivan et al., 2005) and increased free radical formation (Singh et al., 2006; Shao et al., 2006, Davis et al., 2008). Accordingly, increased levels of markers of oxidative stress have been found in human cerebrospinal fluid after TBI (Varma et al., 2003) as well as in controlled cortical impact models of TBI (Opii et al., 2007). Moreover, experimental findings from our group have showed that acute FPI-induced impairment in an animal spatial learning task increased oxidative stress markers and decreased Na^+,K^+-ATPase activity, suggesting that ROS-induced Na^+,K^+-ATPase activity inhibition may contribute to the deficits in spatial learning after TBI (Lima et al., 2008).

The ion pump Na^+,K^+-ATPase is an ubiquitous plasma membrane protein which plays a key role in the maintenance of intracellular electrolyte homeostasis in virtually all tissues. In the Central Nervous System (CNS), Na^+,K^+-ATPase activity accounts significantly for the maintenance of the electrochemical gradient across the plasma membrane underlying resting, action potentials as well as the modulation of neurotransmitter release and uptake. As a consequence, a decrease of Na^+,K^+-ATPase activity directly affects neurotransmitter signaling, neural activity, as well as the whole animal behavior (Jamme et al., 1995; Li and Stys, 2001; Lees et al., 1990). Furthermore, Na^+,K^+-ATPase seems to be particularly sensitive to free radical-induced damage because its inhibition has been associated with alterations in plasma membrane lipid composition (Dencher et al., 2007), in the redox state of regulatory sulphydryl groups (Pari and Murugavel, 2007) and in other amino acid residues caused by free radicals or lipid peroxidation (Siems et al., 1996; Potts et al., 2006).

Maintaining brain health and plasticity throughout life is an important public health goal and, thus, the beneficial effects of exercise on the brain are becoming increasingly evident (Ang and Gomez-Pinilla, 2007). Studies in human and rodents have demonstrated that physical exercise has the capacity to enhance learning and memory (Kramer et al., 2006; Fordyce and Farrar, 1991) under a variety of conditions, from counteracting the mental decline associated with aging (Erickson and Kramer, 2009) to facilitating functional recovery in patients suffering from brain injury and diseases (Bohannon, 1993). Moreover, it has been demonstrated that performing exercise prior to brain trauma produces prophylactic effects on attendant brain damage, such as limiting the infarct size following forebrain ischemia (Endres et al., 2003) and inducing transoperative benefits in animal models of stroke (Hicks et al., 1998).

Among the variety of physical exercises most used in researches involving animals, treadmill running and swimming stand out (Carvalho et al., 2005; Prada et al., 2004). Although there are still doubts in regarding which exercise (swimming or treadmill running) would be the most suitable to avoid unnecessary stress to the animals, the use of swimming rats as a model of exercise presents advantages since swimming is a natural ability of the rats. In this context, studies using swimming as an animal model of training revealed the occurrence of adaptation to physical training similar to those observed in human beings (Voltarelli et al., 2002).

Although it is believed that physical exercise on general health and neurorehabilitation after traumatic brain injury may be useful (Ang and Gomez-Pinilla, 2007), little information is available regarding the prophylactic role of physical exercise on deleterious effects induced by TBI. In this study, we aimed to investigate whether previous swimming training protects against oxidative damage (protein carbonylation and TBARS) and neurochemical alterations (immunodetection of α_1 subunit and activity of Na^+,K^+-ATPase) after TBI.

2. Results

A significant increase in total body weight of sedentary versus trained rats along six weeks of swimming training [$F(1,14)=6.94$; $P<0.05$; Fig. 1A] was observed. In addition, statistical analyses showed a clear stabilization of the blood lactate concentration in the trained group when compared with the sedentary group [$F(1,12)=12.47$; $P<0.04$; Fig. 1B], indicating that the training program increased the aerobic resistance of the animals (Gobatto et al., 2001).

Determination of TBARS content and protein carbonylation levels, 48 h after FPI or sham-operation procedure, are depicted in Figs. 2A and B, respectively. Statistical analysis revealed that FPI induces an increase of TBARS [$F(2,41)=10.69$; $P<0.001$] and protein carbonyl content [$F(2,41)=5.81$; $P<0.006$] in ipsilateral cortex in sedentary animals when compared with sham and naive group. On the other hand, statistical analysis also revealed that physical training protected against the increase of TBARS [$F(2,41)=6.66$; $P<0.03$; Fig. 2A] and protein carbonyl content [$F(2,41)=5.62$; $P<0.007$; Fig. 2B] in the ipsilateral cortex after FPI. Physical training also attenuated TBARS production [$F(2,41)=7.05$; $P<0.05$; Fig. 2A] and protein carbonylation [$F(2,41)=8.27$; $P<0.05$; Fig. 2A] in naive animals when compared with sham-operated animals, indicating that this protocol of physical training induces compensatory responses to oxidative stress in sham-operated animals.

Fig. 3 shows the effect of physical training and FPI on Na^+, K^+-ATPase activity, 48 h after FPI or sham-operation procedure. Statistical analyses revealed that FPI induces a decrease

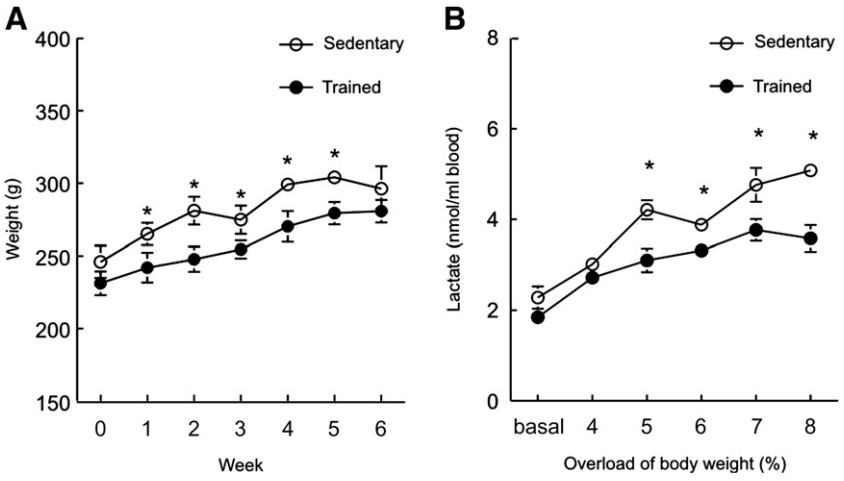

Fig. 1 – Effect of 6 weeks of swimming training on body weight (A) and lactate threshold assay (B). *P<0.05 compared with trained group (*F* test for simple effect). Data mean±S.E.M. for *n*=6 in each group.

in Na^+,K^+-ATPase activity in the ipsilateral cerebral cortex of sedentary animals [$F(2,41)=5.33$; $P<0.009$], and that previous physical training protected against this inhibition [$F(2,41)=4.05$; $P<0.05$]. In order to investigate whether FPI-induced decrease in Na^+,K^+-ATPase activity was due to a decrease in the levels of available enzyme molecules, we measured the immunocontent of the α_1 subunit of Na^+,K^+-ATPase in cerebral cortex. Statistical analysis revealed that FPI decreased the immunocontent of the α_1 subunit of Na^+,K^+-ATPase in the ipsilateral cerebral cortex of sedentary animals [$F(2,41)=7.35$; $P<0.002$, Fig. 3B] and that previous physical training protected against this reduction [$F(2,41)=2.79$; $P<0.05$].

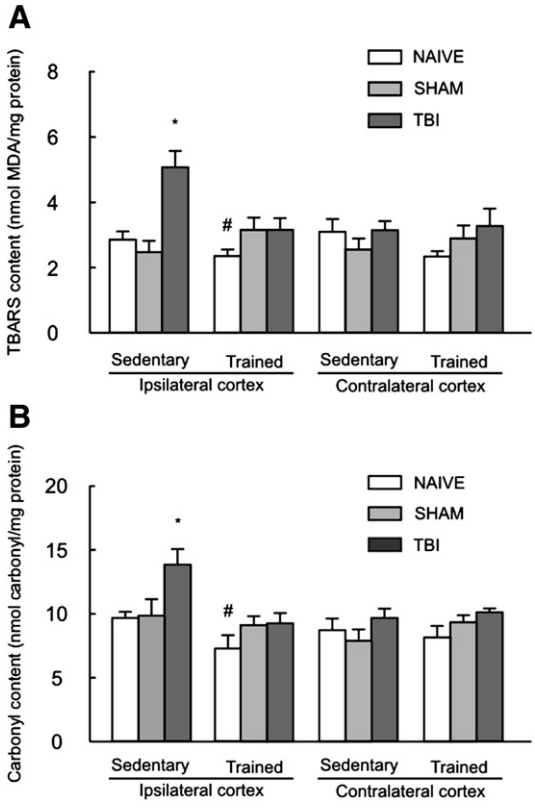

Fig. 2 – Physical training prevents TBI-induced TBARS (A) and protein carbonylation increase (B). Data are mean+S.E.M. for *n*=8 in each group. *P<0.05 compared to sham and naive sedentary group. #P<0.05 compared with trained-FPI and sham trained groups (Student–Newman–Keuls test).

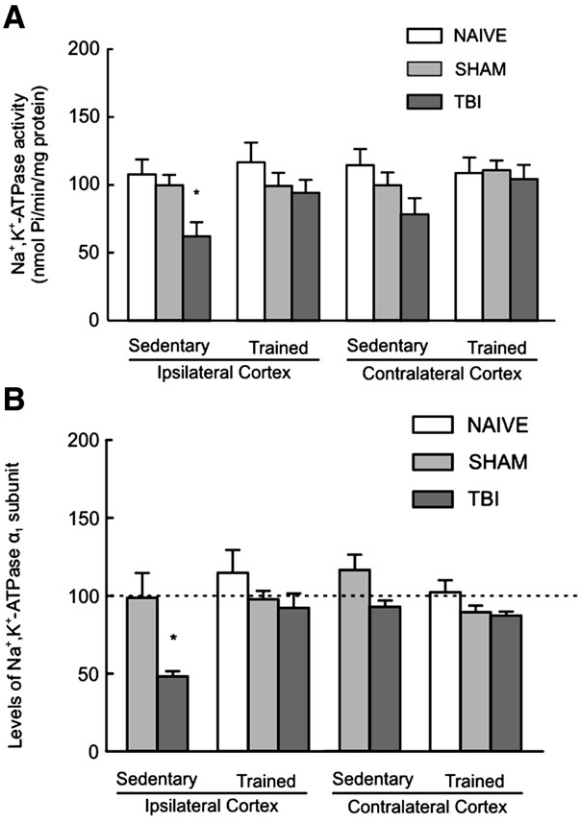

Fig. 3 – Effect of physical training and TBI Na^+,K^+-ATPase activity (A) and α subunit levels (B). Data are mean+S.E.M. for *n*=8 in each group. *P<0.05 compared to sham and naive group. (Student–Newman–Keuls test).

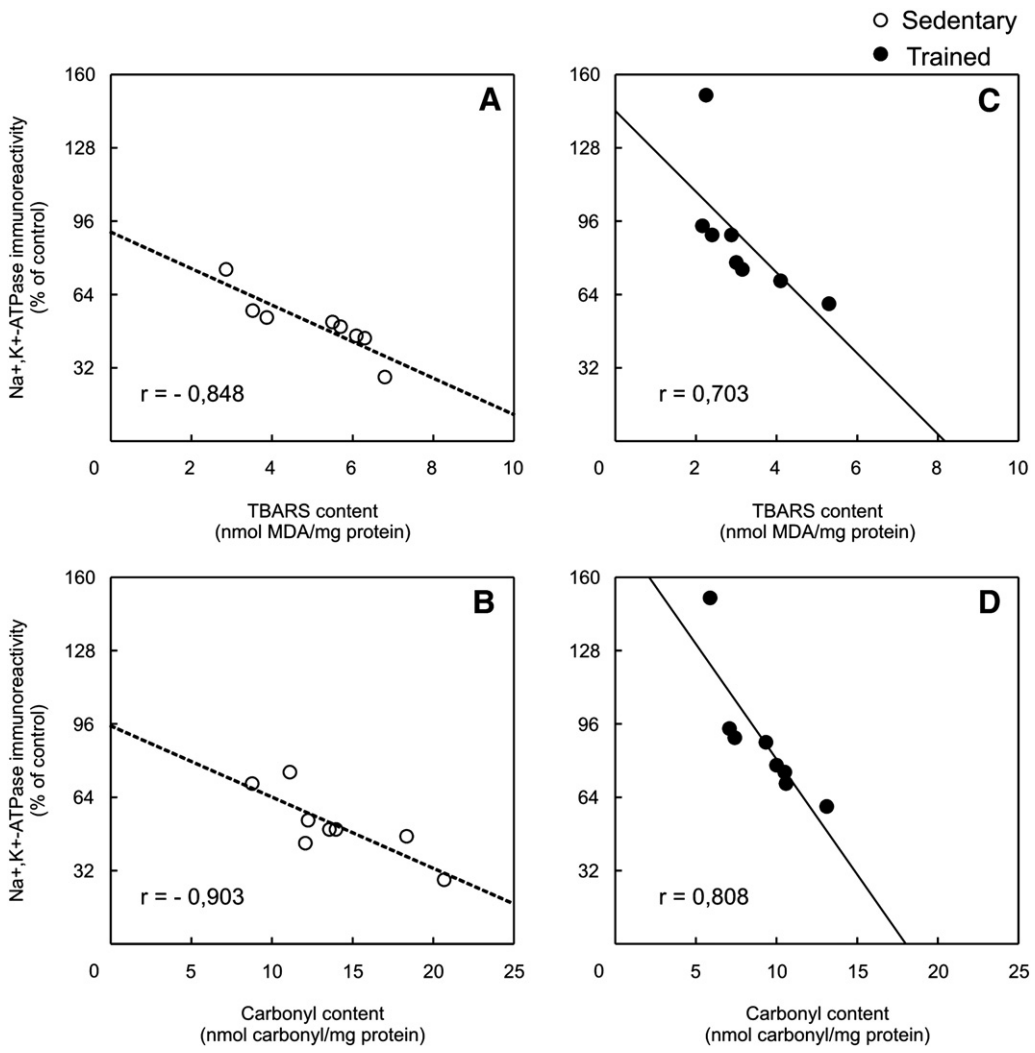

Fig. 4 – Correlation between the level of Na^+,K^+-ATPase α_1 subunit after TBI and TBARS production and protein carbonylation in the ipsilateral cerebral cortex of sedentary animals (A and B) and in trained animals (C and D), respectively.

Considering that Na^+,K^+-ATPase α_1 subunit is one of the targets for ROS and is directly involved in oxidative stress (Dada et al., 2003), we studied whether these events are interconnected in this model of TBI. Correlation analysis (Pearson's correlation analysis) revealed that reduction in the number of Na^+,K^+-ATPase α_1 subunit after FPI correlated with TBARS production ($r=-0.848$; $P<0.009$; Fig. 4A) and protein carbonylation ($r=-0.890$; $P<0.003$; Fig. 4B) in the ipsilateral cerebral cortex of sedentary animals. In addition, the Pearson's correlation revealed negative correlation between TBARS production ($r=0.703$; $P<0.05$) and protein carbonyl content ($r=0.808$; $P<0.008$) with Na^+,K^+-ATPase α_1 subunit in ipsilateral cortex of animals trained after FPI.

3. Discussion

We have confirmed and extended our previous findings assuring that a single FPI episode in rat parietal cortex decreases Na^+,K^+-ATPase activity with concomitant increase in the levels of oxidative stress markers (Lima et al., 2008). Moreover, we have demonstrated a significant correlation between the decrease of Na^+,K^+-ATPase α_1 subunit with the increase of TBARS and protein carbonyl content after FPI. On the other hand, 6 weeks of swimming training protocol affords significant protection against FPI-induced TBARS formation, protein carbonylation and Na^+,K^+-ATPase activity inhibition. These data agree with the assumption that adaptive responses to regular and moderate endurance exercise might reduce production of oxidants and a radical leak during oxidative phosphorylation (Packer et al., 2008). Furthermore, the negative correlation between TBARS and protein carbonyl content with Na^+,K^+-ATPase α_1 subunit in animals trained after FPI reinforces the idea that compensatory responses to oxidative stress elicited by this protocol of physical training protect against the failure of some selected targets, such as Na^+,K^+-ATPase enzyme.

Concerns about increased death frequency after TBI and its potential for secondary injury have led to overprotective measures by many health care professionals (Werner and

Engelhard, 2007). Although a preinjury exercise regimen for humans may not be the most effective treatment since the time of injury cannot be predicted (Vaynman and Gomez-Pinilla, 2005), the protection exerted by physical training in this model of TBI is of particular interest because it supports the idea that physical training may exert prophylactic effects on brain damage after FPI. In line with this view, studies with humans and animals have demonstrated that previous physical training reduces the risk of stroke and heart attacks in men both with and without pre-existing ischemic heart disease (Wannamethee and Shaper, 1992) and reduces post-ischemic infarction and edema in brain of tested Sprague–Dawley rats (Stummer et al., 1994; Wang et al., 2001). Furthermore, preinjury exercise has been shown to have transoperative benefits in a model of Parkinsonism (Smith and Zigmond, 2003; Tillerson et al., 2003). Such studies intend to reduce the levels of initial damage, to limit long-term secondary degeneration, and to support neural repair or behavioral compensation (Kleim et al., 2003).

In the present study, we have showed that parameters that indicate generation of oxidative stress and collapses in ion gradient homeostasis in cortical homogenates were affected after FPI. These data, to some extent, agree with previous studies that have demonstrated that oxidative damage mediated by reactive oxygen and nitrogen species is well recognized as a significant component of the secondary injury cascade that accompanies TBI (Potts et al., 2006, Yilmaz et al., 2007).

It is worth pointing out that ROS generation is a necessary and unavoidable consequence of aerobic metabolism, and the rate of free radical or oxidant generation in biological tissue is closely related to oxygen consumption (Toldy et al., 2005). Consequently, it seems that mitochondria, in addition to being the sources of oxidant production, are also targets of oxidants (Boveris and Navarro, 2008). In this context, a significant number of studies have proposed that post-traumatic sequelae establish conditions of increased metabolic demands on the reduced mitochondrial population, resulting in the depletion of metabolic substrate such as glucose and piruvate (Vink et al., 1994; Bentzer et al., 2000; Stahl et al., 2001) due to a reduction in metabolic enzyme activity (cytochrome oxidase, succinate dehydrogenase) (Hovda et al., 1991).

Considering that the significant adaptive response to regular exercise involves the increase of O_2 consumption and mitochondrial biogenesis (Packer et al., 2008; Boveris and Navarro, 2008), it is plausible to propose that previous physical training may result in improved ability of the neuron to withstand TBI-mediated energy depletion and consequently ROS generation (Soustiel and Sviri, 2007; Opii et al., 2007). This hypothesis has been upheld in animal studies that indicate that significant adaptive response to regular exercise involves greatly increased endurance capacity, permitted by the increase of O_2 consumption and mitochondrial biogenesis (Packer and Cadenas, 2007; Boveris and Navarro, 2008).

Though treadmill running is mentioned as the most used exercise type in experiments with animals (Daggan et al., 2000; Carvalho et al., 2005), the use of swimming rats physical exercise model presents advantages over treadmill running. For instance, swimming is a natural ability of the rat, and this avoids the selection of the animals, which is necessary in experimental protocols using treadmill running (Arida et al., 1999). Moreover, the difficulty for velocity maintenance and presence of electric stimulus as stress factor are additional pitfalls of the treadmill running (Gobatto et al., 2001). Despite such methodological differences, both swimming and treadmill running seems to be good models for investigate the effects of physical training on laboratory animals. In this context, we found a clear stabilization of the blood lactate concentration in trained versus sedentary rats in the lactate threshold test. Since previous studies have suggested that stabilization of blood lactate in trained animals is due to muscle aerobic adaptations leading to lower lactate production and/or increased blood lactate removal for the same relative and absolute workload (Jones and Carter, 2000; Gladden, 2000; Donovan and Pagliassotti, 2000), our results are in agreement with the idea that swimming training is an effective protocol to induce muscle aerobic adaptations in rats, similar to those observed in human beings (Voltarelli et al., 2002).

In the present study, we also revealed that FPI decreased Na^+,K^+-ATPase activity and the immunocontent of the α_1 subunit of Na^+,K^+-ATPase. In addition, the negative correlation between the number of Na^+,K^+-ATPase α_1 subunits with TBARS and protein carbonylation increase in the ipsilateral cerebral cortex of sedentary animals after FPI suggest that Na^+,K^+-ATPase enzyme is one cellular oxidative stress target (Dobrota et al., 1999).

Although it is believed that the Na^+,K^+-ATPase activity can be regulated by changes in catalytic activity brought about by changes in affinity for its major substrates (Ross and Soltesz, 2000), it is plausible to propose that Na^+,K^+-ATPase activity is regulated by endocytosis or exocytosis of Na^+ pump molecules between the plasma membrane and intracellular compartments (Bertorello et al., 1999; Chibalin et al., 1999) after FPI. In line with this view, experimental findings in epithelial cells exposed to hypoxia through mitochondria generated-ROS activate an intracellular signaling pathway, triggering the endocytosis of Na^+,K^+-ATPase molecules through the activation of PKC-ζ and phosphorylation of Ser-18 of the α_1 subunit of Na^+,K^+-ATPase (Dada et al., 2003; Chen et al., 2006). However, further in-depth studies are necessary to definitively establish the mechanism involved.

Experimental findings did not demonstrate directly the definitive target of physical exercise mediated neuroprotection after FPI. However, the strong inverse correlation between TBARS production and protein carbonyl content with Na^+,K^+-ATPase α_1 subunit in trained rats suggests that the prophylactic effect elicited by physical training in this model of TBI was due to compensatory responses to oxidative stress and consequent maintenance of Na^+,K^+-ATPase structure. This is particularly important considering that mitochondrial oxidative damage precedes the onset of neuronal loss after TBI (Singh et al., 2006) and the phosphorylation of the Na^+,K^+-ATPase α_1 subunit mediated by mitochondria-ROS generation constitutes the triggering mechanisms that initiate its endocytosis (Chen et al., 2006). Thus, a reduction of mitochondrial-ROS generation induced by physical training (Boveris and Navarro, 2008) may protect against Na^+,K^+-ATPase inhibition and progressive pathobiological changes induced by TBI (Ross and Soltesz, 2000; Jain, 2008).

It is also important to point out that a significant increase in total body weight in sedentary versus trained rats was observed along the six weeks of the swimming training. The difference in body weight between sedentary and trained rats may be explained by changes in body composition. For instance, a decrease in subcutaneous adipose tissue of trained rats may explain why body mass was lower in this group. Since we have not determined body composition in the present study, this explanation remains speculative in nature, and further studies are necessary to determine the mechanisms involved. In addition, the clear stabilization of the blood lactate concentration in the trained group when compared with sedentary group for the lactate threshold assay suggests that the training program increased aerobic resistance of the animals (Gobatto et al., 2001).

In conclusion, the present study reports that FPI increased the levels of oxidative stress markers followed by inhibition of Na^+,K^+-ATPase activity and α_1 subunit decrease. In addition, the effective protection exerted by previous physical training against these deleterious effects provides a framework to guide further studies to examine the mechanisms by which exercise alters neuronal functions and thus, delay or prevent secondary cascades that lead to long-term cell damage and neurobehavioral disability after TBI.

4. Experimental procedures

4.1. *Animal and reagents*

All experiments involving the animals were conducted in conformance with the policy statement of the European Communities Council Directive (86/609/EEC) and adequate measures were taken to minimize pain and discomfort. In the present study 90-day-old male Wistar rats, weighing 220–260 g at the beginning and 270–320 g at the end of the experimental period were used. During this period, the animals were maintained in a controlled environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All efforts were made to reduce the number of animals used, as well as to minimize their suffering. Reagents were purchased from Sigma (St. Louis, MO).

4.2. *Adaptation to the water*

All rats were adapted to water before the beginning of the experiment. The adaptation consisted of keeping the animals in shallow water at 32 °C between 9:00 and 11:00 a.m. The adaptation period proceeded during the entire experimental period, and the purpose of the adaptation was to reduce stress without promoting a physical training adaptation.

4.3. *Swimming training protocol and lactate threshold assay*

The use of swimming rats as a model of exercise presents advantages over treadmill running, since swimming is a natural ability of the rats. This feature avoids the selection of animals, which is necessary in experimental protocols using treadmill running (Arida et al., 1999). For exercise training, rats were randomly assigned to the following groups: trained/naive, trained/sham craniotomy and trained/TBI. The training period lasted 6 week and consisted of 60-min daily sessions five times per week. Swimming was always performed in water at a temperature of 32 °C between 9:00 and 11:00 a.m. All animals underwent a swimming adaptation period without weights during the first week of training. After the swimming adaptation period, the rats were subjected to swimming training with a workload (5% of body weight) to improve endurance (Gobatto et al., 2001). Together with the training session, sedentary rats were placed in the bottom of a separate tank with shallow water (5 cm) at 32 °C for 30 min, 5 days/week without a workload (5% of body weight). After six weeks of training, a test protocol was used to determine the lactate threshold (LT) in sedentary (n=7) and trained rats (n=7). The LT test was carried out according to the protocol described by Marquezi et al. (2003) and consisted of swimming exercises with progressive overload through weights attached to the animal's tail, corresponding to 4%, 5%, 6%, 7%, and 8% of body weight of each animal for 3-min periods, separated by 1-min resting periods. During the resting periods, 25 μl blood samples were collected from the tail vein into heparinized capillary tubes for determination of lactate concentration. The LT for each animal was calculated based on the point of inflection of the graph when plotting lactate concentration against the corresponding exercise workload.

4.4. *Traumatic brain injury*

After six weeks of training, the sedentary and trained rats underwent lateral fluid percussion injury (FPI). Rats assigned to sham groups were anesthetized and connected to the injury device, but received no injury. The FPI was carried out as described previously (D'Ambrosio et al., 1999; 2004). Briefly, animals were anesthetized with a single i.p. injection of Equithesin (6 ml/kg), a mixture containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%) and placed in a rodent stereotaxic apparatus. 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, taking care to keep the dura mater intact. A plastic injury cannula was placed over the craniotomy with dental cement. When the dental cement hardened, the cannula was filled with Chloramphenicol, closed with a proper plastic cap and the animal was removed from the stereotaxic device and returned to its homecage. After 24 h, the animals were anesthetized with Halothane, and had the injury cannula attached to the fluid percussion device and placed in a heatpad 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 (3.53±0.17 atm) impact was applied against the exposed dura. Pressure pulses were measured extracranially by a transducer (Fluid Control Automação Hidráulica, Belo Horizonte, MG, Brazil) and recorded on a storage oscilloscope (Gould Ltd., Essex, UK). Sham-operated animals underwent an identical

procedure, with the exception of the fluid percussion injury (FPI). Immediately after these procedures, the cannula was removed and the orifice was covered with dental cement. Naive rats underwent randomization with no further intervention.

4.5. *Tissue processing for neurochemical analyses*

Considering that the early free radical generation (3 to 72 h) has been postulated to lead to oxidative damage and adversely affect synaptic function and plasticity after TBI (Singh et al., 2006; Ansari et al., 2008), the animals were killed by decapitation 48 h after TBI and had their brain exposed by the removal of the parietal bone. The cerebral cortex was rapidly dissected on an inverted ice-cold Petri dishand divided in ipsilateral and contralateral cortex. Each tissue sample was homogeneized in cold 10 mM Tris–HCl buffer (pH 7.4) and then divided in aliquots for subsequent neurochemical analyses, as described below.

4.6. *Measurement of TBARS content*

TBARS content was estimated in a medium containing 0.2 ml of brain homogenate, 0.1 ml of 8.1% SDS, 0.4 ml of acetic acid buffer (500 mM, pH 3.4), and 0.75 ml of 0.81% thiobarbituric acid (TBA). The mixture was finally made up to 2 ml with type I ultrapure water and heated at 95 °C for 90 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm (Ohkawa et al., 1979).

4.7. *Measurement of the protein carbonyl content*

Total protein carbonyl content was determined by the method described by Yan et al. (1995), adapted for brain tissue (Oliveira et al., 2004). Briefly, homogenates were diluted to 750–800 μg/ml of protein in each sample, and 1 ml aliquots were mixed with 0.2 ml of 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.2 ml HCl (2 M). After incubation at room temperature for 1 h in a dark ambient, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.8 ml of heptane (99.5%), and 1.8 ml of ethanol (99.8%) were added sequentially and mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1 ml of ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 ml of 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 calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}, as described by Levine et al. (1990).

4.8. *Na^+,K^+-ATPase activity measurement*

Assay of Na^+,K^+-ATPase activity was performed according to Wyse et al. (2000). 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 $MgCl_2$, and 50 μg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 μl. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μl of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and the reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

4.9. *Immunodetection of Na^+,K^+-ATPase α_1 subunit*

In order to investigate whether the possible alteration of Na^+,K^+-ATPase activity after TBI and physical training was due to a decrease or increase in the levels of available enzyme molecules, we performed immunodetection analysis for the α subunit of Na^+,K^+-ATPase in cerebral cortex. Slot blot analysis was used for quantification of Na^+,K^+-ATPase α_1 subunit levels, according to the procedures described by Banjac et al., 2001, with minor modifications. Briefly, cerebral cortex (ipsi and contralateral site) was homogenated in 30 mM Tris–HCl buffer, pH 7.4, and centrifuged at 800 *g* at 4 °C for 10 min. The supernatant was then centrifuged at 16,000 *g* at 4 °C for 20 min. The pellet was re-suspended in 5 mM phosphate-buffered saline (PBS, pH 7.4) and protein content was normalized to 3 μg/ml. Next, 250 μl of these homogenized were loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 4% (w/v) fat-free dry milk in PBS, containing 0.04% (v/v) Tween 20 for 2 h and incubated with a 1:4000 dilution of anti-Na^+,K^+-ATPase α subunit polyclonal antibody in PBS, containing 0.01% (w/v) sodium azide and 0.04% (v/v) Tween 20 (PBS) for 1 h. The membrane was washed three times in PBS and was incubated for 1 h with an anti-alkaline phosphatase secondary antibody diluted in PBS in a 1:8000 ratio. The membrane was washed for three times in PBS for 5 min and developed using the BCIP/NBT method. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).

4.10. *Protein determination*

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as standard.

4.11. *Statistical analyses*

Data from body weight and blood lactate concentration were analyzed by one-way analysis of variance (ANOVA). Data from total protein carbonylation, TBARS, immunodetection of α_1 subunit and Na^+,K^+-ATPase activity determinations *ex vivo* were analyzed by three-way ANOVA. Pos hoc analyses were carried out by the *F* test simple effect or the Student–Newman–Keuls, when appropriate. All data are expressed as mean + S.E.M. Correlation analyses were carried out using the Pearson's correlation coefficient. $P<0.05$ was considered significant.

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REFERENCES

Ang, E.T., Gomez-Pinilla, F., 2007. Potential therapeutic effects of exercise to the brain. Curr. Med. Chem. 14, 2564–2571.

Ansari, M.A., Roberts, K.N., Scheff, S.W., 2008. Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. Free Radic. Biol. Med. 45, 443–452.

Arida, R.M., Scorza, F.A., dos Santos, N.F., Peres, C.A., Cavalheiro, E.A., 1999. Effect of physical exercise on seizure occurrence in a model of temporal lobe epilepsy in rats. Epilepsy Res. 37, 45–52.

Banjac, A., Nedeljkovic, N., Horvat, A., Kanazir, D., Nikezic, G., 2001. Ontogenetic profile of ecto-ATPase activity in rat hippocampal and caudate nucleus synaptic plasma membrane fractions. Physiol. Res./Acad. Sci. Bohemoslovaca 50, 411–417.

Bentzer, P., Davidsson, H., Grande, P.O., 2000. Microdialysis-based long-term measurements of energy-related metabolites in the rat brain following a fluid percussion trauma. J. Neurotrauma. 17, 441–447.

Bertorello, A.M., Ridge, K.M., Chibalin, A.V., Katz, A.I., Sznajder, J.I., 1999. Isoproterenol increases Na+–K+-ATPase activity by membrane insertion of alpha-subunits in lung alveolar cells. Am. J. Physiol. 276, L20–27.

Bohannon, R.W., 1993. Physical rehabilitation in neurologic diseases. Curr. Opin. Neurol. 6, 765–772.

Boveris, A., Navarro, A., 2008. Brain mitochondrial dysfunction in aging, IUBMB Life 60 308–314.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carvalho, J.F., Masuda, M.O., Pompeu, F.A.M., 2005. Method for diagnosis and control of aerobic training in rats based on lactate threshold. Comp. Biochem. Physiol. A 140, 409–413.

Chen, Z., Krmar, R.T., Dada, L., Efendiev, R., Leibiger, I.B., Pedemonte, C.H., Katz, A.I., Sznajder, J.I., Bertorello, A.M., 2006. Phosphorylation of adaptor protein-2 mu2 is essential for Na+, K+-ATPase endocytosis in response to either G protein-coupled receptor or reactive oxygen species. Am. J. Respir. Cell Mol. Biol. 35, 127–132.

Chibalin, A.V., Ogimoto, G., Pedemonte, C.H., Pressley, T.A., Katz, A.I., Feraille, E., Berggren, P.O., Bertorello, A.M., 1999. Dopamine-induced endocytosis of Na+,K+-ATPase is initiated by phosphorylation of Ser-18 in the rat alpha subunit and Is responsible for the decreased activity in epithelial cells. J. Biol. Chem. 274, 1920–1927.

Dada, L.A., Chandel, N.S., Ridge, K.M., Pedemonte, C., Bertorello, A.M., Sznajder, J.I., 2003. Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-zeta. J. Clin. Invest. 111, 1057–1064.

Daggan, R.N., Zafeiridis, A., Dipla, K., Puglia, C.D., Gratz, I., Catalano, E, Kendrick, C.V., 2000. The effects of chronic exercise on anesthesia induced hepatotoxicity. Med. Sci. Sports Exerc. 32, 2024–2028.

D'Ambrosio, R., Maris, D.O., Grady, M.S., Winn, H.R., Janigro, D., 1999. Impaired K(+) homeostasis and altered electrophysiological properties of post-traumatic hippocampal glia. J. Neurosci. 19, 8152–8162.

D'Ambrosio, R., Fairbanks, J.P., Fender, J.S., Born, D.E., Doyle, D.L., Miller, J.W., 2004. Post-traumatic epilepsy following fluid percussion injury in the rat. Brain 127, 304–314.

Davis, L.M., Pauly, J.R., Readnower, R.D., Rho, J.M., Sullivan, P.G., 2008. Fasting is neuroprotective following traumatic brain injury. J. Neurosci. Res. 86, 1812–1822.

Dencher, N.A., Frenzel, M., Reifschneider, N.H., Sugawa, M., Krause, F., 2007. Proteome alterations in rat mitochondria caused by aging. Ann. N. Y. Acad. Sci. 1100, 291–298.

Dobrota, D., Matejovicova, M., Kurella, E.G., Boldyrev, A.A., 1999. Na/K-ATPase under oxidative stress: molecular mechanisms of injury. Cell Mol. Neurobiol. 19, 141–149.

Donovan, M, Pagliassotti, M.J., 2000. Quantitative assessment of pathways for lactate disposal in skeletal muscle fiber types. Med. Sci. Sports Exerc. 32, 772–777.

Endres, M., Gertz, K., Lindauer, U., Katchanov, J., Schultze, J., Schrock, H., Nickenig, G., Kuschinsky, W., Dirnagl, U., Laufs, U., 2003. Mechanisms of stroke protection by physical activity. Ann. Neurol. 54, 582–590.

Erickson, K.I., Kramer, A.F., 2009. Aerobic exercise effects on cognitive and neural plasticity in older adults. Br. J. Sports Med. 43, 22–24.

Faden, A.I., Demediuk, P., Panter, S.S., Vink, R., 1989. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244, 798–800.

Fiske, C.H., Subbarow, Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375–400.

Fordyce, D.E., Farrar, R.P., 1991. Enhancement of spatial learning in F344 rats by physical activity and related learning-associated alterations in hippocampal and cortical cholinergic functioning. Behav. Brain Res. 46, 123–133.

Gladden, L.B., 2000. Muscle as a consumer of lactate. Med. Sci. Sports Exerc. 32, 764–771.

Gobatto, C.A., de Mello, M.A., Sibuya, C.Y., de Azevedo, J.R., dos Santos, L.A., Kokubun, E., 2001. Maximal lactate steady state in rats submitted to swimming exercise. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 130, 21–27.

Hicks, R.R., Boggs, A., Leider, D., Kraemer, P., Brown, R., Scheff, S.W., Seroogy, K.B., 1998. Effects of exercise following lateral fluid percussion brain injury in rats. Restor. Neurol. Neurosci. 12, 41–47.

Hovda, D.A., Yoshino, A., Kawamata, T., Katayama, Y., Becker, D.P., 1991. Diffuse prolonged depression of cerebral oxidative metabolism following concussive brain injury in the rat: a cytochrome oxidase histochemistry study. Brain Res. 567, 1–10.

Jain, K.K., 2008. Neuroprotection in traumatic brain injury. Drug Discov. Today 13, 1082–1089.

Jamme, I., Petit, E., Divoux, D., Gerbi, A., Maixent, J.M., Nouvelot, A., 1995. Modulation of mouse cerebral Na+,K(+)-ATPase activity by oxygen free radicals. Neuroreport 7, 333–337.

Jones, M, Carter, H., 2000. The effect of endurance training on parameters of aerobic fitness. Sports Med. 29, 373–376.

Kleim, J.A., Jones, T.A., Schallert, T., 2003. Motor enrichment and the induction of plasticity before or after brain injury. Neurochem. Res. 28, 1757–1769.

Kramer, A.F., Erickson, K.I., Colcombe, S.J., 2006. Exercise, cognition, and the aging brain. J. Appl. Physiol. 101, 1237–1242.

Lees, G.J., Lehmann, A., Sandberg, M., Hamberger, A., 1990. The neurotoxicity of ouabain, a sodium–potassium ATPase inhibitor, in the rat hippocampus. Neurosci. Lett. 120, 159–162.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.

Lima, F.D., Souza, M.A., Furian, A.F., Rambo, L.M., Ribeiro, L.R., Martignoni, F.V., Hoffmann, M.S., Fighera, M.R., Royes, L.F., Oliveira, M.S., de Mello, C.F., 2008. Na+,K+-ATPase activity impairment after experimental traumatic brain injury: relationship to spatial learning deficits and oxidative stress. Behav. Brain Res. 193, 306–310.

Li, S., Stys, P.K., 2001. Na(+)–K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter. Neuroscience 107, 675–683.

Marquezi, M.L., Roschel, H.A., dos Santa Costa, A., Sawada, L.A., Lancha Jr, A.H., 2003. Effect of aspartate and asparagine supplementation on fatigue determinants in intense exercise. Int. J. Sport. Nutr. Exerc. Metab. 13, 65–75.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Oliveira, M.S., Flavia Furian, A., Freire Royes, L.F., Rechia Fighera, M., de Carvalho Myskiw, J., Gindri Fiorenza, N., Mello, C.F., 2004. Ascorbate modulates pentylenetetrazol-induced convulsions biphasically. Neuroscience 128, 721–728.

Opii, W.O., Nukala, V.N., Sultana, R., Pandya, J.D., Day, K.M., Merchant, M.L., Klein, J.B., Sullivan, P.G., Butterfield, D.A., 2007. Proteomic identification of oxidized mitochondrial proteins following experimental traumatic brain injury. J. Neurotrauma. 24, 772–789.

Osteen, C.L., Moore, A.H., Prins, M.L., Hovda, D.A., 2001. Age-dependency of 45 calcium accumulation following lateral fluid percussion: acute and delayed patterns. J. Neurotrauma. 18, 141–162.

Packer, L., Cadenas, E., 2007. Oxidants and antioxidants revisited. New concepts of oxidative stress. Free Radic. Res. 41, 951–952.

Packer, L., Cadenas, E., Davies, K.J., 2008. Free radicals and exercise: an introduction. Free Radic. Biol. Med. 44, 123–125.

Pari, L., Murugavel, P., 2007. Diallyl tetrasulfide improves cadmium induced alterations of acetylcholinesterase, ATPases and oxidative stress in brain of rats. Toxicology 234, 44–50.

Potts, M.B., Koh, S.E., Whetstone, W.D., Walker, B.A., Yoneyama, T., Claus, C.P., Manvelyan, H.M., Noble-Haeusslein, L.J., 2006. Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. NeuroRx 3, 143–153.

Prada, F.J.A., Voltarelli, F.A., De Oliveira, C.A., Gobatto, C.A., de Mello, M.A., 2004. Condicionamento anaeróbico e estresse oxidativo em ratos treinados por natação em intensidade equivalente ao limiar anaeróbio. R. Bras. Cien. Mov. 12, 29–34.

Ross, S.T., Soltesz, I., 2000. Selective depolarization of interneurons in the early posttraumatic dentate gyrus: involvement of the Na(+)/K(+)-ATPase. J. Neurophysiol. 83, 2916–2930.

Shao, C., Roberts, K.N., Markesbery, W.R., Scheff, S.W., Lovell, M.A., 2006. Oxidative stress in head trauma in aging. Free Radic. Biol. Med. 41, 77–85.

Siems, W.G., Hapner, S.J., van Kuijk, F.J., 1996. 4-hydroxynonenal inhibits Na(+)–K(+)-ATPase. Free Radic. Biol. Med. 20, 215–223.

Singh, I.N., Sullivan, P.G., Deng, Y., Mbye, L.H., Hall, E.D., 2006. Time course of post-traumatic mitochondrial oxidative damage and dysfunction in a mouse model of focal traumatic brain injury: implications for neuroprotective therapy. J. Cereb. Blood Flow Metab. 26, 1407–1418.

Smith, A.D., Zigmond, M.J., 2003. Can the brain be protected through exercise? Lessons from an animal model of parkinsonism. Exp. Neurol. 184, 31–39.

Soustiel, J.F., Sviri, G.E., 2007. Monitoring of cerebral metabolism: non-ischemic impairment of oxidative metabolism following severe traumatic brain injury. Neurol. Res. 29, 654–660.

Stahl, N., Ungerstedt, U., Nordstrom, C.H., 2001. Brain energy metabolism during controlled reduction of cerebral perfusion pressure in severe head injuries. Intensive Care Med. 27, 1215–1223.

Stummer, W., Weber, K., Tranmer, B., Baethmann, A., Kempski, O., 1994. Reduced mortality and brain damage after locomotor activity in gerbil forebrain ischemia. Stroke 25, 1862–1869.

Sullivan, P.G., Rabchevsky, A.G., Waldmeier, P.C., Springer, J.E., 2005. Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death? J. Neurosci. Res. 79, 231–239.

Tillerson, J.L., Caudle, W.M., Reveron, M.E., Miller, G.W., 2003. Exercise induces behavioral recovery and attenuates neurochemical deficits in rodent models of Parkinson's disease. Neuroscience 119, 899–911.

Toldy, A., Stadler, K., Sasvari, M., Jakus, J., Jung, K.J., Chung, H.Y., Berkes, I., Nyakas, C., Radak, Z., 2005. The effect of exercise and nettle supplementation on oxidative stress markers in the rat brain. Brain Res. Bull. 65, 487–493.

Varma, S., Janesko, K.L., Wisniewski, S.R., Bayir, H., Adelson, P.D., Thomas, N.J., Kochanek, P.M., 2003. F2-isoprostane and neuron-specific enolase in cerebrospinal fluid after severe traumatic brain injury in infants and children. J. Neurotrauma. 20, 781–786.

Vaynman, S., Gomez-Pinilla, F., 2005. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil. Neural. Repair 19, 283–295.

Vink, R., Golding, E.M., Headrick, J.P., 1994. Bioenergetic analysis of oxidative metabolism following traumatic brain injury in rats. J. Neurotrauma. 11, 265–274.

Voltarelli, F.A., Gobatto, C.A., de Mello, M.A., 2002. Determination of anaerobic threshold in rats using the lactate minimum test. Braz. J. Med. Biol. Res. 35 (11), 1389–1394.

Wang, R.Y., Yang, Y.R., Yu, S.M., 2001. Protective effects of treadmill training on infarction in rats. Brain Res. 922, 140–143.

Wannamethee, G., Shaper, A.G., 1992. Physical activity and stroke in British middle aged men. BMJ 304, 597–601.

Werner, C., Engelhard, K., 2007. Pathophysiology of traumatic brain injury. Br. J. Anaesth. 99, 4–9.

Wyse, A.T., Streck, E.L., Barros, S.V., Brusque, A.M., Zugno, A.I., Wajner, M., 2000. Methylmalonate administration decreases Na+,K+-ATPase activity in cerebral cortex of rats. Neuroreport 11, 2331–2334.

Yan, L.J., Traber, M.G., Packer, L., 1995. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. Anal. Biochem. 228, 349–351.

Yilmaz, G., Surer, H., Inan, L.E., Coskun, O., Yucel, D., 2007. Increased nitrosative and oxidative stress in platelets of migraine patients. Tohoku. J. Exp. Med. 211, 23–30.