



Short communication

Na⁺,K⁺-ATPase activity impairment after experimental traumatic brain injury: Relationship to spatial learning deficits and oxidative stress

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ABSTRACT

Traumatic brain injury (TBI) is a devastating disease that commonly causes persistent mental disturbances and cognitive deficits. Although studies indicate that oxidative stress and functional deficits occurring after TBI are interrelated events, the knowledge of the mechanisms underlying the development of such cognitive deficits has been limited. Thus, in the present study, we investigated the effect of fluid percussion brain injury (FPI) on a spatial learning task and levels of oxidative stress markers, namely, protein carbonylation and thiobarbituric acid-reactive substances (TBARS) and Na⁺,K⁺-ATPase activity 1 or 3 months after FPI in rats. Statistical analysis revealed that FPI increased the scape latency and mean number of error in Barnes maze test 1 and 3 months after FPI. We also found that protein carbonylation and TBARS content increased in the parietal cortex 1 and 3 months after FPI. In addition, 3 months after FPI, protein carbonylation levels increased both in ipsilateral and contralateral cortices of FPI animals. Indeed, statistical analysis revealed a decrease in Na⁺,K⁺-ATPase activity in the cerebral cortex of 1 month FPI animals. Furthermore, the decrease in enzyme activity found 3 months was larger, when compared with 1 month after FPI. These results suggest that cognitive impairment following TBI may result, at least in part, from increase of two oxidative stress markers, protein carbonylation and TBARS that occurs concomitantly to a decrease in Na⁺,K⁺-ATPase activity.

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Traumatic brain injury (TBI) is one of the major events in civilian life [3] and is a leading cause of disability and death [13]. The patients who survive present severe long-term neurological abnormalities, including deficits in learning and memory [26]. Functional deficits result from both direct, immediate mechanical disruption of brain tissue (the primary injury) and indirect, delayed (secondary) injury mechanisms. Secondary mechanisms are potentially amenable to post-injury therapeutic intervention because of their delayed onset and progression over hours to days and months after the initial trauma and include intracranial hemorrhage, brain swelling, raised intracranial pressure, and hypoxic/ischemic brain

damage, among others [17]. The oxidative damage mediated by reactive oxygen (ROS) and nitrogen species is a significant component of the secondary injury cascade that accompanies TBI [24]. In fact, increased levels of markers of oxidative stress have been found in human cerebrospinal fluid after severe TBI [2,31] and also in animal models of TBI, such as in the fluid percussion and controlled cortical impact models of TBI [23,32]. It is also remarkable that pharmacological treatment with antioxidants can significantly ameliorate motor and cognitive outcomes after TBI. For instance, the antioxidant α -phenyl-*N*-tert-butyl nitron markedly improve neurological reflexes and the performance in Morris water maze test as well as significantly reduced the loss of ipsilateral hemispheric tissue [15]. The results of these studies seem to indicate that oxidative stress and functional deficits occurring after TBI are interrelated events and that the study of the mechanisms involved in this interface can open new avenues to better understand the TBI pathology [5]. Thus, since Na⁺,K⁺-ATPase

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is especially sensitive to oxidative stress [12,18] and is a key enzyme involved in maintenance of brain excitability and its inhibition induces spatial learning deficits [35], we investigated the role of this enzyme in spatial learning deficits induced by experimental TBI. For this purpose, spatial learning performance, levels of oxidative stress markers, namely, protein carbonylation and thiobarbituric acid-reactive substances (TBARS) and Na^+, K^+ -ATPase activity were measured 1 or 3 months after a single episode of fluid percussion in the rat.

Adult male Wistar rats (270–300 g), maintained under controlled light and environment (12:12 h light/dark cycle, $24 \pm 1^\circ\text{C}$, 55% relative humidity), with standard laboratory chow (Guabi, Santa Maria, RS, Brazil) and water *ad libitum*. All reagents were purchased from Sigma (St Louis, MO, USA) except TBA, which was obtained from Merck (Darmstadt, Germany). All experimental protocols designed aiming to keep the number of animals used to a minimum, as well as their suffering. Animal utilization reported in this study has been conducted in accordance with the policies of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996, and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (23081.018516/2006-57).

Fluid percussion brain injury (FPI) was carried out as described previously by D'Ambrosio et al. [6,7]. 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 parietal cortex, 2 mm posterior to the bregma and 3 mm lateral to the midline, taking care to keep the dura mater intact. A plastic injury cap was placed over the craniotomy with dental cement. When the dental cement hardened, the cap was filled with chloramphenicol 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 cap attached to the fluid percussion device and placed in a heat pad maintained at $37 \pm 0.2^\circ\text{C}$.

Traumatic brain injury was induced 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 to model severe TBI. 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 FPI. Immediately after these procedures, the cap was removed and the orifice was covered with dental cement.

The tests for spatial learning assessment as well as neurochemical determinations (oxidative stress parameters and Na^+, K^+ -ATPase activity) were performed at 1 or 3 months after the FPI. For this purpose, sham and FPI animals were randomly assigned in four groups, as follows:

- Experiment 1 (performed at 1 month after FPI): sham ($n = 8$) and FPI ($n = 8-9$).
- Experiment 2 (performed at 3 months after FPI): sham ($n = 8$) and FPI ($n = 8-9$).

To test the performance of the animals in a spatial learning paradigm, we chose the Barnes maze test, described by Barnes [1] and adapted by Oliveira et al. [22]. The apparatus was located in a $4 \text{ m} \times 4 \text{ m}$ 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 one maze hole.

Briefly, on the first day of experiment, the rats were moved to the testing room and left undisturbed for 60 min. Following this habituation period, the 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 center of the maze. With light on, the start box was removed and the rat allowed exploring freely and finding the escape box. A maximum latency of 180 s to find it was allowed. Each rat was given three trials per day, over four consecutive days. In each trial we scored the time to reach the escape tunnel and the number of wrong holes visited. The arena, as well as boxes, was wiped clean using distilled water both between each training session for a given rat and between each rat. Immediately after the Barnes maze test, the animals were transferred to the central area of a round open field (56 cm in diameter), which had its floor divided into 10 equal areas. The number of areas crossed was recorded for 20 min.

After the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. The ipsilateral cortex was rapidly dissected on an inverted ice-cold Petri dish. The contralateral cortex was subjected to the same procedure and served as internal control. Each tissue sample was homogenized in cold 10 mM Tris-HCl buffer (pH 7.4) and then divided in aliquots for subsequent neurochemical analyses, as described below.

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 [21]. Total protein carbonyl content was determined by the method described by Yan et al. [34], adapted for brain tissue [27]. Assay of Na^+, K^+ -ATPase activity was performed according to Wyse et al. [33]. Protein content was measured colorimetrically by the method of Bradford [4], using bovine serum albumin (1 mg/ml) as standard. Statistical analysis was carried out by the Kruskal-Wallis test. Post hoc analysis was carried out, when appropriate, by the followed by Mann-Whitney test. Correlation analyses were carried out using the Pearson's correlation coefficient.

Fig. 1A and B shows the effect of FPI on the rat performance in the Barnes maze test. The first set of experiments was performed 1 month after FPI, and FPI animals showed an increased latency for escape at the second day of test, and number of errors at the second and third days of test when compared with sham animals, indicating a decreased performance of FPI animals in this spatial learning paradigm. There were no significant differences between sham and FPI animals in the latency for escape and number of errors in the first and in the fourth days of test. A decreased performance of FPI animals in the Barnes maze test was also found at 3 months after FPI, since FPI animals showed an increased latency for escape at the second and third days of test, and an increased number of errors at the third day of test, when compared with sham animals. We did not detect any motor deficit in the animals by analyzing the number of crossings in the open field, suggesting that a impaired performance in the Barnes maze is not related to motor disabilities.

In order to evaluate the association between spatial learning deficits and levels of oxidative stress markers 1 or 3 months after FPI, we measured the levels of reactive species-induced damage to lipids (TBARS) and proteins (protein carbonylation). TBARS content increased in parietal cortex of FPI animals at 1 and 3 months, both in the ipsilateral and contralateral sides. Protein carbonylation levels are shown in Fig. 2B. One month after FPI, protein carbonylation

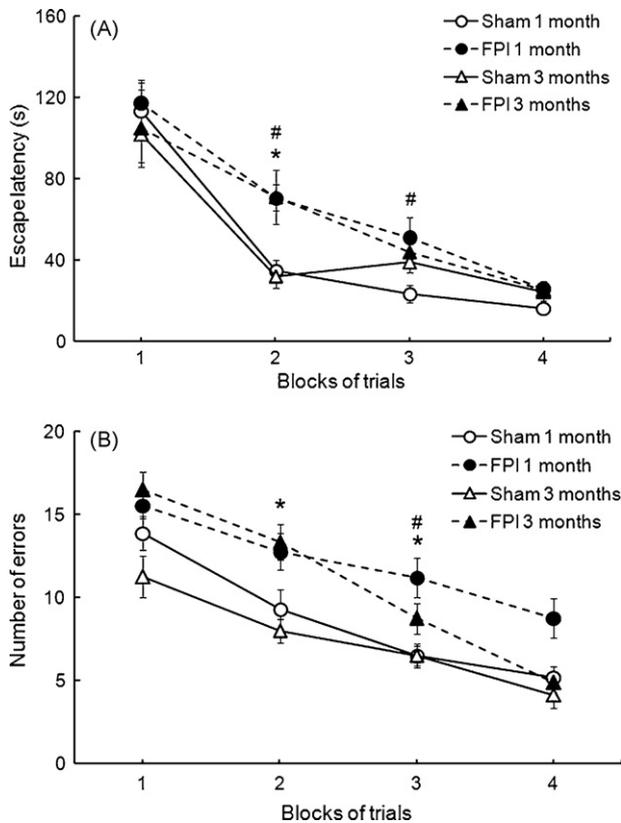


Fig. 1. Effect of fluid percussion brain injury (TBI) on the (A) escape latency and (B) number of errors in the Barnes maze test. Data are the mean \pm S.E.M. for $n=8-9$ animals in each group. * $P < 0.05$ compared with 1 month sham group. # $P < 0.05$ compared with 3 months sham group.

levels increased only in the ipsilateral cortex of FPI animals, in comparison with sham animals. However, 3 months after FPI, protein carbonylation levels increased both in ipsilateral and contralateral cortices of FPI animals, when compared with sham animals. Such increase in protein carbonylation levels in FPI animals was larger at 3 months than at 1 month after FPI, indicating that protein carbonylation increases over time after FPI, both in ipsilateral and contralateral cortices.

Since Na^+, K^+ -ATPase is especially sensitive to oxidative stress, we evaluated the effect of FPI on Na^+, K^+ -ATPase activity (Fig. 2C). Statistical analyses revealed a decrease in Na^+, K^+ -ATPase activity in the ipsilateral cerebral cortex of FPI animals both at 1 and 3 months after FPI, when compared with sham animals. Furthermore, the decrease in enzyme activity 3 months after FPI was larger, when compared with decrease at 1 month after FPI, indicating that FPI-induced decrease in Na^+, K^+ -ATPase activity is time-dependent.

Considering that a decrease in Na^+, K^+ -ATPase activity plays role in learning and memory deficits [35], we decided to investigate whether Na^+, K^+ -ATPase activity in the ipsilateral cerebral cortex correlated with animal performance (escape latency) in the Barnes maze at the second day of test (in which the learning deficits were more evident). Statistical analysis demonstrated that there is no correlation between these parameters, either in sham (Pearson's coefficient = 0.267; $P > 0.05$ for $n=17$) or FPI (Pearson's coefficient = 0.089; $P > 0.05$ for $n=17$) animals (data not shown).

In this study we showed that a single FPI episode in rat parietal cortex decreases Na^+, K^+ -ATPase activity with a concomitant increase in levels of oxidative stress markers, protein carbonylation and TBARS. Moreover, animals showed an impaired ability to

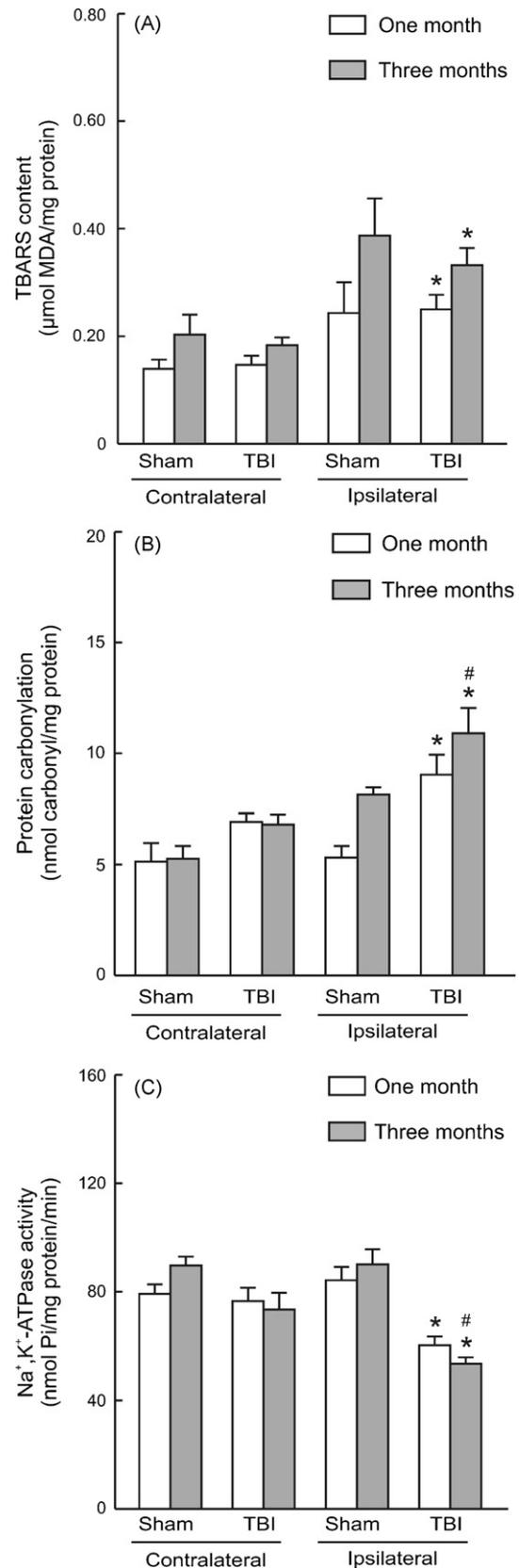


Fig. 2. Effect of fluid percussion brain injury (TBI) on (A) TBARS content, (B) protein carbonylation levels and (C) Na^+, K^+ -ATPase activity in rat parietal cortex. Data are the mean \pm S.E.M. for $n=8-9$ animals in each group. * $P < 0.05$ compared with respective sham group. # $P < 0.05$ compared with 1 month after TBI group.

perform the Barnes maze, a spatial learning task. These alterations were found both at 1 or 3 months after FPI.

Chronic cognitive impairments such as deficits in attention, slow processing speed, or learning and memory are frequently reported as a consequence of TBI in humans [26] and experimental animals [5]. Although the cognitive impairments are among the most debilitating outcomes in patients, yet, to date, no treatment can prevent or counteract these effects in TBI patients [14,20,30]. Moreover, the mechanisms underlying the development of such cognitive deficits remain elusive. While neuronal death has been considered to be a major factor, the pervasive memory, cognitive and motor function deficits suffered by many mild TBI patients do not always correlate with cell loss. In this context, it has been demonstrated that pharmacological treatment with neurotrophin-4/5 significantly protects against hippocampal cell death in mice, but not improves animal performance in three hippocampal-dependent behavioral tests [25] suggesting that cognitive impairment following TBI may result, at least in part, from alterations in surviving neurons [5].

In this study we showed that FPI induces a decrease in Na^+, K^+ -ATPase activity. These results are in full agreement with the view that FPI-induced neurochemical alteration, such Na^+, K^+ -ATPase activity inhibition, might be a factor contributing to a decreased rat performance in the Barnes maze task. Accordingly, the intracerebroventricular injection of ouabain, a selective inhibitor of Na^+, K^+ -ATPase impairs rat performance in the Morris water maze, another spatial learning and memory test [35]. Moreover, transgenic mice lacking the α subunit of Na^+, K^+ -ATPase have severe learning and memory deficits, as determined using the Morris water maze [19]. Altogether, these data may suggest that the presently reported deficits in spatial learning and decrease in Na^+, K^+ -ATPase activity after TBI are interrelated events, and one might suggest that decreased Na^+, K^+ -ATPase activity may contribute to the currently observed deficits in spatial learning after TBI. However, one may consider the fact that Na^+, K^+ -ATPase activity in the ipsilateral cerebral cortex did not correlate with animal performance in the Barnes maze at the second day of test, suggesting that other factors may also contribute to impaired spatial learning performance after TBI.

The mechanisms underlying the presently observed decrease in Na^+, K^+ -ATPase activity may include oxidative modification, since in the present study we showed that increase in protein carbonylation and TBARS occurs concomitantly to a decrease in Na^+, K^+ -ATPase activity. In concordance with this view, it has been demonstrated that oxidative damage, mediated by reactive oxygen and nitrogen species, including the superoxide ion, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite, is well recognized as a significant component of the secondary injury cascade that accompanies TBI [24]. Furthermore, while free-radical-induced damage has been proposed to be involved in traumatic cell injury and cell death, free radical scavengers, such catalase and glutathione peroxidase (GSH-Px) are associated with partial amelioration of traumatic injury [11,16,29].

Nevertheless, whether the presently reported increase in protein carbonylation and TBARS levels reflects a decrease in antioxidant defenses, an increase in reactive species formation or both remains to be determined, but it is interesting to point out that the increase in these markers of oxidative stress occur in parallel with a decrease in Na^+, K^+ -ATPase activity. It has long been known that the Na^+, K^+ -ATPase activity is particularly sensitive to reactive species, which alter plasma membrane lipid composition [12] and the redox state of regulatory sulfhydryl groups [18] as well as other amino acid residues [28]. In addition, oxidative and nitrosative stress induce protein carbonylation [9], which is related to increased susceptibility to proteolysis and marked impairment of protein functionality [8]. Moreover, Dobrota et al. [10] suggested

that the most important consequence of oxidative modification is that Na^+, K^+ -ATPase molecules have lost the ability to interact with one another, decreasing oligomeric structure formation and leading to subsequent hydrolysis rate suppression.

In summary, the present study we showed that a single episode of FPI induces impairment in animal performance in a spatial learning and memory paradigm, an increase of the oxidative stress markers and a decrease in Na^+, K^+ -ATPase activity. Furthermore, these findings add a new insight into the neurochemical mechanism involved in the TBI-induced spatial learning impairment. Therefore, while the exact mechanism underlying of secondary injury after TBI is poorly understood, we think that these data may help in the understanding of TBI pathology.

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