BMB
Reports

BMB Rep. 2022; 55(10): 512-517
www.bmbreports.org

The effects of early exercise in traumatic brain-injured rats with changes in motor ability, brain tissue, and biomarkers

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Traumatic brain injury (TBI) is brain damage which is caused by the impact of external mechanical forces. TBI can lead to the temporary or permanent impairment of physical and cognitive abilities, resulting in abnormal behavior. We recently observed that a single session of early exercise in animals with TBI improved their behavioral performance in the absence of other cognitive abnormalities. In the present study, we investigated the therapeutic effects of continuous exercise during the early stages of TBI in rats. We found that continuous low-intensity exercise in early-stage improves the locomotion recovery in the TBI of animal models; however, it does not significantly enhance short-term memory capabilities. Moreover, continuous early exercise not only reduces the protein expression of cerebral damage-related markers, such as Glial Fibrillary Acid Protein (GFAP), Neuron-Specific Enolase (NSE), S100β, Protein Gene Products 9.5 (PGP9.5), and Heat Shock Protein 70 (HSP70), but it also decreases the expression of apoptosis-related protein BAX and cleaved caspase 3. Furthermore, exercise training in animals with TBI decreases the microglia activation and the expression of inflammatory cytokines in the serum, such as CCL20, IL-13, IL-1α, and IL-1β. These findings thus demonstrate that early exercise therapy for TBI may be an effective strategy in improving physiological function, and that serum protein levels are useful biomarkers for the predicition of the effectiveness of early exercise therapy. [BMB Reports 2022; 55(10): 512-517]

INTRODUCTION

Traumatic brain injury (TBI) has long been a major cause of death and disability worldwide and continues to have a devastating impact on patients and their families, creating a significant public health burden (1). Based on national statistics reports, the death rate from traffic accidents in Korea is decreasing over time; however, the rate of injuries is continuing to increase, and the rate of industrial accidents remains higher than that of Organization for Economic Cooperation and Development member countries. The number of falls in the elderly population is also increasing due to the increase in the elderly population (2); therefore, the number of TBI cases is estimated to increase. Although the number of deaths due to TBI has decreased with the development of medical treatment methods, the number of patients with disabilities is continuing to increase. Indeed, there is great interest in early treatment which will minimize disability and ensure proper social return (2).

However, there remains scarce literature on early exercise treatment for patients with traumatic brain injury. It is well known that exercise can help to improve brain function. Neurons, synapses, and increased blood vessels have been observed within the brains of exercised rats, and blood flow increases with the formation of blood vessels in the motor cortex and cerebellum (3-5). However, clinical studies on exercise therapy for traumatic brain injury still remain limited. Although improvement in functioning was reported when exercise therapy was performed in patients with traumatic brain injury several months after their injury, no clinical studies of early exercise therapy for patients within the acute phase of injury have been reported upon (6, 7).

In the studies by Humm *et al*. and Griesbach *et al*., which are most often cited as the basis for animal experiments dealing with the adverse effects of early exercise therapy in trauma-

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https://doi.org/10.5483/BMBRep.2022.55.10.097

Received 13 June 2022, Revised 6 July 2022, Accepted 17 August 2022

Keywords: Apoptosis, Biomarker, Early exercise, Exercise therapy, Traumatic brain injury

ISSN: 1976-670X (electronic edition)

tic brain injury, only histological changes in the hippocampus had been observed; however, changes in other parts of the brain, particularly at the periphery of the lesion, were not evaluated. In addition, behavioral assessments, such as memory or motor function assessments, were also not performed (8, 9). On the other hand, Lippert-Grüner *et al*. reported that in a TBI animal model, rotarod exercise for 15 minutes for 5 days, with environmental enrichment from Day 1 after trauma, improved overall motor function and reduced neuron damage (10).

In a recent reanalysis study on the effectiveness of a large-scale AVERT (A Very Early Rehabilitation Trial) conducted amongst stroke patients, early rehabilitation treatment was seen as effective; however, recovery was reduced as the treatment time increased (11). This result is noteworthy; however, the application of the results of this study on TBI has still remain limited. As mentioned above, the inconsistent animal test results of early exercise therapy for TBI have resulted in a decrease in clinical studies on exercise therapy for patients within the acute phase of recovery. Therefore, a well-designed study which can determine the proper timing of exercise therapy in TBI remains an urgent need.

In TBI, various substances, such as inflammatory cytokines, are secreted from the cells of damaged tissues. Various studies have reported upon the use of inflammatory cytokines as biomarkers for predicting the degree of damage and prognosis. The secretion of S100β and glial fibrillary acid protein (GFAP) from astrocytes is further increased by brain damage. Additionally, neuron-specific enolase (NSE) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels are increased through neuronal damage. These substances spread to the peripheral blood through disruption of the blood-brain barrier (BBB) which is caused by brain damage, and are the main targets for blood biomarker research (12). Several studies have reported that the increased concentrations of S100β, GFAP, NSE, and UCH-L1 in the blood after traumatic brain injury have been associated with poor prognosis (13-15). The heat shock protein 70 (Hsp70), which is secreted immediately upon damage to neurons, astrocytes, microglia, or endothelial cells, is also a known biomarker of traumatic brain injury (16).

Damaged brains may be susceptible to excitation during the acute phase of TBI, and some reports have demonstrated that early exercise after TBI aggravates brain damage and interferes with functional recovery. Although many studies have reported that exercise can improve disability after TBI, the treatment time window for exercise early after receiving TBI still remains controversial. In Yoon & Kim's study, low-intensity early (3 days after TBI) exercise exhibited a beneficial effect on behavioral performance without causing cognitive deficits, unlike the effects of high-intensity exercise (17). These authors found that strenuous exercise in the early stages of injury raises body temperature, increases cellular level metabolism, and also causes changes in various acute inflammatory hormones and neurotransmitters, which then adversely affect sensitive brain cells' recovery during the acute stage.

However, no studies have been conducted using biomarkers to evaluate the effectiveness of early exercise therapy in TBI. In this current study, we have attempted to determine whether early exercise therapy would help to improve motor and cognition functions, and we aimed to evaluate the appropriate markers of early exercise therapy for TBI.

RESULTS

Effects of early exercise on locomotion performance in TBI in rats

The animals were trained on an animal treadmill three times per day, for three consecutive days, prior to TBI using a controlled cortical impact device and then performed treadmill exercise from Day 1 after TBI (Fig. 1A). In order to confirm the effect of early exercise therapy on motor function, we then performed the rotarod test 1, 3, 7, and 14 days after TBI. Rats with TBI were randomly divided into two groups: (1) non-exercise (TBI) and (2) low-intensity exercise (10 m/minutes for 30 minutes) (TBI-E). We observed that the latency was more significantly improved in the TBI-E group when compared to that of the TBI group (Fig. 1B). However, the memory function measured by the Y-maze and passive avoidance tests was not significantly different between the two groups (Fig. 1C, D). During treatment, neither weight loss nor behavioral changes were observed in either the TBI or TBI-E groups (Fig. 1E). These observations thus suggest that low-intensity exercise in the early period of TBI helps to improve behavioral function.

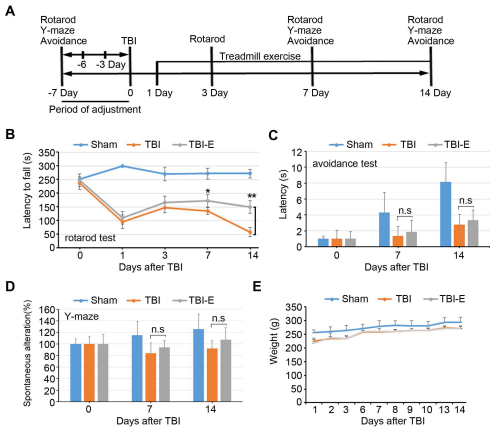

Fig. 1. Early exercise therapy improves locomotion performance after TBI. (A) A timeline showing the establishment of a mouse model of TBI, early exercise therapy, and the assessment of motor and memory function through rotarod, Y-maze, and passive avoidance tests. (B) The rotarod tests were performed up to 14 days following the induction of TBI in order to evaluate motor function. *P < 0.05, **P < 0.01 versus TBI group (sham, n = 6; TBI, n = 7; TBI-E, n = 7). (C, D) The memory functions were measured through passive avoidance and Y-maze tests performed after TBI. (E) The time-dependent alterations in rat bodyweight after TBI.

Effect of early exercise on brain parenchymal loss and expression of apoptotic protein after TBI

In order to evaluate the effect of exercise therapy on brain parenchymal volume loss, we performed the H&E staining of rat brain tissues. Fourteen days post-TBI, the volume of brain injuries showed no significant difference between the TBI and TBI-E groups (Fig. 2A, B), thereby suggesting that the volume of parenchyma was not associated with improved behavior in TBI rats with early exercise. We then further evaluated cell death in brain tissues 3, 7, and 14 days after post-TBI both with and without early exercise. In the 7-day samples after TBI, TUNEL staining showed that early exercise decreased TUNEL-positive apoptotic cells when compared to brain tissue from the TBI group (Fig. 2C, D). Also, TUNEL co-staining with neuron and glial cells further demonstrated that both TUNEL-positive cells decreased by early exercise (Supplementary Fig. 1). In order to then investigate the effect of early exercise on neuronal cell death, we measured the expression of apoptosis-related proteins (BAX and cleaved caspase-3) through immunoblotting. Both BAX and cleaved caspase-3 expression levels in the early exercise group were significantly lower than those of the TBI group on days 7 and 14 (Fig. 2E, F). However, there was no significant change in BAX or cleaved caspase-3 protein expression on day 3 post-TBI. Thus, the results show that early exercise may effectively inhibit the induction of apoptosis-related proteins by TBI, which may result in improved behavioral function.

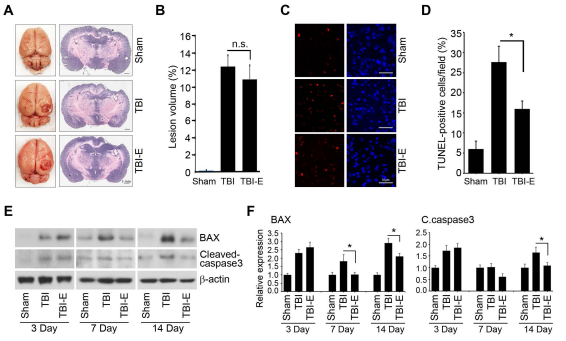

Fig. 2. Effect of early exercise on brain damage and apoptosis after TBI in the rat brain. (A) The representative pictures of whole brain and brain slice sections of Sham, TBI, TBI-E animals. The brain sections were stained with hematoxylin and eosin on day 14 after TBI. Scale bar, 1 mm. (B) The relative percent volume of damaged brain area is presented in the form of bar graphs. (C) The representative fluorescence images demonstrating TUNEL-positive nuclei (red color) on rat brain 14 days after TBI (scale bar, 50 μm). (D) The relative percentage of TUNEL-positive cells is presented through bar graphs. (E) The expression of BAX and cleaved caspase-3 was detected through immune blot analysis on days 3, 7, and 14 after TBI, and the β-actin was used as loading control. (F) The relative protein expressions of BAX and cleaved caspase-3 are presented through bar graphs. The data are presented as mean ± SE of three independent experiments. *P < 0.05.

Effect of early exercise on expression of brain damage biomarkers after TBI

A previous study showed that various biomarkers, such as GFAP, NSE, PDP9.5, HSP70, and S100β, rapidly increased after brain blood-barrier disruption in patients with TBI (18, 19). Hence, we hypothesized that early exercise therapy may inhibit injury-related proteins in the tissues affected by TBI, and thus improve the behavioral function in TBI in animals. In order to test the changes in damage-related protein expression following early exercise treatment, we examined the expression of TBI biomarkers with western blotting. The protein levels of GFAP, PGP9.5, NSE, and S100β were reduced in the aftermath of early exercise 14 days after TBI; however, there was no significant difference in the level of HSP70 between the TBI and TBI-E groups following early exercise treatment at 7 and 14 days post TBI (Fig. 3A, B). We also confirmed the TBI biomarker expression in brain tissues through immunohistochemistry testing at 3 and 14 days after TBI (Fig. 3C). The protein levels of GFAP, NSE, and S100β were significantly increased in both the TBI and TBI-E groups when compared to those in the sham group. Additionally, there was no significant change in the levels

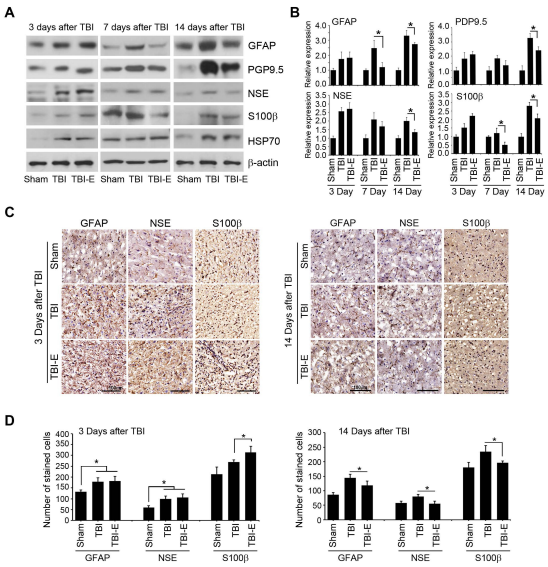

Fig. 3. Effects of early exercise on protein levels related to TBI-induced injury. (A) The expression of GFAP, PDP9.5, NSE, HSP70, and S100β was detected by immune blot analysis 3, 7, and 14 days after TBI, and the β-actin was used as a loading control. (B) The intensity of the bands was quantified using Image J software and then normalized with β-actin. Data are shown as mean ± SE of the three independent experiments. *P < 0.05 versus TBI group. (C) The histological comparison of brain injury marker expression was performed on sham, TBI, and TBI-E brain tissue. The histological sections of brain tissues were stained with antibodies against GFAP, NSE, and S100β. Scale bar, 100 μm. (D) The protein expression of the brain injury markers was presented as the number of positive cells per high power field (HPF). The values shown here are mean ± SE. *P < 0.05.

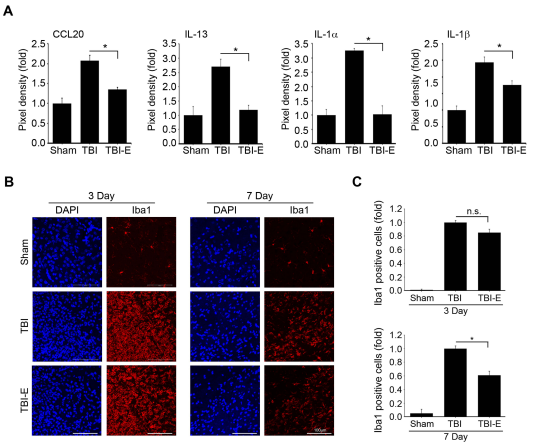

Fig. 4. Effect of early exercise on inflammation cytokine array and microglial activation after TBI. (A) A tat cytokine array performed of blood serum samples collected in sham, TBI, and TBI-E groups. The protein expression of CCL20, IL13, 1L-1α, and IL-1β was quantified through scanning densitometry. The values shown are mean ± SE. *P < 0.05. (B) The brain tissues were stained with Iba1 antibody (red) using samples 3 and 7 days after TBI. Scale bar = 100 μm. (C). The Iba1 positive cells were presented as the number of positive cells per high power field (HPF). The values shown here are mean ± SE. *P < 0.05.

of these biomarkers between the TBI and TBI-E groups 3 days after injury occurrence. However, our data showed that the expression of GFAP, NSE, and s100β was significantly downregulated in the TBI-E group when compared to that of the TBI group 14 days after TBI (Fig. 3D). These data indicate that brain injury-related proteins were effectively inhibited due to early exercise from 7 days after therapy.

Effect of early exercise on inflammatory cytokine secretion and microglia activation of animals with TBI

In TBI, the investigation of the appropriate markers in the blood could be useful to help diagnose the disease. Previous reports have demonstrated that TBI induces the expression of CCL20, IL13, IL-1α, and IL-1β within blood serum (20, 21). Therefore, in order to confirm the change of inflammatory proteins in serum due to early exercise, we performed a cytokine array analysis using animal serum 48 hours after TBI. The cytokine array data showed that the level of inflammatory cytokines (CCL20, IL-13, IL-1α, and IL-1β) was increased in the TBI group, whereas there was a significantly decrease in the initial exercise treatment group (Fig. 4A). The secretion of inflammatory cytokines was essential for microglia-mediated inflammation. Thus, we next investigated whether the effect of early exercise is associated with microglia (Iba1) infiltration and activation within the brain tissue. Compared with the TBI group, the TBI-E group exhibited a reduced number of Iba1-positive cells 7 days after TBI but not in 3 days after TBI (Fig. 4B, C). These results indicated that early exercise reduces inflammatory cytokines and microglia activation and thus may improve the therapeutic effect of TBI.

DISCUSSION

Many studies amongst rodents and humans indicate that exercise improves brain function. Among the studies in this field, there are many reports focused on the hippocampus, a brain region which is important for learning and memory. Based on these reports, the structure and function of the hippocampus may be modified by exercise (22). Using a translational rodent model of concussion, Mychasiuk *et al*. reported the effects of voluntary exercise on concussion recovery and investigated the effects of exercise related to post-concussion syndrome (PCS) and gene expression changes (bdnf, dnmt1, Igf1-a, Tert) within the prefrontal cortex and hippocampus.

Results suggest that exercise, which started within 1-3 days of concussion, greatly improved exercise and cognitive function; however, its efficacy was limited to the treatment of emotional impairments (23). On the other hand, Weinstein *et al*. reported mood changes in ambulatory individuals with chronic TBI (> 6 months post-injury) after 12 weeks of vigorous aerobic exercise. The greatest improvement in overall mood changes was observed after 12 weeks of exercise training, with improvement seen as early as 4 weeks (24). Therefore, there remains a difference in the pattern of recovery depending on the timing of treatment for TBI.

Injury caused by trauma can be divided into primary injuries which occur at the moment of trauma as a result of mechanical forces which disrupt the structural integrity of the brain, and secondary injuries caused by biomechanical and cellular changes (25). Amongst rats with TBI, we studied the ways in which early exercise therapy influences behavior, cognition, and primary brain structure in the aftermath of trauma, and its impact on secondary hematologic changes. Within this study, we showed that motor function performed by the rotarod test 1, 3, 7, and 14 days after sustained traumatic injury was significantly improved within the low-intensity exercise (10 m/min for 30 min) group (TBI-E) when compared to the non-exercise group (TBI), but the memory function measured by the Y-maze and passive avoidance tests was not significantly different between the two groups. These observations suggest that low-intensity exercise within the early period of TBI improves overall behavioral function. Similar results have been observed in Yoon & Kim's study, in which low-intensity early (3 days after TBI) exercise showed a beneficial impact on behavioral performance without inducing cognitive deficit, unlike the effects of high-intensity exercise (17). Regarding these results, these authors stated that intense exercise in the early stage of injury caused an increase in body temperature, resulting in increased metabolism at the cellular level, changes in various acute inflammatory hormones, and changes in neurotransmitters, which all adversely affected sensitive brain cells in the acute phase of injury.

We also found that the volume of brain injuries showed no

significant difference between the TBI and TBI-E groups, suggesting that the volume of parenchyma was not associated with the improved behavior of rats with TBI with early exercise. Generally, spectroscopic studies performed shortly after TBI have shown consistent imaging patterns which manifest as increased choline and decreased N-acetylaspartate in the regions of the adult brain that are known to be most susceptible to shear injuries (26). Within this study, we evaluated the expression levels of apoptosis-related proteins (BAX and cleaved caspase-3) and various biomarkers, such as GFAP, NSE, PGP9.5, HSP70, and S100β, on day 3, 7, and 14 post-TBI by immunoblotting. Necrosis and apoptosis are the two major mechanisms of cell death within the central nervous system, with distinct physiological and pathological characteristics, biochemical pathways, and histological explanations (27). Necrosis is generally activated through a calpain-mediated cell death pathway, and apoptosis is caused by a tightly regulated biochemical cascade involving the activation of caspases (28). BCl-2-related X-protein (BAX) from cytoplasmic to mitochondrial membranes leads to the onset of cytoplasmic cascades within mitochondria, and caspase-3 is involved in the cleavage of specific cytoskeletal proteins which play an important role in cell death amongst neurological and neurodegenerative diseases (29). Within our study, both BAX and cleaved caspase-3 expression levels in the early exercise group were significantly lower than within the TBI group on Days 7 and 14. However, there was no significant change in BAX or the cleaved caspase-3 protein expression on Day 3 post-TBI. These results reveled that early exercise may down-regulate these apoptotic proteins. Previous studies on apoptosis in TBI have also caspase-3 upregulation in brain tissue at an early stage; however, they did not investigate the effects of early exercise on this phenomenon (30, 31). Clark *et al*. reported that the cleavage of caspase-1 was up-regulated; however, BAX did not increase in tissues from patients with TBI when compared with controls (32). Many protein biomarkers have been extensively studied for the diagnosis and prognosis of different degrees of TBI severity. The HSP70 levels did not change with early exercise between the TBI and TBI-E groups at 14 days; however, the protein levels of GFAP, PGP9.5, NSE, and S100β did decrease. These data indicate that brain injury-related proteins were effectively inhibited through early exercise.

Microglia and macrophages play an essential role in immune regulation within the brain after TBI (33). Continuous microglia activation accelerates neuronal cell damage through the production of many inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, which all play an important role in neurodegenerative diseases (34). For example, IL-1β promotes the development of secondary damage after TBI, thereby resulting in neuronal damage and consequent loss of motor function. On the other hand, Ilknur *et al*. reported that he neutralizing of IL-1β improves functional recovery following experimental TBI through TBI-induced microglia activation (35). Our study also indicated that the level of inflammatory cytokines (CCL20, IL-13, IL-1α, and IL-1β) were elevated at 48 hours in TBI group. Cytokine array data showed that four inflammatory cytokines decreased within the early exercise (TBI-E) group when compared with the TBI group. Additionally, early exercise effectively decreased the microglia cell number and brain infiltration rate through the inhibiting of inflammatory cytokines. These results suggest that inflammatory cytokines modulated by early exercise may serve as diagnostic markers for TBI.

Based on these findings, we thus conclude the following. Early exercise therapy for TBI may be a desirable strategy for the improvement of physiological function. Additionally, serum protein levels are useful biomarkers for the prediction of the safety and effectiveness of early exercise therapy.

MATERIALS AND METHODS

Study approval

Animal studies and all research processes were approved by the Sungkyunkwan University Institutional Animal Care and Use Committee (IACUC) with approval number SKKUIACUC 2019-08-09-1.

Further detailed information is provided in the Supplementary Information.

ACKNOWLEDGEMENTS

This work was supported by National Research Foundation of Korea (NRF) grants funded by the Korean Government (MSIP) (NRF-2016R1A5A2945889, 2017R1A2B4010100, 2018 R1D1 A1B07050274, and NRF-2020R1F1A1073261).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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