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Treadmill exercise inhibits traumatic brain injury-induced hippocampal apoptosis

Dong-Hyun Kim [a,b], Il-Gyu Ko [a], Bo-Kyun Kim [a], Tae-Woon Kim [a,b], Sung-Eun Kim [a], Mal-Soon Shin [a], Chang-Ju Kim [a], Hong Kim [c], Kyeong-Mi Kim [d], Seung-Soo Baek [e,*]

[a] Department of Physiology, College of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea
[b] School of Sport Science, Sungkyunkwan University, Kyunggi-do 440-746, Republic of Korea
[c] Department of Oriental Sports Medicine, College of Health & Therapy, Daegu Haany University, Gyeongbuk 712-715, Republic of Korea
[d] Department of Occupational Therapy, Inje University, Gyeongnam 621-749, Republic of Korea
[e] Department of Physical Education, College of Arts and Physical Education, Sang Myung University, Seoul 110-743, Republic of Korea

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ABSTRACT

Traumatic brain injury (TBI) occurs when an outside force impacts the brain. The main problem associated with TBI is neuronal cell death of the brain, and the outcome of TBI ranges from complete recovery to permanent disability, and sometimes death. Physical exercise is known to ameliorate neurologic impairment induced by various brain insults. In the present study, we investigated the effects of treadmill exercise on short-term memory and apoptosis in the hippocampus following TBI in rats. TBI was induced by an electromagnetic-controlled cortical impact. The rats in the exercise group were forced to run on a treadmill for 30 min once daily for 10 consecutive days, beginning 2 days after induction of TBI. For the current study, a step-down avoidance task, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, Western blot for Bcl-2 and Bax, and immunohistochemistry for caspase-3 were conducted. The present results revealed that TBI impaired short-term memory, and increased DNA fragmentation and caspase-3 expression in the hippocampus. Induction of TBI also enhanced expression of pro-apoptotic factor Bax protein and suppressed expression of anti-apoptotic factor Bcl-2 protein in the hippocampus. Treadmill exercise alleviated short-term memory impairment, and decreased DNA fragmentation and caspase-3 expression in the hippocampus. In addition, treadmill exercise remarkably suppressed expression of Bax protein and slightly increased expression of Bcl-2 protein in TBI-induced rats. The present study showed that treadmill exercise might overcome TBI-induced apoptotic neuronal cell death, thus facilitating recovery following TBI.
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1. Introduction

Traumatic brain injury (TBI) occurs when an outside force impacts the brain. TBI induces skull fractures, intracranial injuries, loss of consciousness, post-traumatic amnesia, and motor functional deficits. Greater than 10 million people are injured annually worldwide, and at least 1.5 million people currently suffer from TBI-related disabilities. In previous studies, 40%–50% of patients suffer cognitive dysfunction after TBI [1]. The main problem associated with TBI is neuronal cell death of the central nervous system. Such neuronal cell death after TBI is attributed to direct mechanical injury or post-traumatic secondary injury that occurs from weeks to months following TBI [2]. Recent evidence revealed that apoptosis is a key mechanism that is involved in secondary or delayed neuronal cell death [3].

Apoptosis is a programmed cell death that serves to eliminate dying cells in proliferating or differentiating cell populations. Apoptosis plays a crucial role in the development and maintenance of homeostasis in multicellular organisms. However, inappropriate or excessive apoptosis is implicated in many diseases [4]. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay detects the characteristic feature of apoptotic cell death (DNA fragmentation) [5]. DNA fragmentation detected by TUNEL staining occurs as a pattern consistent with apoptosis following TBI [6]. Another important characteristic feature of apoptosis is activation of caspases. Caspase-3 is one of the most widely studied caspases, and it is a key executor of apoptosis [7]. Expression of activated caspases was increased after TBI [8]. In addition to caspases, Bcl-2 family proteins also play a pivotal role in the regulation of apoptosis. The Bcl-2 family is classified into anti-apoptotic proteins and pro-apoptotic proteins based on function. The balance between pro-apoptotic and anti-apoptotic Bcl-2 family members determines the mitochondrial response to apoptotic stimuli [9]. Graham et al. [10] showed that expression of various Bcl-2 family proteins was altered following induction of TBI.

In animal and human studies, exercise both forced and voluntary exercise types, improved cognitive function, learning ability, and memory capability [11,12]. Some studies have suggested that regular

* Corresponding author. Department of Physical Education, College of Arts and Physical Education, Sang Myung University, #7 Hongji-dong, Jongno-gu, Seoul 110-743, Republic of Korea. Tel.: +82 2 2287 5133; fax: +82 2 2287 0075.
E-mail address: ssoop@smu.ac.kr (S.-S. Baek).

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voluntary exercise can be considered a therapeutic tool for TBI [13,14]. Although it is believed that voluntary exercise is useful for neurorehabilitation after TBI [13], the effect of forced treadmill exercise on TBI-induced memory loss in relation to apoptotic neuronal cell death has not been evaluated.

In the present study, we investigated the effect of forced treadmill exercise on short-term memory and apoptotic neuronal cell death in the hippocampus after TBI in rats. For this study, a step-down inhibitory avoidance task, the TUNEL assay, immunohistochemistry for caspase-3, and Western blot analysis for Bcl-2 and Bax were performed.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats, weighing 210 ± 10 g (7 weeks), were obtained from a commercial breeder (Orient Co., Seoul, Korea) for the experiments. The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. The rats were housed under controlled temperature (23 ± 2 °C) and lighting (07:00 h–19:00 h) conditions with food and water available *ad libitum*. The rats were randomly divided into the following four groups: the control group ($n = 10$), the control exercise group ($n = 10$), the TBI-induced group ($n = 15$), and the TBI-induced and exercise group ($n = 15$).

2.2. Induction of TBI

TBI was induced using a previously described procedure [15]. In brief, the rats were anesthetized with Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). A midline incision was made on the skin and underlying fascia. A circular craniotomy (5.0 mm) was performed using a Dremel motor tool and a specially designed drill bit that prevented damage to the meninges and cortex (2.4 mm lateral to the midline, and 4.2 mm posterior to the coronal suture). The contusion injury was created with an electromagnetic contusion device (Impact One™, Stereotaxic Impactor; MyNeurolab, St. Louis, MO) using a sterile stainless steel impactor tip (3.0 mm diameter) that was activated at a velocity of 5.00 m/sec. The impactor tip was positioned above the cortex, and resulted in a 2.5 mm compression to the cortex. The rats in the control (sham operation) group and in the control exercise group were treated in an identical manner; however, a brain contusion was not induced.

2.3. Treadmill exercise protocol

The rats in the exercise groups were forced to run on a treadmill for 30 min once daily for 10 consecutive days, starting 2 days after TBI induction. The exercise load for the exercise groups consisted of running at a speed of 2 m/min for the first 5 min, 5 m/min for the next 5 min, and then at a speed of 8 m/min for the last 20 min, with a 0° inclination. These settings represent a low intensity treadmill exercise. The rats in the non-exercise groups were left in the treadmill without running for the same period as the rats in the exercise group.

2.4. Step-down avoidance task

The latency of the step-down avoidance task was determined to evaluate the short-term memory capability. The rats were trained in a step-down avoidance task 12 days after induction of the TBI. Two hours after training, the latency (sec) of the animals in each group was determined. The rats were placed on a 7 × 25 cm platform, which was 2.5 cm high. The platform faced a 42 × 25 cm grid of parallel 0.1 cm-caliber stainless steel bars spaced 1 cm apart. In training sessions, the animals received a 0.5 mA scramble foot shock for 2 s immediately upon stepping down. The interval between the rats stepping down and placing all four paws on the grid was defined as the latency time. A latency over 180 s was counted as 180 s.

2.5. Tissue preparation

The rats were sacrificed immediately after determining the latency of the step-down avoidance task. The animals were anesthetized using Zoletil 50® (10 mg/kg, i.p.). Blood from each rat was collected by cardiac puncture. Corticosterone levels in plasma samples were assayed by using a commercial CS RIA kit (DPC) as described previously [16]. The rats were transcardially perfused with 0.05 M phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 0.5 M phosphate buffer (PB; pH 7.4). Brains were dissected, post-fixed in the same fixative overnight, and transferred to 30% sucrose for cryoprotection. Forty μm thick coronal sections were made using a freezing microtome (Leica, Nussloch, Germany). Ten slice sections on average in the hippocampus were collected from each rat. The sections of 2.5 mm to 2.7 mm posterior from the bregma were used for immunohistochemistry.

2.6. TUNEL staining

To visualize DNA fragmentation, TUNEL staining was performed using an In Situ Cell Death Detection Kit® (Roche, Mannheim, Germany) according to the manufacturer's protocol [17]. The sections were post-fixed in ethanol–acetic acid (2:1) and rinsed. The sections were then incubated with proteinase K (100 μg/ml), rinsed, and incubated in 3% H_2O_2, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. The sections were rinsed and visualized using Converter-POD with 0.03% 3,3′-diaminobenzidine (DAB). Mayer's hematoxylin (DAKO, Glostrup, Denmark) was used as a counterstain, and the sections were mounted onto gelatin-coated slides. Slides were air-dried overnight at room temperature, and coverslips were mounted using Permount®.

2.7. Caspase-3 immunohistochemistry

To visualize caspase-3 expression, caspase-3 immunohistochemistry was performed as the previously described method [17]. The sections were selected from each brain and incubated overnight with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), then with biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h. The secondary antibody was amplified with the Vector Elite ABC kit® (1:100; Vector Laboratories). Antibody-biotin–avidin–peroxidase complexes were visualized using 0.03% DAB, and sections were mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount®.

2.8. Western blot analysis

The hippocampi were collected, then immediately frozen at −70 °C. The tissues were homogenized with lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2.6H_2O$, 1 mM EGTA, 1 mM PMSF, 1 mM Na_2VO_4, and 100 mM NaF, then ultracentrifuged at 50,000 rpm for 1 h. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA). Protein (40 μg) was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse actin antibody (1:2000; Santa Cruz Biotechnology), mouse Bax antibody (1:1000; Santa Cruz Biotechnology), and mouse Bcl-2 antibody (1:1000; Santa Cruz Biotechnology) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibodies against actin, Bax, and Bcl-2 (1:2000; Amersham Pharmacia