Supplementary Data

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

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SUPPLEMENTARY MATERIALS AND METHODS

TBI animal model

Animal studies and all research processes were approved by the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee (IACUC). The therapeutic effects of exercise were examined in an animal model of TBI in vivo. There were 7 rats per group. Briefly, 8-week-old male rats were housed in laminar-flow cabinets under specific pathogen-free conditions. The animals were anesthetized with isoflurane (2% isoflurane in a 1:2 mixture of O_2/N_2O) via a mask and held in a stereotaxic frame with an ear bar. The controlled cortical impactor was carried out using a pinpoint PCI 3000 precision cortical impactor (Hatteras Instruments, Cary, NC, USA) to generate the TBI model. Unilateral 4-mm craniectomy was performed on the right side using a high-speed drill. The tip of the impactor was centered over the injury site (2.0 mm posterior to bregma and 1.5 mm right from midline), and the injury was produced using the following conditions: the depth was set at 1.5 mm, and the velocity was set at 3.0 m/s to achieve moderate brain injury. In the sham group, rats underwent craniotomy without CCI injury.

Treadmill exercise

All rats were pretrained on a motorized treadmill at a speed of 10 m/min for 30 min three times prior to the TBI operation. The rats in the sedentary group were placed on a stationary treadmill (without running).

Behavior test

The rotarod test was used to evaluate motor function and balance. The animals were placed on a 6 cm diameter of the cylinder, and the duration that the animal walked on the rotarod was automatically recorded. The speed was slowly increased from 4 to 40 rpm within 180 s and performed three times per day once a week for the analysis. The animals were trained three times per day for three consecutive days before surgery. Long-term memory was assessed using a passive avoidance chamber (avoidance system, B. S. Technolab Inc.) divided into white and illuminated compartments, and black and dark compartments. For the acquisition trial, the animals were placed in a white and illuminated chamber; meanwhile, 30 s later, a door to a dark enclosed chamber with a stainless grid floor was opened. As soon as the animals entered the dark chamber, the door closed, and the animals received an electric foot shock (0.3 mA, 15 s). After electric shock, the animals were removed from the chamber. The latency to cross the dark chamber was recorded automatically. In order to evaluate retention, the animals were again placed in an illuminated chamber, and the time to cross the dark chamber was recorded. The maximal latency time was limited to 300 s, and no electric foot shock was administered during the retention trial. The spontaneous alternation behavior of the animals in the Y-maze was used to evaluate working memory (or short-term memory) in a new environment. The Y-maze consisted of three black painted arms (45x10x35 cm,), and these arms were positioned at an equal angle (120° from one another). The evaluation was compromised in one trial. At the beginning of the trial, the rats were placed at the end of the arm and allowed to freely explore the three arms for 10 min. The number of arm entries was counted such that the rats entered the less recently visited arm. The discrimination preference value was calculated as

follows: Alternation (%) = (Alternation frequency/Max alternation frequency) × 100. The number of entries was measured and analyzed from video recordings with Etho Vision XT (Noldus Information Technology Inc., Leeburg, USA). The rats were allowed to enter an arm with all four paws to count the number of arm entries. The maze was cleaned between each use by the animals with 70% ethanol and dried to eliminate traces of odor. The Y-maze test was performed once a week for 28 days.

Antibodies and reagents

The following antibodies were used: anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NSE, anti-S100 β, anti-PDP9.5, anti-NeuN, anti-GFAP (Abcam, Cambridge, UK), anti-GFAP, anti-HSP70, anti-BAX, anti-cleaved caspase3; (Cell Signaling Technology, Danvers, MA, USA). The Proteome Profiler Rat XL Cytokine Array was purchased from R&D Biosciences (ARY030).

Western blot analysis

3, 7, and 14 days after CCI, three rats from each group were sacrificed, and the brain was removed. Brain samples around the contusion site in the TBI group and around the craniotomy site in the sham group were dissected and lysed with RIPA buffer (Cell Signaling Technology). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and incubated with primary antibodies for 16 h at 4 ° C. After washing, the membranes were incubated with Horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. A detection kit solution (GWVITEK, Seoul, South Korea) was applied to the membranes, and the signals were detected using X-ray films (AGFA, Mortsel,

Belgium) or a detection system (JP-33, JPI Healthcare, Seoul, South Korea). Bands were quantified using the ImageJ software and normalized to β-actin.

TUNEL assay

TUNEL was performed on cryostat sections using an in situ cell death detection kit (TMR red; Roche, NJ, USA) based on the manufacturer's instructions. The nuclei were counterstained with DAPI stain. Immunostained images were acquired using a laser scanning confocal microscope (LSM 710; Carl Zeiss, Germany).

Immunohistochemistry

The rats were sacrificed with intracardiac perfusion with phosphate-buffered saline and 4.0%

Paraformaldehyde (PFA). The brains were sliced coronally at 1.0-mm thickness. The sliced brains were fixed with 4.0% PFA overnight and transferred to 20% sucrose in PBS until tissue sinks. Fixed brain tissues were embedded in OCT compound for immunohistochemistry (IHC). Each block was sliced into 8-μm-thick sections and mounted on poly L-lysine-coated slides.

Specimens were stained with antibodies against GFAP (1:100), NSE (1:100), and S100β (1:100) for 24 h at 4°C, followed by biotinylated secondary antibodies (Vector Laboratories, Orton, Southgate, UK) at 1:100 dilution for 1 h, and avidin-biotin complex (Elite ABC; Vector Laboratories) for 1 h. Non-specific IgG antibodies were used as negative controls. Diaminobenzidine tetrahydrochloride was used as the enzyme substrate to observe specific antibody localization, and Harris hematoxylin was used as a nuclear counterstain.

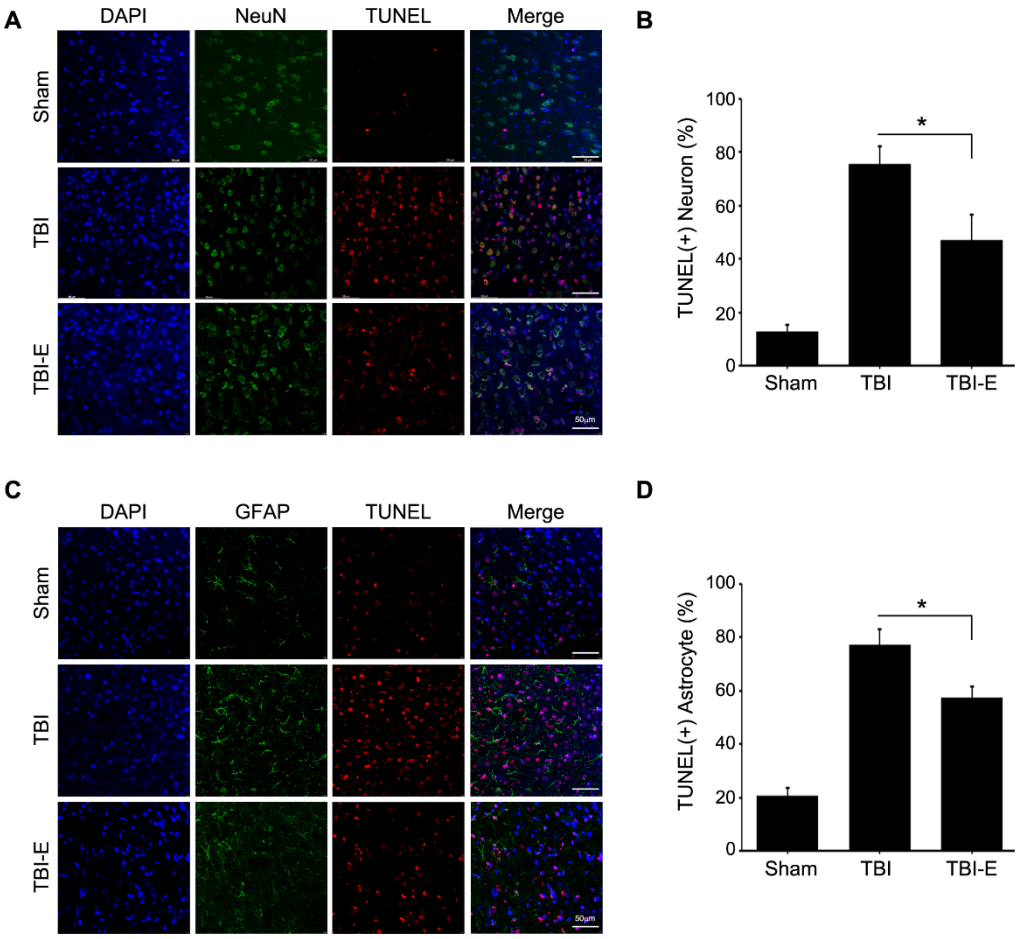
Rat Cytokine array

The rat serum is collected from whole blood without anticoagulation treatment. A rat-specific cytokine antibody array XL series (R&D, MN, USA) consisting of 79 different cytokine antibodies spotted in duplicate onto two membranes in blood was analyzed based on the manufacturer's instructions.

In brief, cytokine array membranes were blocked with blocking buffer and incubated with blood serum for 16 h at 4 °C. The cytokine array membranes were washed three times with wash buffer I for 10 min at RT. The membranes were incubated for 1 h at RT with primary biotin-conjugated antibody, followed by horseradish peroxidase (HRP)-conjugated streptavidin at a 1:000-fold dilution, which was added to each membrane, and the mixture was incubated for 30 min. Protein spots were detected using the ECL Western Blotting detection reagents (Promega, Madison, WI, USA). The mean chemiluminescence of all positive spots per membrane was calculated and used for normalization.

Statistics

Data are presented as the mean ± SEM, and analyses were performed using one-way ANOVA with Tukey's post hoc test. A $P < 0.05$ was considered statistically significant.



Supplementary Figure S1. Effects of early exercise on apoptosis in neuron and glial cells after TBI

(A) Representative fluorescence images demonstrating TUNEL-positive neuron cells on rat brain 14 days after TBI. (B) The relative percentage of TUNEL-positive neurons is presented as bar graphs. (C) Representative fluorescence images demonstrating TUNEL-positive glial cells on rat brain 14 days after TBI. (D) The relative percentage of TUNEL-positive glial cells is presented as bar graphs. *, P ⟨0.05. scale bar, 50 μm.