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RESEARCH ARTICLE

Repetitive magnetic stimulation affects the microenvironment of nerve regeneration and evoked potentials after spinal cord injury

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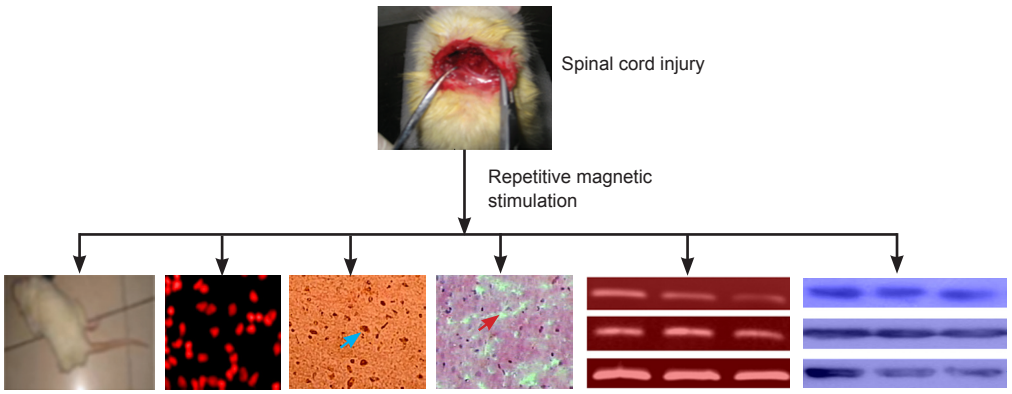
Graphical Abstract



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Abstract

Repetitive magnetic stimulation has been shown to alter local blood flow of the brain, excite the corticospinal tract and muscle, and induce motor function recovery. We established a rat model of acute spinal cord injury using the modified Allen's method. After 4 hours of injury, rat models received repetitive magnetic stimulation, with a stimulus intensity of 35% maximum output intensity, 5-Hz frequency, 5 seconds for each sequence, and an interval of 2 minutes. This was repeated for a total of 10 sequences, once a day, 5 days in a week, for 2 consecutive weeks. After repetitive magnetic stimulation, the number of apoptotic cells decreased, matrix metalloproteinase 9/2 gene and protein expression decreased, nestin expression increased, somatosensory and motor-evoked potentials recovered, and motor function recovered in the injured spinal cord. These findings confirm that repetitive magnetic stimulation of the spinal cord improved the microenvironment of neural regeneration, reduced neuronal apoptosis, and induced neuroprotective and repair effects on the injured spinal cord.

Key Words: *nerve regeneration; spinal cord injury; repetitive magnetic stimulation; motor function; rats; rehabilitation; plasticity; regenerative microenvironment; neural regeneration*

Introduction

The potential of magnetic stimulation for treating spinal cord injury (SCI) has gradually received greater attention by experts in the field. Magnetic stimulation has been shown to improve cough and respiratory function in SCI patients, improve bowel functions and muscular atrophy of the lower extremities in paraplegic patients, and reduce deep vein thrombosis (Fitch et al., 1999; Hallett, 2007; Paim et al., 2013; Yin et al., 2013). Amar and Levy (1999) verified that microenvironment at the injury site was significantly improved by repetitive magnetic stimulation to the spinal cord, and secondary nerve injury was effectively reduced. Pulses of magnetic stimulation increase blood flow in the capillary bed. Pulse accumulation promotes angiogenesis and indirectly contributes to growth of nerve fibers because of vascular tropism (Crowe et al., 1997; Ohta et al., 2005). Magnetic stimulation reduces Ca^{2+} concentrations, increases Mg^{2+} content, and regulates ion imbalances in the injured spinal cord. Ca^{2+} and Na^{+}-K^{+}-ATPase activities are important regulatory factors for gene expression in neurons

(Pommerenke et al., 1996). However, very little is known about how repetitive magnetic stimulation to the spinal cord improves the microenvironment of the injury site and promotes nerve repair, as well as its precise mechanisms of action. The present study explored related indicators of the microenvironment in the injured rat spinal cord using repetitive magnetic stimulation.

Materials and Methods

Ethics statement

This study was approved by the Animal Ethics Committee of China-Japan Union Hospital, Jilin University of China. The animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

Establishment of SCI models

Sixty-seven specific pathogen-free, adult, female, Sprague-Dawley rats, aged 1 month and weighing 250–290 g, were purchased from the Animal Laboratory of Tianjin Medcial University of China (license No. SCXK (Jin) 20070001). The rats were housed at 25°C with 40–60% relative humidity under natural light. Forty-seven rats were intraperitoneally anesthetized with 10% chloral hydrate and fixed on the bench in the prone position. After the lower back was shaved, a median incision was made on the back using the $T_{8–9}$ spinous processes as a center to expose the $T_{7–10}$ spinous processes and the lamina. The $T_{8–9}$ spinous processes and part of the lamina tissue were removed. This exposed dura mater served as the lesion area. Based on the method established by Allen (1940), but with some modifications, a 10-g object was vertically dropped from a 2.5-cm height, which directly impacted the dura mater and $T_{8–9}$. The wound was washed with hydrogen peroxide, and the tissue was sutured layer by layer. Extrusion was conducted 2–3 times daily to assist with urination until the micturition reflex was recovered. Paralysis of the lower limbs was observed along with tail swinging and spasms. These responses confirmed successful establishment of the model. Model establishment failed in three rats and four rats were excluded because of death. The remaining 40 rats were equally and randomly assigned to the SCI group and repetitive magnetic stimulation group. Twenty rats in the sham surgery group were not exposed to Allen's injury, but the spinal cord was exposed.

Repetitive magnetic stimulation to the spinal cord

Four hours after injury, rats in the repetitive magnetic stimulation group received repetitive magnetic stimulation using a Magstim Rapid2 magnetic stimulator (Magstim, Woburn, MA, USA), with a maximum output intensity of 2.2 T and a 25-mm diameter butterfly coil. The rats received repetitive magnetic stimulation in a supine position and were fixed in a wooden box. The center of the coil was placed at $T_{6–7}$. Stimulus intensity was 35% of the maximum output intensity. Stimulation was given at a frequency of 5 Hz, with 5 seconds for each sequence, an interval of 2 minutes for 10 sequences, once a day, 5 days in a week, for 2 consecutive weeks.

Evaluation of motor function

Motor functions were assessed before injury, and at 1 and 3 days, and 1, 2, 3, and 4 weeks after injury in the three groups using the modified Tarlov scoring system, the Basso-Beattie-Bresnahan (BBB) locomotor rating scale, and the inclined plane test.

The inclined plane test: rats were horizontally placed on a smooth tiltboard with their heads to the front. The angle was increased every 5°, and the maximum angle at which the rats stayed on the board for 5 seconds was recorded (Wang et al., 2013).

BBB locomotor rating scale: the BBB scores ranged from 0 to 21, where 21 = normal and 0 = complete paralysis. The blind method was used (Wang and Zhang, 2015).

Modified Tarlov scoring system: 0, the lower limbs cannot move or bear weight; 1, the lower limbs can move, but cannot bear weight; 2, the lower limbs can move freely or powerfully, but cannot bear weight; 3, the lower limbs can support weight and walk one or two steps; 4, can walk with mild disturbance; 5, normal walking (Wang and Zhang, 2015).

Determination of apoptotic cells

Three days after injury, five rats from each group were anesthetized with chloral hydrate. After the chest was opened, aortic cannulation was conducted through the left ventricle. Tissue was fixed with 4% paraformaldehyde and a 2-cm long segment of the spinal cord was resected with the injury site as the center. The spinal cord was fixed again in paraformaldehyde, embedded in wax, and sliced into 20-μm thick sections. The sections were then dewaxed. Under an inverted microscope (Leica, Tokyo, Japan), the apoptotic cells were quantified in ten 200× fields. The mean value was then calculated.

Reverse transcription-polymerase chain reaction (RT-PCR)

Three days after injury, five rats from each group were randomly selected. Spinal cord tissue (50 mg) was obtained from the injury site at $T_{8–9}$. In accordance with Trizol reagent instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA), total RNA was extracted from the spinal cord. RNA content was measured with an ultraviolet spectrophotometer (Olympus, Tokyo, Japan). Using the two-step RT-PCR kit (TaKaRa, Dalian, China), mRNA was reverse-transcribed into cDNA, and cDNA was amplified using PCR. Primer sequences were as follows: matrix metalloproteinase (MMP)2 (414 bp): upstream 5′-TTT TTG TGC CCA AAG AAA GG-3′, downstream 5′-ATG TCA GAC AAC CCG AGT CC-3′; MMP9 (379 bp): upstream 5′-GGT TTC TGT CCA GAC CAA GG-3′, downstream 5′-TGC AAG GAT TGT CAT CTG GA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAP-

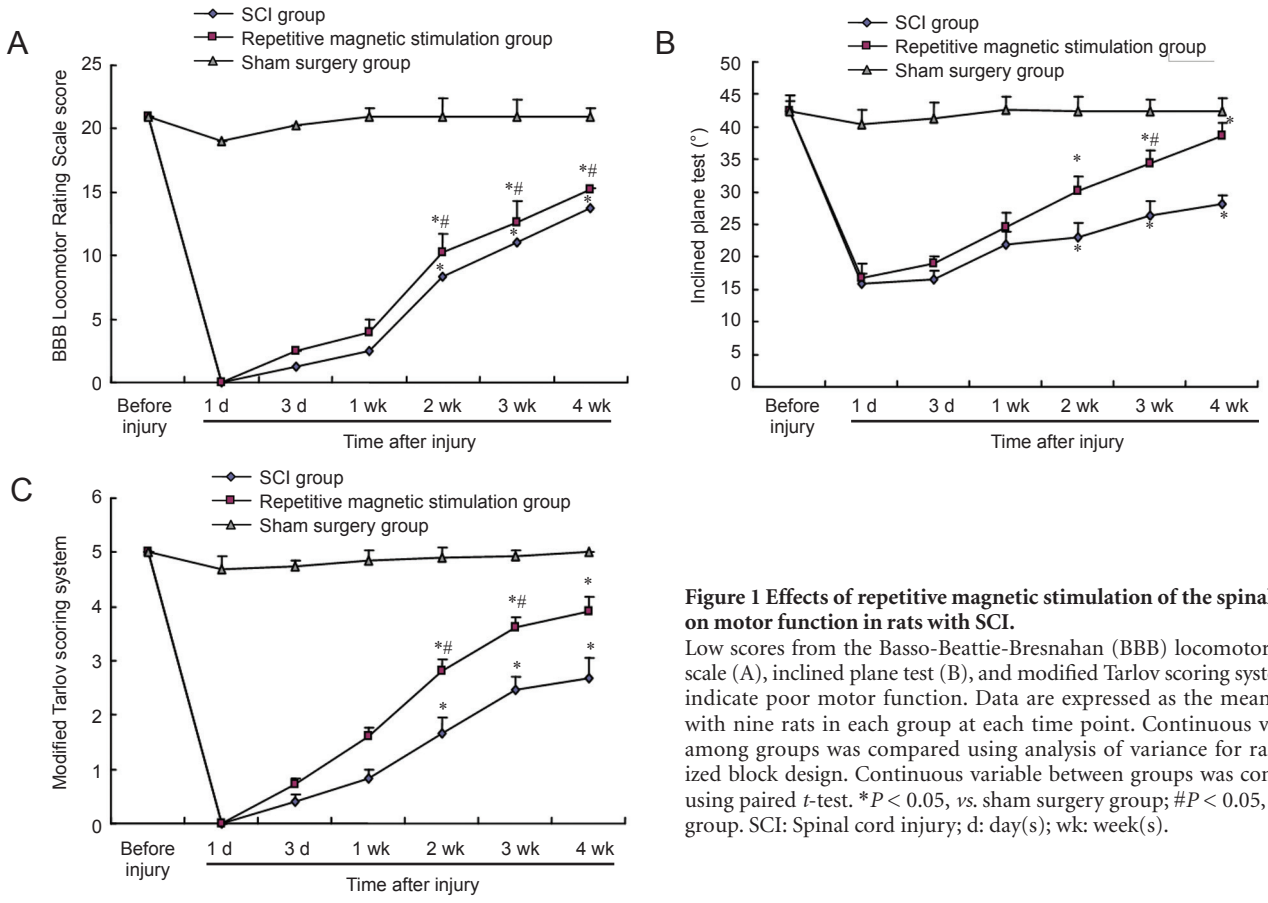

Figure 1 Effects of repetitive magnetic stimulation of the spinal cord on motor function in rats with SCI.
Low scores from the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (A), inclined plane test (B), and modified Tarlov scoring system (C) indicate poor motor function. Data are expressed as the mean ± SD, with nine rats in each group at each time point. Continuous variable among groups was compared using analysis of variance for randomized block design. Continuous variable between groups was compared using paired *t*-test. **P* < 0.05, *vs.* sham surgery group; #*P* < 0.05, *vs.* SCI group. SCI: Spinal cord injury; d: day(s); wk: week(s).

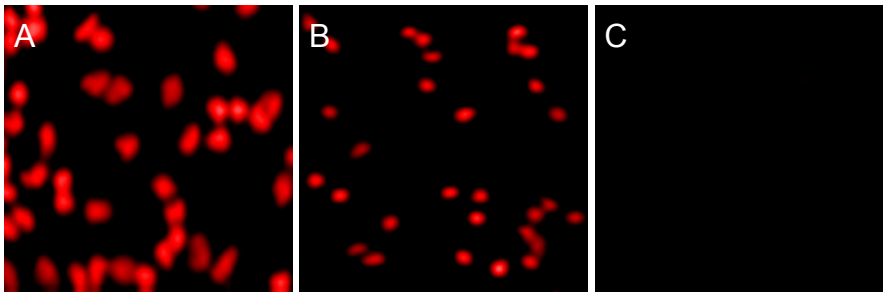

Figure 2 Effects of repetitive magnetic stimulation on apoptosis in the injured spinal cord of rats (terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, × 200).
(A) Abundant apoptotic cells are observed in the injured spinal cord of rats in the spinal cord injury group. (B) The number of apoptotic cells is apparently reduced in the injured spinal cord of rats from the repetitive magnetic stimulation group. (C) No apoptotic cells are detected in the spinal cord of rats from the sham surgery group.

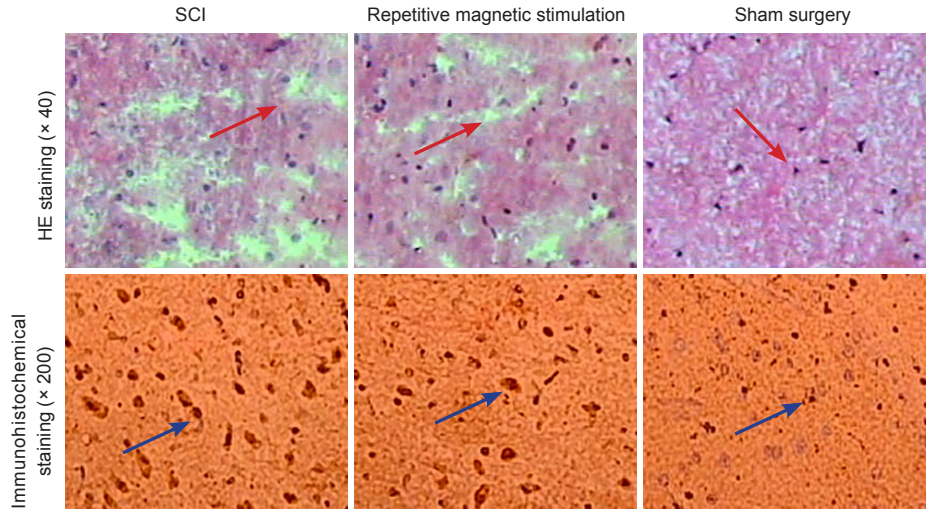

Figure 4 Effects of repetitive magnetic stimulation on repair of the injured spinal cord in rats.
Compared with the sham surgery group, a cavity is visible in the SCI group, with a decreased number of nestin-positive cells. In the repetitive magnetic stimulation group, the cavity is smaller, and the number of nestin-positive cells is increased. Red arrows show cavity. Blue arrows show nestin expression. SCI: Spinal cord injury; HE: hematoxylin-eosin.

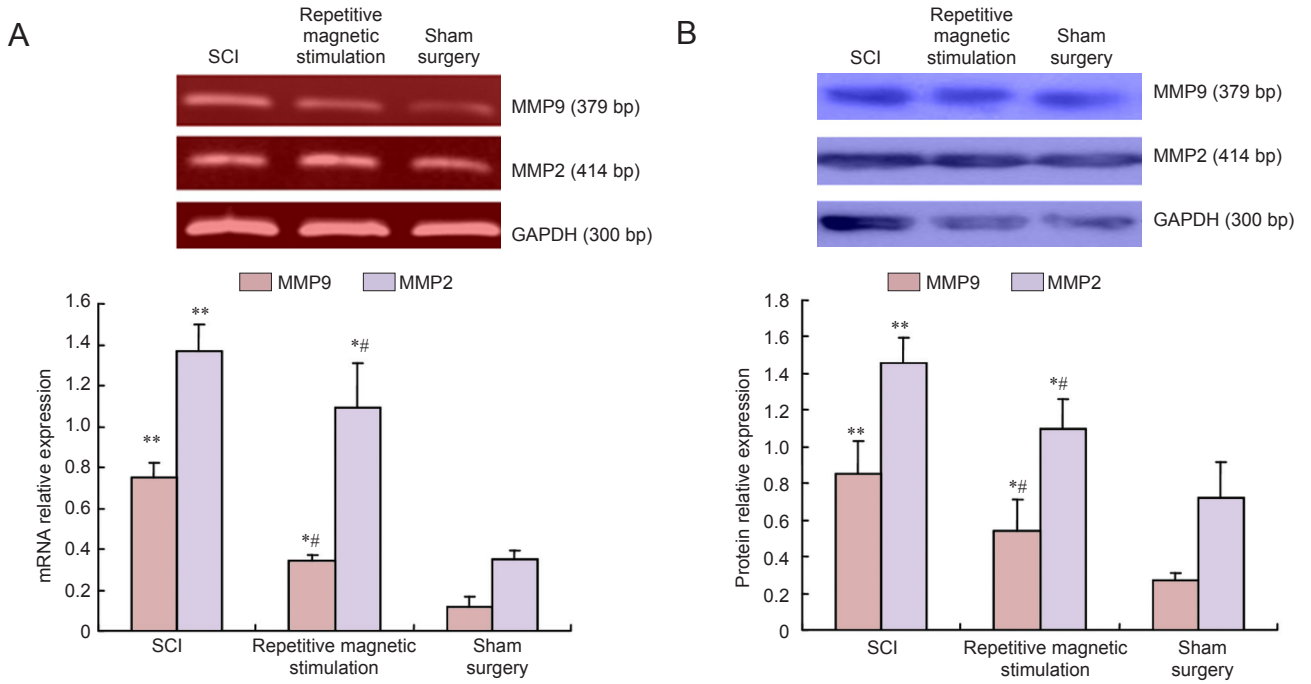

Figure 3 Effects of repetitive magnetic stimulation on MMP 9/2 mRNA (A) and protein (B) expression in the spinal cord of rats with SCI.
The relative mRNA expression levels are expressed as the ratio of integral optical density of MMP9/2 to GAPDH. The relative expression level of MMP9/2 protein is expressed as the ratio of optical density of MMP9/2 to GAPDH. Data are expressed as mean ± SD, with five rats in each group at each time point. Continuous variable among groups was compared using analysis of variance for randomized block design. Continuous variable between groups was compared using paired *t*-test. **P* < 0.05, ***P* < 0.01, *vs.* sham surgery group; #*P* < 0.05, *vs.* SCI group. MMP: Matrix metalloproteinase; SCI: spinal cord injuny; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

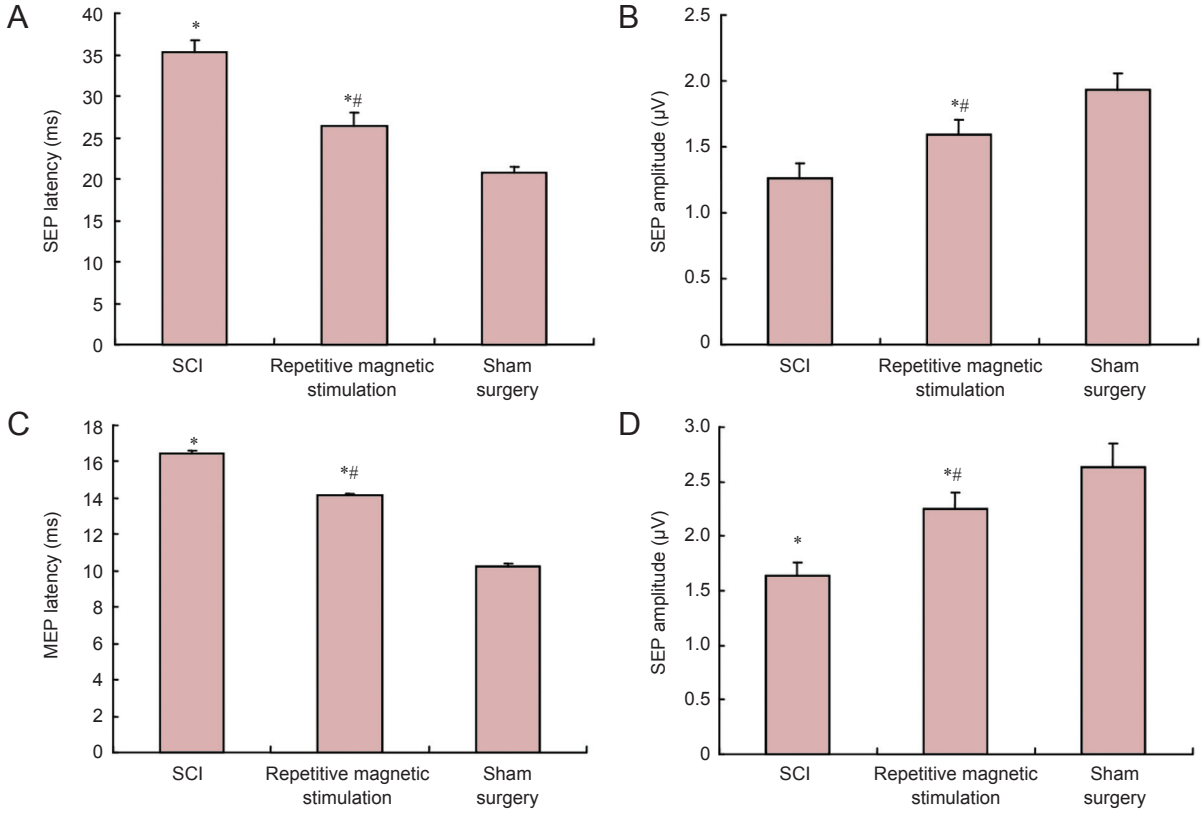

Figure 5 Effects of repetitive magnetic stimulation of the spinal cord on nerve conduction in the injured spinal cord of rats.
Data are expressed as the mean ± SD, with five rats in each group at each time point. Continuous variable among groups was compared using analysis of variance for randomized block design. Continuous variable between groups was compared using paired *t*-test. **P* < 0.05, *vs.* sham surgery group; #*P* < 0.05, *vs.* SCI group. MEP: Motor-evoked potential; SEP: somatosensory-evoked potential; SCI: spinal cord injury.

DH) (300 bp): upstream 5′-GAG GAC CAG GTT GTC TCC TG-3′, and downstream 5′-GGA TGG AAT TGT GAG GGA GA-3′. Amplified products were electrophoresed. Integral optical density analysis of electrophoresis results was conducted using a gel image analysis system (Media Cybernetics, WA, USA). The ratio of integral optical density of MMP9/2 to GAPDH was calculated as the relative expression levels of MMP9/2 mRNA.

Western blot assay

Following RNA extraction in RT-PCR, the remaining sample was centrifuged at 1,500 r/min for 30 minutes and the supernatant was collected. The total protein concentration was measured using the Bradford protein assay. Samples were separated by electrophoresis on a 5% stacking gel at 40 V for 1 hour and 10% separating gel at 60 V for 3.5 hours. The separated proteins were transferred onto a membrane using the wet method at 14 V for 14 hours. The membrane was blocked in a swinging bed at 37°C for 2 hours, and washed three times for 10 minutes each. The membranes were incubated with rabbit anti-rat MMP9/2 polyclonal antibody (1:500; Sigma, St. Louis, MO, USA) and rabbit anti-rat GAPDH polyclonal antibody (1:500; Sigma) at room temperature for 60 minutes, followed by three washes with tris-buffered saline/Tween-20 (TBST) for 10 minutes each. The membranes were then incubated with alkaline phosphatase-labeled goat anti-rabbit IgG (1:2,000; Gibco BRL, Gaithersburg, MD, USA) at room temperature for 60 minutes, followed by three washes with TBST for 10 minutes each, followed by tris-buffered saline for 10 minutes. The samples were visualized with 3,3′-diaminobenzidine (Beijing CellChip Biotechnology, Co., Ltd., Beijing, China). Optical density was analyzed using Quantity One analysis software (Hyclone, Logan, UT, USA). The ratio of optical denstiy of MMP9/2 to GAPDH was considered the relative expression level of MMP9/2 protein.

Immunohistochemical staining and hematoxylin-eosin (HE) staining

Four weeks after injury, five rats from each group were anesthetized with 10% chloral hydrate (350 mg/kg) for euthanasia.

Immunohistochemical staining: sections were placed at room temperature for 30 minutes, blocked with fetal bovine serum for 1 hour, washed three times with PBS for 5 minutes each, incubated with mouse anti-rat nestin antibody (1:5,000; Roche) at 4°C overnight, following goat anti-mouse IgG-FITC (1:100) at 37°C for 1 hour, and mounted onto glass slides. The sections were dehydrated through a graded alcohol series, permeabilized with xylene, and mounted with neutral resin. The number of nestin-positive cells was observed in ten 200× fields using an inverted microscope.

HE staining: after anesthesia, the chest was opened to expose the heart. Ascending aortic cannulation was performed. The right auricle was cut open, washed with physiological saline, and fixed with 4% paraformaldehyde. A 1-cm section of spinal cord tissue at the site of injury was collected, dehydrated through a graded alcohol series, and sliced into 20-μm thick longitudinal frozen sections. The sections were then stained with hematoxylin for 5 minutes, washed with running water, differentiated with hydrochloric acid in ethanol for 10 seconds, washed with running water for 10 minutes, stained with eosin for 7 minutes, washed with running water, dehydrated through a graded alcohol series, permeabilized with xylene, and mounted with neutral resin.

Detection of somatosensory-evoked potential (SEP) and motor-evoked potential (MEP)

Four weeks after injury, five rats each from the sham surgery group, SCI group, and repetitive magnetic stimulation group were intraperitoneally anesthetized with 10% chloral hydrate (350 mg/kg). SEP and MEP were measured using a Keypoint 4-evoked Potential System (Beijing Weidi Kangtai Medical Instrument Co., Ltd., Beijing, China).

SEP: The rats were placed in a horizontal plane. The hind limbs were stimulated with a stimulating electrode. A recording electrode was placed at the intersection of the coronal suture and sagittal suture healing line under the scalp (*i.e.*, hindlimb cortical sensory area). A reference electrode was placed 0.5 cm posterior to the hindlimb cortical sensory area. A direct current, square wave, and electrical pulses were given until the hind limb exhibited a slight tic. The conditions were as follows: current intensity of 5–15 mA, pulse width of 0.2 ms, frequency of 3 Hz, superimposed: 50–60 times. SEP latency and amplitude were recorded.

MEP: The stimulating electrodes were placed 2 mm anterior to the coronal suture and 2 mm lateral to the sagittal suture under the scalp (*i.e.*, motor cortex). The electrodes were stimulated as follows: stimulus intensity of 40 mA, pulse width of 0.1 ms, frequency of 1 Hz, sensitivity of 5 μV/D, scanning speed of 5 ms/D; superimposed: 300–500 times. MEP latency and amplitude were recorded.

Statistical analysis

All data were expressed as the mean ± SD, and analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). Continuous variable among groups was compared using analysis of variance for randomized block design. Continuous variable between groups was compared using paired *t*-test. An alpha of 0.05 with a two-tailed test was used.

Results

Repetitive magnetic stimulation improved motor funcion in SCI rats

There was no significant difference in BBB locomotor rating scale scores, inclined plane test scores, or modified Tarlov scoring system scores in all rats prior to model establishment. BBB locomotor rating scale scores, inclined plane test scores, and modified Tarlov scoring system scores were significantly greater in the repetitive magnetic stimulation group than in the SCI group (2–4 weeks after injury; $P < 0.05$). The above scores were significantly less in the SCI and repetitive magnetic stimulation groups

than in the sham surgery group at 2–4 weeks after injury ($P < 0.05$; **Figure 1**).

Repetitive magnetic stimulation of the spinal cord reduced apoptosis in the injured spinal cord

TUNEL assay results revealed significantly less apoptotic cells in the repetitive magnetic stimulation group (13.67 ± 2.32/200× field) than in the SCI group (32.76 ± 3.44/200× field) ($P < 0.05$). No apoptotic cells were identified in the sham surgery group (**Figure 2**).

Repetitive magnetic stimulation of the spinal cord reduced MMP9/2 mRNA and protein expression in the injured spinal cord

RT-PCR and western blot assay results demonstrated significantly increased MMP9/2 mRNA and protein expression at 72 hours after injury ($P < 0.01$). MMP9/2 mRNA and protein expression in the repetitive magnetic stimulation group was less than in the SCI group ($P < 0.05$), but more than in the sham surgery group ($P < 0.05$; **Figure 3**).

Repetitive magnetic stimulation of the spinal cord improved pathomorphology and increased the number of nestin-positive cells in the injured spinal cord

Four weeks after injury, hematoxylin-eosin and immunohistochemical staining revealed a complete and clear structure spinal cord, with no cavity and densely arranged nerve fibers, in the sham surgery group. A large number of nestin-positive cells were quantified (30.42 ± 3.83/200× field). In the SCI group, the spinal cord tissue exhibited a loose structure, with a visible cavity and a large number of necrotic neurons. The nerve fibers were loosely arranged and appeared shorter and less compared with the repetitive magnetic stimulation group. There were fewer nestin-positive cells in the SCI group (5.83 ± 1.72/200× field) than in the sham surgery group ($P < 0.05$). In the repetitive magnetic stimulation group, loose spinal cord tissue and a small cavity were visible, with a recovered density of nerve fibers. The number of nestin-positive cells was slightly higher in the repetitive magnetic stimulation group (19.24 ± 2.20/200× field) than in the SCI group ($P < 0.05$; **Figure 4**).

Repetitive magnetic stimulation of the spinal cord improved electrophysiological function in the injured spinal cord

After model establishment, the evoked potential waveform disappeared in the repetitive magnetic stimulation and SCI groups. Four weeks later, SEP and MEP were slightly recovered in the SCI group compared with the sham surgery group ($P < 0.05$). SEP and MEP were significantly recovered, latencies were shorter, and amplitudes were higher in the repetitive magnetic stimulation group than in the SCI group ($P < 0.05$; **Figure 5**).

Discussion

Repetitive magnetic stimulation of the spinal cord can reverse synaptic function at the site of injury, improve neuronal plasticity, and protect neurons against external factor-induced degeneration and necrosis. Long-term repetitive magnetic stimulation can increase mRNA expression of brain-derived neurotrophic factor (Muller et al., 2000) and reduce c-fos protein levels at the injury site (Hausmann et al., 2000), which suggests a neuroprotective effect of repetitive magnetic stimulation of the spinal cord. Consequently, the neuroprotective effect of repetitive magnetic stimulation has been shown to indirectly reduce proliferation of reactive astrocytes, as well as decrease axonal sprouting, synaptic reconstruction, and excitatory loop formation (Avoli, 1996; Muller et al., 2000; Kudo et al., 2005).

Following central nervous system injury, MMP2 and MMP9 disrupt the tight connections between capillaries, basement membranes, and the blood brain barrier, and induce vasogenic edema of the central nervous system (Liu and Shubayev, 2011; Yang et al., 2013; Lee et al., 2014). Results from the present study suggested that repetitive magnetic stimulation effectively reduced edema-related gene and protein expression at the injury site, which could also be responsible for reducing the degree of edema. Repetitive magnetic stimulation of the spinal cord also effectively reduced neuronal apoptosis at the injury site. Results from the BBB locomotor rating scale, inclined plane test, and modified Tarlov scoring system showed that repetitive magnetic stimulation of the spinal cord improved motor function of the hind limbs in rats. Repetitive magnetic stimulation shortened SEP and MEP latencies and increased amplitudes in SCI rats, and effectively contributed to functional recovery of the injured spinal cord. This study provides a new theoretical basis for repetitive magnetic stimulation in SCI repair.

Author contributions: *SFW and JLJ conceived and designed the study, provided data and ensured the integrity of the data. SQZ analyzed the data, wrote the paper, obtained the funding, provided technical or material support and served as a principle investigator. XDG and XGW were in charge of paper authorization and statistical analysis. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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