Effects of repetitive magnetic stimulation on motor function and expression of GAP-43 and 5-HT in rats with spinal cord injury

Hao Liu

Western Command

Deqi Xiong

The Second People's Hospital Of Yibin

Wenchun Wang (✉ wwc1977biology@163.com)

the General Hospital of Western Theater Command <https://orcid.org/0000-0001-9742-9089>

Li Yuan

the General Hospital of Western Theater Command

Rizhao Pang

Western Command

Qian Deng

Southwest Jiaotong University

Nianyi Sun

Shengjing Hospital of China Medical University

Jinqi Zheng

the General Hospital of Western Theater Command

Jiancheng Liu

Western Command

Wu Xiang

the General Hospital of Western Theater Command

Zhesi Chen

the General Hospital of Western Theater Command

Jiachun Lu

Cheng Du Eighth People's Hospital

Anren Zhang

Shanghai Fourth People's Hospital Affiliated To Tongji University School Of Medicine

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Abstract

Objectives: Spinal cord injury (SCI) is a disastrous central nervous system (CNS) disorder. The aim of this study was to explore the effects of repetitive trans-spinal magnetic stimulation (rTSMS) act on different segments of the spinal cord on movement function and expression of GAP43 and 5-HT in rats after acute cord injury and to preliminarily discuss the best treatment site of rTSMS, so as to provide theoretical foundation and experimental evidence for the clinical application of rTSMS in spinal cord injury.

Methods: In the present study, we used a rat model of T10 laminectomy with transient violent oppression by aneurysm clip. The rats were classified into A group (sham surgery), B group (acute SCI without stimulation), C group (T6 segment stimulation), D group (T10 segment stimulation) and E group (L2 segment stimulation).

Results: In vivo the magnetic stimulation was found to protect motor function and alleviate myelin sheath damage, decrease the expression levels of NgR and Nogo-A, increase the expression levels of growth-associated protein-43 (GAP43) and 5-hydroxytryptamine (5-HT), and inhibit TUNEL-positive cells as well as the expressions of apoptosis-related protein of rats following 8 weeks post-operation.

Conclusions: This study suggests that rTSMS can promote the expression of GAP-43 and 5-HT and axonal regeneration in the spinal cord which is beneficial to the recovery of motor function after acute spinal cord injury.

1. Introduction

Spinal cord injury (SCI) is a severely disabling disease caused by various reasons including traffic accident and natural disaster, characterized by the destruction of spinal cord normal anatomic structure and function leads to the sensory and motor dysfunction (1–3). The incidence rate of SCI shows a trend of increasing year by year and becoming younger (4, 5). In addition, as one of the most important and prominent functional disorders of SCI, motor dysfunction greatly affects the life and work of patients. Therefore, the promotion of motor function recovery after SCI is one of the most important and urgent research topics. However, there is still no definite and effective method for the treatment of spinal cord injury in clinical practice.

Since the theory of spinal cord plasticity was proposed, it has become an important direction of SCI treatment (6). In recent decades, magnetic stimulation (MS) technology has gradually attracted wide attention from researchers and clinicians for its safe and effective characteristics. Studies have shown that transcranial magnetic stimulation (TMS) can change the excitability of nerve cells, accelerate the regeneration of nerve cells, induce axon regeneration and lateral bud growth, enhance the remodeling of nervous system and promote the recovery of motor function (7, 8). In addition, repetitive transcranial magnetic stimulation (rTMS) as a non-invasive new technique, can induce an electrical current in the cerebral cortex, change the action potential of cortical nerve cells, and produce long-term plasticity in the spinal cord (9). Other studies have found that 10 Hz rTMS can improve the motor function and relieve

muscle spasm in SCI rats, and that neurotransmitter levels are significantly changed after MS treatment (10). rTMS not only affects the central nervous system, but magnetic stimulation of peripheral nerves can also cause excitatory changes of nerve cells. It has been reported that rTMS has an obvious therapeutic effect on post-SCI respiratory dysfunction (11) and neurogenic rectum (12).

Furthermore, the domestic and foreign studies have reported that the repetitive trans-spinal magnetic stimulation (rTSMS) also can induce axon regeneration and functional reconstruction of spinal nerve cells, and promote the recovery of motor function after SCI (13). However, the stimulus parameters, stimulus sites and intervention time selected in previous studies are not consistent, and corresponding comparative studies are not available. Thus, it is still unclear what the effect of rTSMS on which segment of spinal cord can most significantly improve motor function after acute SCI. To solve the clinical problem, we intend to study the effect of rTSMS on motor function recovery after acute SCI in different spinal segments, and preliminarily explore the best therapeutic sites of this treatment technology.

2. Materials And Methods

2.1 Animals

Prior to the start of the study, approval had been obtained from the Ethics/Review Committee of experimental animal Use and Care in Chengdu General Hospital of the Military Region (2013YG-B005). 80 SD rats (half male and half female; 285 ± 25 g) were purchased from Chengdu Dossy Experimental Animals Co. Ltd. (License number SCXK (Sichuan) 2015-030). The experimental protocol was approved by the Local Ethics Committee for Animal Experiments in General Hospital of Western Theater Command (China) for the care and use of laboratory animals. All animals were maintained in the animal rooms where controlled conventional conditions at temperatures of $24 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 15\%$, 12 h light-dark cycle, and allowed free water and standard diet. The leader of the project promised that the members of this project had complied with the ethical principles of experimental animals, paid attention to animal welfare, cared for animals, and minimized the damage to animals.

2.2 Establishment of a rat model of acute spinal cord injury

After anesthetized with 3% pentobarbital sodium (30 mg pentobarbital sodium /kg rat body weight) by intraperitoneal injection (14), the rats were fixed on surgery table. The surgical site was shaved and sterilized. Briefly, a longitudinal incision of 3 cm was made to expose the T9, T10 and T11 vertebral plate. Then, the laminectomy was performed at the T10 level to expose the dorsal epidural of spinal cord. The triangular needle was clamped with a needle-holding device so that the blunt end passes through the space between the ventral dura and the vertebral body completely to avoid spinal cord injury and pulling. The aneurysm clip (Yasargil Titan Mini Clips, FT228T, Closing force 70 g) was fixed with the application clip, and after opening the clip, the aneurysm clip was perforated from T10 through the channel to the opposite side to ensure that the aneurysm clip completely crossed the spinal cord. Then the application clip was suddenly released, and the spinal cord was compressed by instant violence, causing spasmodic tremor and spasmodic oscillation in the rat body. After the clip was removed gently for 10 s, subdural

congestion or hematoma could be seen, with obvious clip marks outside the dural (15). The wound was then sutured layer by layer. After the surgery, the rats were injected with penicillin for 7 successive days. Auxiliary urination by rubbing the bladder of rats at 4 h after the operation and at 8 am and 8 pm every day until the rats resumed spontaneous urination. For the sham-surgery controls, the rats underwent a T10 laminectomy without transient violent oppression by aneurysm clip.

2.3 Animal grouping and intervention method

80 SD rats were randomly divided into five groups ($n = 16$ for each), including A group (T6 segment stimulation), B group (T10 segment stimulation), C group (L2 segment stimulation), D group (acute SCI without stimulation), E group (sham surgery). Our magnetic stimulation program follows established safety guidelines for repetitive magnetic stimulation (16, 17). Rats in groups A, B and C were treated with rTSMS corresponding to their different segments from 4 d postoperative using aMagstim Rapid Stimulator (Magstim Co. Ltd, Whitland, Wales, UK), where, group A corresponding to Segment T6, group B corresponding to segment T10, and group C corresponding to segment L2. The center of the "8" shape coil cooled by vacuum was fixed close to the spine and the axis of the coil was perpendicular to the rat spinal segment to be stimulated. During the stimulus in group E (sham surgery), the magnetic coil was placed under other conditions, but the magnetic stimulus was not transmitted. However, the magnetic coil vacuum cooling system still simulated some typical auditory stimuli that occurred during the real stimulus. The rats underwent 20 min of baseline measurement, and 10 min of post-stimulation measurement. During the 20-minute baseline measurement, rats were continuously stimulated by rTSMS or sham stimulation. Treatment in daily at 3 pm, stimulating parameters are as follow: 5 Hz, 75% maximum output intensity (1.5 T), each sequence 5 s, 2 s intermittent time, every time-continuous stimulation 10 series. Duration: 1 time a day, five times a week, continuous treatment for 8 weeks.

2.4 Collection of spinal cord tissue specimens

After 8 weeks, 8 rats were randomly removed from each group and anesthetized by intraperitoneal injection with an excess of 10% chloral hydrate (6 ml/1 kg). The dorsal skin of the rats was then snipped off. With the spinal cord at T10 as the center, the surrounding vertebral plates and spinous processes were cut off, the spinal cord was completely exposed, and the spinal nerve roots on both sides were removed and cut off. With the damaged area as the center, spinal cord tissue about 1 cm length was taken, and 4 samples were randomly selected from each group and then placed in the cryopreservation tube. After being labeled, the samples were put into liquid nitrogen. The remaining 4 samples in each group were prefixed in 3% glutaraldehyde solution and observed by TEM. After the samples were stored, 4 rats were randomly selected from each group and spinal tissue was extracted by cardiac perfusion. The spinal tissue about 1 cm length was removed from the injured area. 10% chloral hydrate solution (3 ml/1 kg) was injected intraperitoneally. After successful anesthesia, the abdomens of rats were cut along the costal margin. The pericardium was removed to fully expose the heart and the ascending aorta. The left ventricle (apex) was rapidly perfused with 150 ml of normal saline. Subsequently, the ascending aorta was perfused with a universal tissue fixator (neutral, 4% paraformaldehyde + PBS). Perfusion was stopped when the liver turned white and the limbs twitched and writhed. According to the modeling method, the skin, fascia, muscle and other tissues on the back of rats were cut along the spinal cord

direction to expose the damaged spinal cord. The spinal cord was fully exposed. The spinal nerve roots on both sides of the spinal cord were carefully dissected with the glass minute needle, and the spinal nerve roots about 1 cm length were cut off. Finally, the tissues were fixed in the prepared 4% paraformaldehyde fixative for 24 h, then dehydrated, paraffin-embedded and sliced.

2.5 Quantification of Basso, Beattie, and Bresnahan (BBB) Scores

The BBB 22-point of open-field locomotor rating scale was used for evaluating hind limb motor function. 0 point indicates the total paralysis of the hind limbs, and 21 points are fully functioning. Behavioral analyses were conducted by two investigators who were blind as to the treatment at the indicated time points. Briefly, the BBB scores range from 0 points (complete paralysis) to 21 points (normal locomotion). 0 point indicates the complete paralysis of the hind limbs, and 21 points indicate normal locomotion. The rats were evaluated at 1d before surgery and 1 w, 2 w, 4 w, 6 w and 8 w after surgery.

2.6 Material and specimen handling

At 8 weeks post-operation, 8 rats were randomly selected from each group, the rats were anesthetized with 3% pentobarbital sodium (30 mg pentobarbital sodium /kg rat body weight) by intraperitoneal injection, and sections of spinal cord (1 cm long) were harvested from the epicenter of the injury. Four specimens preserved at -80°C for molecular experiment, the other four specimens were fixed in 3% glutaraldehyde for transmission electron microscopy assay. Then, 4 rats were randomly selected from each group, the rats were anesthetized with 3% pentobarbital sodium (30 mg pentobarbital sodium /kg rat body weight) by intraperitoneal injection, and the chest was opened and aortic cannulation was carried out using normal saline via the left ventricle of the rat until liquid became clear. Following this, 4% paraformaldehyde in PBS (300 ml) was used for continuous perfusion, and the perfusion was stopped when the liver was white and the limbs and trunk were stiff. Then, the spinal cord (1 cm long) was harvested from the epicenter of the injury and fixed with 4% paraformaldehyde for 24 h for immunohistochemistry (IHC) staining.

2.7 Transmission electron microscopy (TEM)

The spinal cord tissues (1 cm long) originally stored in 3% glutaraldehyde solution were removed and trimmed according to the specification of 0.5 ~ 1.0 mm². Then, the spinal cord (0.5 ~ 1.0 mm²) was fixed with 3% glutaraldehyde for 2 h, followed by dehydration in a series of acetone solutions. The dehydrated tissues were successively treated with penetrant dehydrating agent: epoxy resin at the ratios of 3:1, 1:1 and 1:3, respectively, for 30 to 60 minutes at each step. Next, tissue was embedded for ultrathin sectioning by electron microscopy. The sections were stained with uranyl acetate and lead citrate for 15 min at room temperature respectively, and then observed under Hitachi H-600IV transmission electron microscope (Hitachi, Japan) and photographed.

2.8 Western Blot assay

The total protein was extracted by using radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd.). The protein was quantified with a BCA Protein Assay kit (NanJing KeyGen Biotech Co., Ltd.). The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE), and then transferred to a poly-vinylidene fluoride (PVDF; Millipore, USA) membrane. Then, the PVDF membrane was sealed with 5% BSA at room temperature for 2 h and incubated with rabbit anti-Nogo-A (ab180847; 1:1,000; abcam, USA), rabbit anti-Nogo (ab184556; 1:10,000; abcam, USA), mouse anti-GAP43 (ab129990; 1:1,000; abcam, USA), rabbit anti-Caspase-3 (ab13847; 1:500; abcam, USA), rabbit anti-Caspase-7 (ab255818; 1:1,000; abcam, USA), rabbit anti-Caspase-9 (ab2013; 1:1,000; abcam, USA), rabbit anti-Cleaved Caspase-3 (ab214430; 1:5000; abcam, USA), rabbit anti-Cleaved Caspase-7 (ab256469; 1:1,000; abcam, USA), rabbit anti-Cleaved Caspase-9 (ab2324; 1:1,000; abcam, USA), rabbit anti-Bax (ab263897; 1:1,000; abcam, USA), mouse anti- β -actin (ab8226; 1:5,000; abcam, USA), goat anti-mouse IgG (ab205719; 1:10,000; abcam, USA) and goat anti-rabbit IgG (ab205718; 1:5,000; abcam, USA). β -actin was used as inner loading control. Protein bands were visualized using an ECL chemiluminescence kit (EMD Millipore) and analyzed by Image-Pro Plus software.

2.9 Immunohistochemical assay

Immunohistochemical staining was performed according to the manufacturer's instructions. Briefly, paraffin-embedded spinal cord tissues were cut into 4 μ m and deparaffinized with xylene. Then, antigen retrieval by 3% methanol hydrogen peroxide at room temperature for 10 min, and sealed with goat serum at room temperature for 20 min. The sections were incubated with mouse anti-GAP43 (ab129990; 1:500; abcam, USA) at 4°C overnight and incubated with goat anti-mouse IgG (ab205719; 1:5,000; abcam, USA) for 30 min at 37°C. Then, the sections were incubated with DAB (Boster, Wuhan, China) for 2 min, and counterstained with hematoxylin. Positive immune staining was presented as brown or yellow granules in cytoplasm or nucleus. PBS was adopted to substitute for primary antibody as negative control group. Five visual fields were randomly selected and assessed for immunoreactive areas at x400 magnification using BA200Digital image system. The image was analyzed by Image-Pro Plus software.

2.10 Immunofluorescence staining of 5-HT

The spinal cord tissues were dissected from rats, rapidly frozen and used for the preparation of longitudinal section (20 μ m) by using cryostat (Leica, Wetzlar, Germany). Pretreatment, blocking, and primary and secondary antibody reactions were performed as described previously (18). Briefly, samples were incubated with rabbit anti-5-HT (ab221181; 1:400; abcam, USA) primary antibodies. Fluorochrome-conjugated anti-rabbit (ab221181; 1:1000; abcam, USA) secondary antibodies were added to the primary antibody-probed sections and incubated. After successive washes, the images were analyzed under a fluorescence microscope ((Nikon, Kona, Japan).

2.11 TUNEL staining

Apoptosis of spinal cord tissues from rats was measured by TUNEL staining using a TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology, Inc). TUNEL stained apoptotic nuclei; DAPI and fluorescein-dUTP stained all nuclei. The apoptotic index (AI) = the number of TUNEL-positive cells/ the total number of cells. AI was evaluated in 15 randomly selected fields.

2.12 Statistical analysis

Statistical analysis was performed using SPSS19.0 software (IBM Corp., Armonk, NY, USA). Kolmogorov–Smirnov test was used to test the normal distribution of measurement variables, and the normal distribution data were described as mean $\pm$ standard error. Behavioral data were compared by repeated-measures ANOVA. The other data among multiple groups were compared by one-way analysis of variance (ANOVA) with Dunnett's post-tests or two-way ANOVA with Bonferroni's post-tests. The differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

3. Results

3.1 The treatment of rTSMS increased BBB scores in rats after acute spinal cord injury

The BBB scores were performed to examine the motor function of the post-surgery rat. As shown in Fig. 1, BBB scores of all rats were 21 at 1d before surgery. After SCI, BBB scores of rats in groups B, C, D and E were rapidly reduced to 0–2 points, and all of them showed typical paraplegia and invisible hind limb movement during crawling. At 2, 4, 6 and 8 weeks after SCI, BBB scores of group C and group D were significantly higher than those of group B ($P < 0.05$). At 4, 6 and 8 weeks after SCI, BBB score of group E was significantly higher than that of group B ($P < 0.05$). BBB scores of group C and group D were higher than those of group E at 4 weeks after SCI ($P < 0.05$). However, BBB scores of group E were higher than those of group C and group D at 6 and 8 weeks after SCI ($P < 0.05$). These results showed that the motor function recovery of rats was more obvious when the magnetic stimulation was applied to T6 and T10 segments at 0 to 6 weeks after the surgery, while the motor function recovery of rats was more obvious when the magnetic stimulation was applied to L2 spinal segment at 6 to 8 weeks after the surgery.

3.2 rTSMS alleviates myelin sheath damage of rats after acute spinal cord injury

Morphological changes in the spinal cord were measured with TEM. As shown in Fig. 2A, the myelin sheath structure in group A was complete and clear, and the arrangement between myelin sheath and axon was normal, and the mitochondria are evenly distributed and clearly structured. In group B, a huge gap was formed between myelin sheaths, and the axons with normal structure basically disappeared, demyelinating, myelin swelling and structural disorder were observed; myelin was disintegrated, broken and lost, and demyelinating was serious. In group E, the arrangement between myelin sheath and axon was relatively dense and regular, with mild demyelination. In group D, large gaps were formed between myelin sheaths, and myelin was swollen, with disordered structure, unclear, and demyelinating. In group C, myelin showed loose lamellar structure, swelling, myelin structure changes, and mild demyelination. These results indicated that rTSMS can alleviate the pathological injury of spinal cord tissue after ASCI to a certain extent, and the effect is the best when magnetic stimulation acts on L2 segment (Fig. 2A).

Studies have confirmed that Nogo-A and NgR expression levels are increased after spinal cord injury (19). In addition, Nogo-A affects the recovery of nerve function after spinal cord injury in primates (20). Therefore, how to reduce the expressions of Nogo-A and NgR is the key to promote the recovery of injured spinal cord. As shown in Fig. 1B and C, the expression levels of Nogo-A and NgR in group B, C, D and E

were all significantly higher than that in group A ($P < 0.05$). In addition, the expression levels of these two proteins in group C, D and E were significantly lower than those in group B ($P < 0.05$) (Fig. 2B-C).

3.3 rTSMS increased the 5-HT expression of rats after acute spinal cord injury

Immunohistochemistry and immunofluorescence staining were used to detect the expression level of 5-HT in spinal cord tissues. Immunohistochemical staining results showed that, after 8 weeks of SCI, compared with group E, the levels of 5-HT protein in the spinal cord of groups C and E were significantly increased ($P < 0.05$). Compared with group B, the expression levels of 5-HT protein in groups C and E were significantly increased ($P < 0.05$) (Fig. 3A). Further, immunofluorescence staining results showed that, compared with group A, the expressions of 5-HT in groups B, C, D and E were significantly increased ($P < 0.05$). While compared with group B, the expressions of 5-HT in groups C, D and E were significantly decreased ($P < 0.05$) (Fig. 3B).

3.4 rTSMS increased the GAP43 expression of rats after acute spinal cord injury

As shown in Fig. 4A, after 8 weeks of SCI, a large number of yellow or brown-yellow positive cells were found in the cytoplasm of the injured spinal cord neurons in groups B, C, D and E. Compared with group A, the levels of GAP43 protein in the spinal cord of groups B, C, D and E were significantly increased ($P < 0.05$). Compared with group B, the expression levels of GAP43 protein in group C and group E were significantly increased ($P < 0.05$). The expression level of GAP43 protein in group E was higher than that in groups C and D ($P < 0.05$). The expression level of GAP43 protein in group C was higher than that in group D ($P < 0.05$). As shown in Fig. 4B, after 8 weeks of SCI, compared with group A, the relative expression levels of GAP43 protein were significantly increased in the spinal cord tissues of groups B, C, D and E ($P < 0.05$). The relative expression level of GAP43 protein in groups C, D and E was significantly higher than that of group B ($P < 0.05$).

3.5 rTSMS inhibited apoptosis of rats spinal cord injury areas

TUNEL-positive cells and apoptosis-related proteins were detected in injured spinal cord tissue of rats. As shown in Fig. 5A and B, after 8 weeks of SCI, compared with group A, the TUNEL-positive cells of the spinal cord tissues in groups B, C, D and E were significantly increased ($P < 0.05$). Compared with group B, the TUNEL-positive cells of the spinal cord tissues in groups C, D and E were significantly decreased ($P < 0.05$). In addition, as shown in Fig. 5C and D, after 8 weeks of SCI, the expression levels of apoptosis-related proteins like Bax, caspase 3, cleaved-caspase 3, caspase 7, cleaved-caspase 7, caspase 9 and cleaved-caspase 9 in groups B, C, D and E were significantly higher than those in group A ($P < 0.05$), while these proteins in group C, D and E were significantly lower than those in group B ($P < 0.05$).

4. Discussion

Spinal cord injury can cause severe dysfunction and even death, which is a kind of traumatic disease with high disability (21). SCI is characterized by high incidence, high disability rate, high cost of treatment and low age (22–24). Therefore, it is of great significance to find effective treatment for patients themselves, their families and society. In recent decades, studies at home and abroad have found that

spinal cord magnetic stimulation can promote the recovery of SCI rats' motor function. Mally et al. (25) reported that 9 patients with thoracic spinal cord injury were treated with low-frequency magnetic stimulation on motor cortex, spinal cord segments above the injury site and lumbar nerve roots. After 6 months, motor function of some patients was significantly improved, and some patients could even stand on their own and walk short distances with walking aids. Ahemd et al. (26) found that 1 Hz magnetic stimulation given to ASCI rats can effectively improve the motor function of rats. Michael et al. (27) also found that on the basis of skill training, using 15 Hz magnetic stimulation to directly act on spinal cord injury segments to treat T13 spinal cord contusion mice can effectively improve the motor function of the mice. At present study, magnetic stimulation was applied to different spinal cord segments of ASCI rats, and behavioral observation over a period of 8 weeks confirmed that rTSMS could effectively promote the recovery of motor function in ASCI rats, which was consistent with previous reports. In addition, the results of transmission electron microscopy showed that, compared with the SCI group, the T6, T10, and L2 segment stimulation group all showed different degrees of improvement in spinal cord ultrastructure injury, suggesting that rTSMS can alleviate the pathological changes of spinal cord after ASCI to some extent.

After spinal cord injury, the functional recovery ability of the spinal cord is very limited, which is mainly caused by the severe inhibition of the regeneration of the axon at the broken end after the injury. With the development of research on spinal cord injury, some factors related to inhibiting axon regeneration have been found. At present, some research results have confirmed that Nogo-A has an inhibitory effect on axonal regeneration, and NgR mediates this effect of Nogo-A (28). In addition, studies have confirmed that Nogo-A and NgR expression levels are increased after spinal cord injury (19). In addition, Nogo-A affects the recovery of nerve function after spinal cord injury in primates (20). Therefore, how to reduce the expressions of Nogo-A and NgR is the key to promote the recovery of injured spinal cord. In this study, it was found that Nogo-A and NgR proteins were expressed in the spinal cord tissues of the sham group, and the results were consistent with those of earlier studies (28). The physiological significance of the expression of nerve fiber growth inhibitor protein in normal tissues may be to act as a boundary to the grown central nerve fibres, so as to prevent the subsequent growth of the lateral branches into the well-formed fibers. In this experiment, it was found that after spinal cord injury, Nogo-A and NgR protein expression increased in both the injury group and the treatment group. With the stimulation of rTSMS on different sites of spinal cord, the expression levels of Nogo-A and NgR decreased, indicating that rTSMS can effectively reduce the expression of Nogo-A and NgR after spinal cord injury, so as to promote the recovery of the injured spinal cord function in rats.

The recovery mechanisms of lower limb motor function after spinal cord injury include sprouting and compensation of the survival descending pathway system, regeneration of the descending pathway of injury, and compensation mechanism of the spinal cord intrinsic loop, namely the increasingly concerned central pattern generator (CPG) theory (29, 30). Normal rats can accept the commands from the nerve centers above the spinal cord, while rats with complete spinal cord injury are more dependent on the activation of spinal cord locomotor CPG, suggesting that the compensatory mechanism of CPG is particularly important for the improvement of motor function in rats with complete spinal cord injury (31).

During exercise, spinal motor neurons are simultaneously regulated by a variety of neurotransmitters, such as 5-HT and norepinephrine (32). Medullary serotonergic neurons originate from the raphe nuclei of medulla oblongata and globus pallidus, which can extend to the motor neurons in the anterior horn of the spinal cord to regulate the activity of neurons (33). 5-HT released by spinal cord intrinsic 5-HT neurons after spinal cord injury can also regulate the electrophysiological activities of spinal cord neurons, affecting the activation of motor neurons and the output of signals (34). At present study, it revealed that rTSMS was significantly increased the 5-HT expression of rats after acute spinal cord injury. The enhanced expression of 5-HT may enhance the continuous inward current by regulating the amplitude and threshold of persistent inward current in neurons, so as to amplify the synaptic input signal, enhance the activation of CPG related neurons and promote the recovery of motor function (35).

Regeneration and budding of axons are related to many factors, among which growth-related protein-43 (GAP43) plays a key role in this process. In the field of neuroscience, GAP43 is known as a marker for axon regeneration and new connection formation of nerve cells (36). Therefore, the changes of GAP43 in the spinal cord tissue can accurately reflect the growth of nerve cells in the damaged spinal cord. In present study, we found the expression of GAP43 protein was significantly increased in SCI rats compared with sham group, indicating that GAP43 was highly expressed again after nerve injury to promote the rapid growth of axon (37). In addition, our study showed that rTSMS could effectively promote the expression of GAP43 protein in the spinal cord injury area of rats, and L2 segment stimulation had the best effect. This may be related to the fact that L2 segment is located in the CPG ring of rats. Under the effect of repeated magnetic stimulation, spinal cord locomotor CPG is activated, which induces post-intervention plasticity and promotes the recombination of regenerated axons with spinal cord neuron circuits (38, 39). Increased intracellular Ca^{2+} concentration is a necessary pathway for all cell death and one of the initiating factors for the secondary pathological changes of spinal cord injury (40). However, the content of Mg^{2+} , a natural antagonist of Ca^{2+} , decreased rapidly after spinal cord injury, which further aggravated the secondary pathological changes of spinal cord tissues (41, 42). Therefore, we speculated that the improvement of motor function and the promotion of axon regeneration after spinal cord injury by rTSMS might be related to the improvement of Ca^{2+} and Mg^{2+} imbalance in the microenvironment of spinal cord injury area.

The pathophysiological changes and injury mechanism after spinal cord injury is a complex cascade reaction process, and irreversible injury (apoptosis) will occur to the nerve cells of the spinal cord after injury (43). At present, the exact molecular mechanism of apoptosis is not clear, but apoptosis involves the activation of a series of apoptotic proteins in the cytoplasm and the cleavage of substrates, and the current research focus is the Caspase protein family and the Bax family proteins. Bax, Caspase-3, Caspase-7 and Caspase-9 are all genes closely related to apoptosis, which have been confirmed to be involved in the regulation of apoptosis in different studies. The Caspase family plays a key role in apoptosis (44). In addition, as a component of ion channels on the mitochondrial membrane, Bax protein can activate caspase-9, which is involved in initiating apoptosis, and further activate caspase-3, which is involved in executing apoptosis (45). All these factors can lead to cell apoptosis. According to the

detection results of TUNEN-positive cells and apoptosis related proteins, rTSMS could effectively inhibit apoptosis in the spinal cord injury area of rats.

Conclusion

In summary, our results demonstrated that rTSMS acts on L2 segment lead to the most obvious improvement of motor function and the highest expression level of GAP43 in rats with spinal cord injury. Our study provides experimental basis for exploring the optimal stimulation site of rTSMS and theoretical support for the clinical application of rTSMS in spinal cord injury. However, since this experiment only selected the site of spinal cord injury and its upper and lower segments for comparative study, it is possible to have better treatment segments. Therefore, a more clear optimal treatment site and its mechanism need to be further studied.

Abbreviations

SCI: Spinal cord injury; CNS: Central nervous system; rTSMS: repetitive trans-spinal magnetic stimulation; GAP43: growth-associated protein-43; 5-HT: growth-associated protein-43 (GAP43) and 5-hydroxytryptamine; MS: magnetic stimulation; TMS: transcranial magnetic stimulation; rTMS: repetitive transcranial magnetic stimulation

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Local Ethics Committee for Animal Experiments in General Hospital of Western Theater Command (China) for the care and use of laboratory animals (Ethical approval number: Protocol Number 2013YG-B005).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HL, DQX, ARZ, and WCW conceived and designed the experiments. LY, RZP, QD, NYS and JQZ performed the experiments. JCL, WX, ZSC and JCL analyzed the data and contributed to the acquisition of reagents and materials. HL and ARZ wrote the manuscript.

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Figures

Figure 1

BBB scores of rats in each group at different time points. (A-G) The BBB 22-point of open-field locomotor rating scale was used for evaluating hind limb motor function in each group at different time points. A: sham surgery group, B: SCI group, C: T6 segment stimulation group, D: T10 segment stimulation group, E: L2 segment stimulation group. * $P < 0.05$, compared with A group; $\Delta P < 0.05$, compared with B group; $\square P < 0.05$, compared with D group; $\blacktriangle P < 0.05$, compared with E group.

Figure 2

Effects of rTSMS on the myelin sheath damage of rats after acute spinal cord injury. (A) Images of dorsal spinal cord tissues about 1 cm in length of rats were observed by TEM following 8 weeks post-operation. Scale bars are 1 μ m (x6000). The red arrow represents demyelinating; the green arrow represents myelin disintegration, rupture, and loss; the blue arrows indicate clear mitochondrial structure. (B-C). WB assay was performed to detect the expression level of NgR and Nogo-A following 8 weeks post-operation. A: sham surgery group, B: SCI group, C: T6 segment stimulation group, D: T10 segment stimulation group, E: L2 segment stimulation group. * $P < 0.05$, compared with A group; $\Delta P < 0.05$, compared with B group.

Figure 3

Effects of rTSMS on the 5-HT protein expression of rats after acute spinal cord injury. (A) Immunohistochemical assay was performed to detect the expression level of 5-HT following 8 weeks post-operation. (B) Immunofluorescence staining was performed to detect the expression level of 5-HT following 8 weeks post-operation. A: sham surgery group, B: SCI group, C: T6 segment stimulation group,

D: T10 segment stimulation group, E: L2 segment stimulation group. *P < 0.05, compared with A group; Δ P < 0.05, compared with B group.

B

A B C D E group

GAP43

β-actin

Group	GAP43 protein expression level
A	1.0
B	~1.7*
C	~2.0*Δ
D	~2.0*Δ
E group	~2.1*Δ

Figure 4

Effects of rTSMS on the GAP43 expression of rats after acute spinal cord injury. (A) Immunohistochemical assay was performed to detect the expression level of GAP43 following 8 weeks post-operation. Scale bars are 40 μm (×200). (B) WB assay was performed to detect the expression level of GAP43 following 8 weeks post-operation. A: sham surgery group, B: SCI group, C: T6 segment stimulation group, D: T10 segment stimulation group, E: L2 segment stimulation group. *P < 0.05,

compared with A group; $\triangle P < 0.05$, compared with B group; $\blacktriangle P < 0.05$, compared with E group; $\boxtimes P < 0.05$, compared with D group.

Figure 5

Effects of rTSMS on TUNEL-positive cells of rats after acute spinal cord injury. (A-B) TUNEL assay was used to detect the TUNEL-positive cells of rats following 8 weeks post-operation. Scale bars are 50 μm ($\times 400$). (C-D) WB assay was performed to detect the expression level of apoptosis-related protein

following 8 weeks post-operation. A: sham surgery group, B: SCI group, C: T6 segment stimulation group, D: T10 segment stimulation group, E: L2 segment stimulation group. * $P < 0.05$, compared with A group; $\Delta P < 0.05$, compared with B group.