

Low-frequency pulsed electromagnetic field promotes functional recovery, reduces inflammation and oxidative stress, and enhances HSP70 expression following spinal cord injury

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Abstract. Low-frequency pulsed electromagnetic fields (LPEMFs) have been reported to be protective for multiple diseases. However, whether the administration of LPEMFs inhibits inflammation and oxidative stress following spinal cord injury requires further investigation. In the current study, a contusion spinal cord injury model was used and LPEMFs administration was applied to investigate the molecular changes, including inflammation, oxidative stress and heat shock protein 70 (HSP70) levels. The results revealed that LPEMFs significantly promoted functional recovery following spinal cord injury, as demonstrated by an increased Basso, Beattie and Bresnahan score. The results demonstrated that LPEMFs decreased the expression of inflammatory factors, including tumor necrosis factor- α , interleukin-1 β and nuclear factor- κ B. Additionally, LPEMFs exposure reduced the levels of inducible nitric oxide synthase and reactive oxygen species, and upregulated the expression of catalase and superoxide dismutase. Furthermore, treatment with LPEMFs significantly enhanced the expression of HSP70 in spinal cord-injured rats. Overall, the present study revealed that LPEMFs promote functional recovery following spinal cord injury, potentially by modulating inflammation, oxidative stress and HSP70.

Introduction

Spinal cord injury (SCI) causes severe damage to the central nervous system, resulting in irreversible motor and sensory dysfunction below the injury area (1). In modern society, the global incidence of SCI varied from 8 to 246 per million depending on the country or region (2), and an increasing number of paralyzed patients survive longer, experiencing a variety of complications (3,4). Currently there are no effective therapeutic treatments for SCI. Methylprednisolone, which was previously used as first-aid measure for acute SCI, is no longer recommended in the guidelines of AANS/CNS as of 2013 due to an increased occurrence of complications and no strong evidence of clinical efficacy (5). Rehabilitation, the only proven effective treatment for SCI, is limited for early application as it is only suitable for less serious injuries and requires active cooperation from patients (6). Thus, novel noninvasive treatments are required as early interventions for patients with SCI.

The pathological process of SCI can be divided into two stages; the primary and secondary injury (7). The primary injury is the original tissue breakdown caused by contusion or compression, which occurs immediately after SCI. The secondary injury is a series of pathologic changes following the primary injury, including an increase in the permeability of the blood spinal cord barrier, the infiltration of inflammatory cells, excitotoxicity, demyelination and neuronal apoptosis, which lasts for days to months. Two of the most important components of the secondary injury in its early stages are inflammation and oxidative stress. The inflammation includes increased expression of pro-inflammatory factors, including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), and a reduction in inflammatory mediators (8). The oxidative stress involves an increase in the levels of nitric oxide (NO) and reactive oxygen species (ROS) (9).

The use of low-frequency pulsed electromagnetic fields (LPEMFs) is a noninvasive therapeutic method for various diseases. Recent evidence has demonstrated that LPEMFs can prevent inflammation and oxidative stress. LPEMF stimulation can suppress the production of IL-1 β and TNF- α in cultured nucleus pulposus cells (10). Furthermore, LPEMFs can reduce

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ROS levels and enhance antioxidative stress responses in osteoblasts (11). LPEMFs exhibit strong neuroprotective effects in the nervous system. In ischemic stroke, LPEMFs can promote functional recovery by activation of the brain derived neurotrophic factor/tropomyosin receptor kinase B/protein kinase B signaling pathway (12). Additionally, LPEMFs can modulate the expression of microRNAs and stimulate tissue regeneration in *in vitro* models of Alzheimer's disease (13). Recent studies have revealed that extremely low-frequency magnetic fields reduce iron-induced tissue damage following SCI (14). However, whether the administration of LPEMFs inhibits the early-stage reaction of SCI secondary injury, inflammation and oxidative stress, requires further exploration. In the present study, neuroprotective effects of LPEMF stimulation on SCI model rats were investigated. The changes in inflammation and oxidative stress molecular markers were then compared, and whether this protective effect was modulated by targeting heat shock protein 70 (HSP70) was examined. The results of the current study may provide a noninvasive alternative therapeutic method for the early treatment of SCI.

Materials and methods

Experimental animals. Adult female Wistar rats (230±20 g; n=60) used in the current study were all provided by Tianjin Medical University Animal Research Center (Tianjin, China; permission no. SCXK-2012-0004). All animal experiments were approved by the Animal Welfare Committee of Tianjin Medical University (Tianjin, China), which is based on the NIH Guide for the Care and Use of Laboratory Animals (15). The rats were randomly distributed into three groups: Sham group, SCI group and LPEMF group. In the sham group, rats underwent laminectomy, and the spinal cord was not injured. In the SCI group, the spinal cord was injured using the same procedure used in the LPEMF group on an identical electromagnetic device, but without the application of LPEMFs. In the LPEMF group, Wistar rats received the LPEMFs treatment for 1 h per day from 24 h after SCI. Animals were sacrificed on days 3, 7 and 14 after SCI, and the spinal cords were harvested for further analysis.

Contusion SCI model. The standard New York University impactor machine was used to induce a spinal cord contusion injury model as described previously (16). Rats were anesthetized with chloral hydrate (300 mg/kg), and a laminectomy was performed to expose the T10 spinal cord. A metal rod (10 g, 25 mm) was dropped onto the back side of the spinal cord. A surveillance system was used to control the compression force and velocity to maintain uniformity between animals. Following the operation, the bladders of these rats were manually emptied.

LPEMFs treatment. The BG100A-2 pulsed magnet field therapeutic apparatus (Concord Beijing Medical Equipment Co., Ltd., Beijing, China; patent no. ZL00101667.9) was used in the current study. The apparatus contains seven coils arranged end-to-end beneath the treatment table and a magnetic line of force was positioned across the rats longitudinally. The frequency, power and duty cycle are all adjustable and the apparatus can be monitored and controlled by computer

system. In the LPEMFs group, rats were exposed to LPEMFs (frequency, 50 Hz; power, 2.5 mT; duty cycle, 40%) at 24 h after SCI. Rats were placed into a transparent plastic chamber with ventilation and were given time to explore for 1 h per day (8:00-9:00 a.m.) for 14 days. In the SCI group, animals were placed in the same chambers on the treatment table for 1 h per day without exposure to LPEMFs.

Assessment of locomotor activity. Functional recovery of the animals was evaluated using the Basso Beattie Bresnahan (BBB) locomotor rating scale (17). The BBB was observed by two groups, and the observers were blinded to the design of current experiment (18). The BBB scores of rats were recorded prior to contusion operation and at 1, 3, 5, 7 and 14 days post-injury (dpi).

Enzyme-linked immunosorbent assay (ELISA). The spinal cord tissue (10 mm block of spinal cord surrounding the lesion center) was collected and homogenized at 14 dpi. Then, the tissue homogenate was centrifuged at a speed of 10,000 x g at 4°C for 20 min and the supernatant was collected for determining protein concentration using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The commercially available ELISA kits were obtained from the following companies: TNF-α ELISA kit (cat. no. ab46070; Abcam, Cambridge, UK), IL-1β ELISA kit (cat. no. RA20422; Bio-Swamp, Wuhan, China), superoxide dismutase (SOD) ELISA kit (cat. no. 706002; Cayman Chemical Company, Ann Arbor, MI, USA), catalase (CAT) ELISA kit (cat. no. 11363727001; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Western blot analysis. After 14 days of treatment or SCI, the spinal cord tissue around the lesion center was collected, harvested and homogenized in radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Shanghai, China). The concentration of protein was detected using bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein samples (50 μg) from three individual animals in each group were resolved using SDS-PAGE (12% gel) for separation and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk and incubated with anti-inducible nitric oxide synthase (iNOS; 1:250; cat. no. ab15323; Abcam) and anti-β-actin (1:2,000; cat. no. ab8227; Abcam) overnight at 4°C. Then, the membrane was incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:10,000; cat. no. ab205718; Abcam) at 37°C for 1 h. BeyoECL Star (cat. no. P0018AM; Beyotime Institute of Biotechnology) was used to develop the HRP signal. Signals were captured using a ChemiDoc MP System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified using ImageJ software version 1.32 (National Institutes of Health, Bethesda, MD, USA). The expression levels of iNOS were determined following normalization to β-actin levels.

Immunohistochemistry analysis. Spinal cords were collected 14 days after injury and samples were quickly frozen at -40°C immersed in 4% paraformaldehyde. Transverse

10 μm thick sections of spinal cord were used for immunohistochemistry analysis. Following permeabilization in 0.25% Triton X-100/PBS for 10 min at room temperature, sections were blocked with 10% goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 60 min at room temperature. Sections were stained using anti-nuclear factor- κB (NF- κB) p65 (1:2,000; cat. no. ab16502; Abcam) and anti-HSP70 (1:100; ab79852; Abcam) at 4°C overnight. Samples were incubated with secondary HRP-conjugated goat anti-rabbit antibody (1:10,000; cat. no. ab205718; Abcam) at 37°C for 1 h. The DAB Horseradish Peroxidase Color Development kit (cat. no. P0202; Beyotime Institute of Biotechnology) was used for signal development (sections were incubated at room temperature for 25 min). Then, 3 fields were randomly selected for each sample, and the positive area in each field was collected and quantified using ImageJ software version 1.32 (National Institutes of Health).

ROS assay. The spinal cord tissue (5 mm block of spinal cord surrounding the lesion center) was collected and homogenized at 14 dpi. Subsequently, the tissue homogenate was centrifuged (1,500 \times g at 4°C for 20 min), and the supernatant was collected. A ROS assay kit (cat. no. D6883; Sigma-Aldrich; Merck KGaA) was used to determine the production of ROS. The supernatant was incubated with 2,7-dichlorodihydrofluorescein diacetate for 1 h and then washed twice with PBS in ice. Fluorescence levels were detected at 480/530 nm.

Statistical analysis. The experimental data are expressed as the mean \pm standard error. A one-way analysis of variance followed by Tukey's post hoc tests for multiple comparisons were used to analyze data (SPSS 19.0 software; IBM Corp., Armonk, NY, USA). $P < 0.05$ were considered to indicate a statistically significant difference.

Results

Protective effects of LPEMFs on locomotor recovery following SCI in rats. To evaluate whether the LPEMF treatment has protective effects on motor function in SCI rats, the BBB locomotor rating scale was used to measure behavior for 2 weeks. As presented in Fig. 1, all the animals exhibited full marks (21 points) in BBB scoring prior to the injury. At dpi 1, the BBB scores dropped to 0. Following SCI, rats exhibited spontaneous functional recovery over time and there were no significant differences between the SCI group and the LPEMF group before dpi 7. However, the rats exhibited better motor function recovery after 7 dpi under LPEMF treatment compared with SCI rats, and the BBB scores were significantly different at dpi 7 and 14 ($P < 0.05$). These results suggested that LPEMF exposure can promote locomotor recovery in SCI rats (Fig. 1).

Protective effect of LPEMFs on expression of pro-inflammatory cytokines in SCI rats. To determine whether LPEMFs suppressed inflammatory reaction by decreasing the secretion of pro-inflammatory cytokines in the injured spinal cord, the expression levels of TNF- α and IL-1 β were assessed. Following SCI, the inflammation markers TNF- α and IL-1 β were significantly increased compared with the Sham group (Fig. 2A and B). However, after 2 weeks of LPEMF

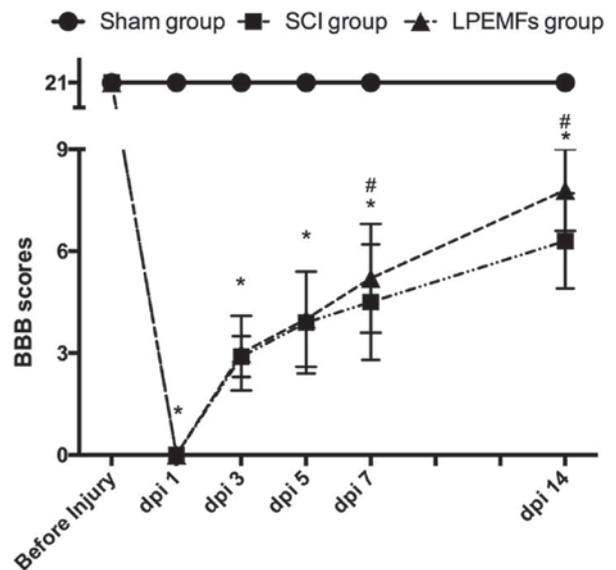


Figure 1. BBB scores in Sham group, SCI group and LPEMF group at different time points. * $P < 0.01$ vs. Sham group; # $P < 0.05$ vs. SCI group. BBB, Basso Beattie Bresnahan; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

treatment, the expression of TNF- α and IL-1 β were decreased in comparison with the SCI group (Fig. 2A and B). These results indicated that LPEMF treatment, to a certain extent, can alleviate the inflammatory reaction following SCI.

Suppressive effect of LPEMFs on NF- κB expression in injured spinal cord. NF- κB is an important transcription factor that stimulates inflammation (19). To determine whether LPEMF treatment could suppress the expression of NF- κB in the injured spinal cord, particularly in the ventral horn, NF- κB protein levels were evaluated using immunohistochemistry (Fig. 3A). In the sham group, the NF- κB was difficult to detect (Fig. 3B). However, after 2 weeks of injury, there was a strong, positive NF- κB signal in the ventral horn of the spinal cord, which contains motor neurons (Fig. 3C). By contrast, the administration of LPEMFs significantly reduced the immunoreactivity of NF- κB in SCI rats (Fig. 3D). The quantified result was consistent with observation of sections (Fig. 3E).

Suppressive effect of LPEMFs on iNOS expression in injured spinal cord. To determine the iNOS expression following SCI, the iNOS was detected using western blot analysis. Significantly higher levels of the iNOS protein were detected following SCI compared with the Sham group. By contrast, the LPEMF treatment suppressed the iNOS protein expression in the injured spinal cord (Fig. 4A and B). The decreased iNOS expression indicated that the LPEMFs can alleviate oxidative stress following SCI.

Protective effect of LPEMFs on ROS production in SCI rats. To investigate the protective effect of LPEMFs on ROS production in SCI rats, the ROS levels between groups were measured. As demonstrated Fig. 5, the injury induced strong ROS production in spinal cord tissue compared with those in the uninjured Sham group. However, the LPEMF administration reduced the ROS level significantly compared with

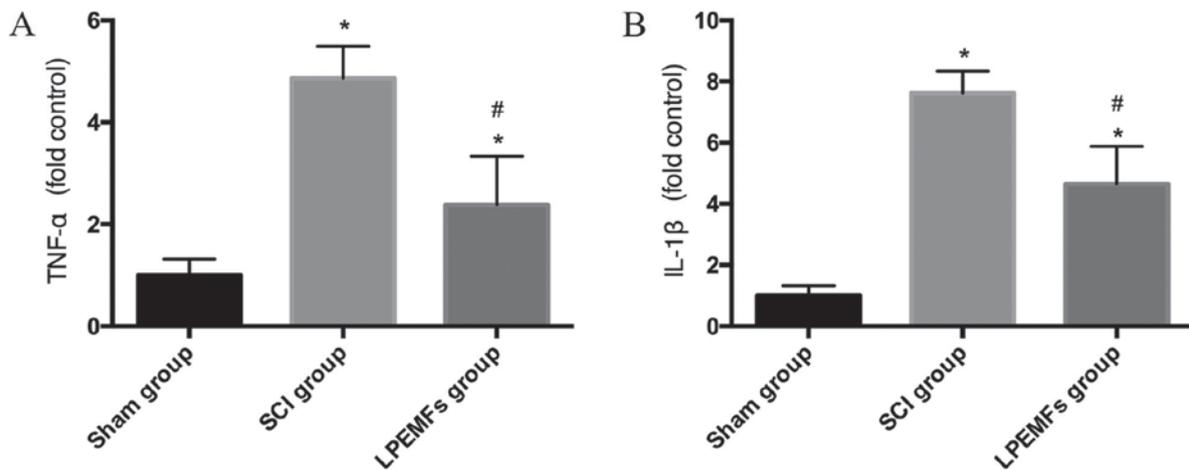


Figure 2. ELISA analysis of expression levels of TNF- α and IL-1 β in the spinal cord. The ELISA was conducted at 14 days post-injury. (A) Expression levels of TNF- α . (B) Expression levels of IL-1 β . * P <0.01 vs. Sham group; # P <0.05 vs. SCI group. TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

SCI rats. This result indicated that LPEMFs can alleviate the oxidative stress by reducing ROS production following SCI.

Protective effect of LPEMFs on expression of antioxidant enzymes following SCI. To explore whether LPEMFs can alleviate oxidative stress through upregulation of antioxidant enzymes, the expression of SOD and CAT in spinal cord was measured using ELISA. Compared with the intact spinal cord, the injured tissue exhibited decreased expression of SOD and CAT. By contrast, the treatment of LPEMFs can reversed this reduction to a certain extent (Fig. 6A and B). These results provided evidence that the protective effect of LPEMFs on oxidative stress may be attributed to the upregulation of antioxidant enzymes.

Protective effect of LPEMFs on expression of HSP70 SCI rats. HSP70 is deemed as the protective agent for inflammation and oxidative stress during tissue damage. In the current study, the expression of HSP70 in the spinal cord following injury with and without the administration of LPEMFs was examined. Following SCI, the expression of HSP70 became scattered in the ventral horn compared with the spinal cord in the Sham group. However, the LPEMF treatment significantly increased the expression of HSP70 in motor neurons (Fig. 7A-D). The quantified result was consistent with observations (Fig. 7E). These results indicated that the anti-inflammatory and anti-oxidative stress effects of LPEMFs may associated with the high expression of HSP70.

Discussion

Electromagnetic fields (EMFs) have long been deemed relevant for human health (20), and recent studies have indicated that LPEMFs exhibit protective effects in multiple pathologies, including Alzheimer's disease (13), stroke (12), wound healing (21) and pain (22). The definition of low frequency is <300 Hz and research has demonstrated that pulsed EMFs exhibited greater therapeutic effects when applied with an amplitude <3 mT and frequencies <100 Hz (23). In the current study, LPEMFs with a 50 Hz frequency and 2.5 mT

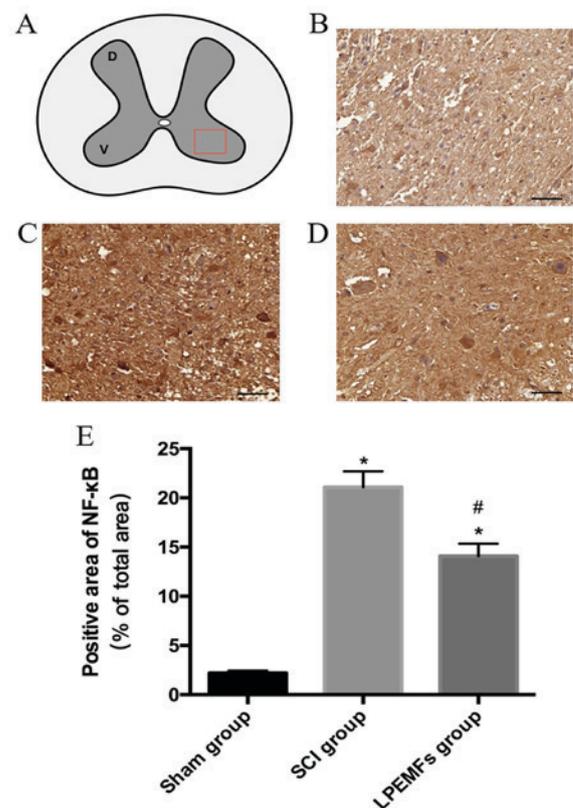


Figure 3. Expression of NF- κ B in the ventral horn of the spinal cord. The NF- κ B was detected at 14 days post-injury. (A) Diagram of the spinal cord. Representative image of NF- κ B expression in the ventral horn of the spinal cord in the (B) sham group, (C) SCI group and (D) LPEMF group. Scale bar, 50 μ m. (E) Statistical comparison among the three groups. * P <0.01 vs. Sham group; # P <0.05 vs. SCI group. NF- κ B, nuclear factor- κ B; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

amplitude were applied, which are suitable parameters for the exploratory research. However, to the best of our knowledge, the current study was first administration of LPEMFs in a contusion SCI model, which is more clinically relevant than the transection model (24,25). Unlike other disorders, the pathological changes following SCI are complex due to

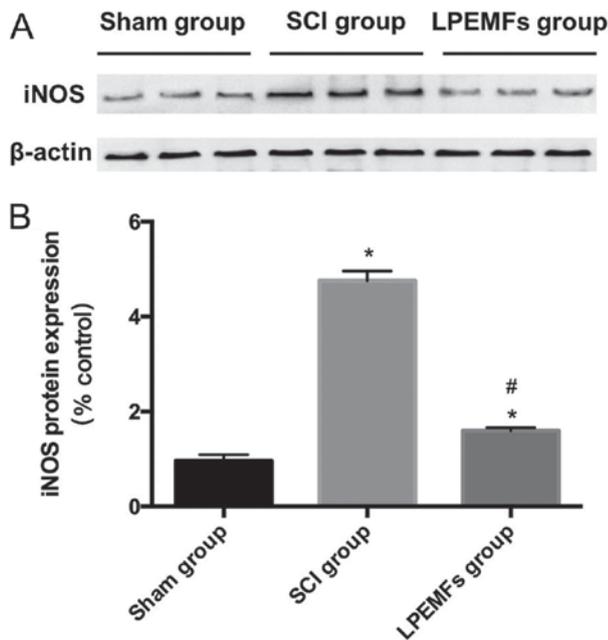


Figure 4. Effect of LPEMFs on the iNOS expression following SCI. The iNOS was detected at 14 days post-injury. (A) Western blot and (B) quantification was used to detect the expression of iNOS in sham, SCI and LPEMF groups. *P<0.01 vs. Sham group; #P<0.05 vs. SCI group. SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields; iNOS, inducible nitric oxide synthase.

the microenvironment comprised of neurons, astrocytes, oligodendrocytes, microglial cells and vascular endothelial cells (26). It is well established that the secondary injury is far more important than primary injury, due the crucial role of the microenvironment in tissue preservation and regeneration (7). In the current study, LPEMF treatment improved the recovery of motor function in the SCI rats and alleviated the inflammation and oxidative stress in the damaged spinal cord, which indicated that LPEMFs promote functional recovery, which may be associated with reduced secondary injury following SCI.

Inflammatory cascades are activated during secondary injury following SCI. High levels of pro-inflammatory factors are released from spinal cord tissue (astrocytes and microglia) and peripheral cells (neutrophils, monocytes and macrophages) to increase vascular permeability. TNF- α and IL-1 β pathophysiological signaling pathways are two of the most important components in SCI inflammatory cascades. The increased expression of TNF- α and IL-1 β can suppress cell survival and lead to cell death. The NF- κ B signaling pathway has been well established as the center of the pathophysiology of inflammatory reactions induced by SCI (27). The NF- κ B can be elevated by the pro-inflammatory cytokines and chemokines following injury. The activation of NF- κ B, as a transcription factor, is required for the upregulation of TNF- α and IL-1 β . Therefore, this positive feedback amplifies the inflammatory reaction and exacerbated the microenvironment following SCI (28). Thus, the results of the current study suggest that LPEMFs can reduce the expression of TNF- α , IL-1 β and NF- κ B to alleviate the inflammatory reaction induced by SCI, and this supports previous reports demonstrating LPEMF exposure exhibits anti-inflammatory effects in synoviocytes, chondrocytes and

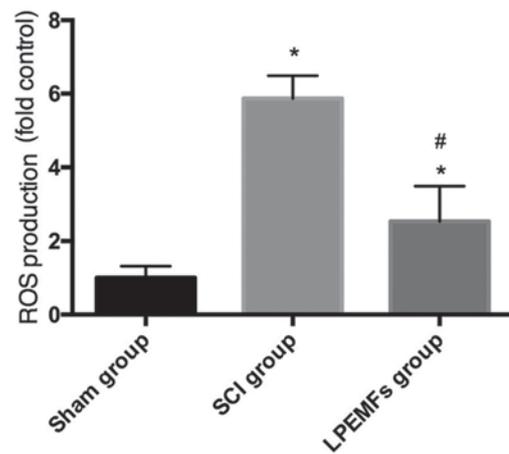


Figure 5. Effect of LPEMFs on the ROS level following SCI. ROS were detected at 14 days post-injury. ROS assay kit was used to determine the production of ROS. *P<0.01 vs. Sham group; #P<0.05 vs. SCI group. ROS, reactive oxygen species; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

osteoblasts (29-31). However, in the current study, inflammation was only detected at 14 dpi. It will be necessary to use other time points to validate these results.

Following SCI, the production and elimination of oxidative species are imbalanced, which results in tissue oxidative stress (32). ROS and NO are two main end-products of oxidative stress. Superoxides, hydroxylradicals, hydrogen peroxides and peroxynitrites are the principal components of ROS (33). NO is synthesized by NOS, and the most abundant isoform is the iNOS, which is located in a variety of cell types in the spinal cord and can represent the production of NO (34). Antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), act to reduce oxidative stress. However, damage of the spinal cord impairs their protective ability (35). In the current study, the administration of LPEMFs significantly reduced the levels of ROS and iNOS in the injured spinal cord. By contrast, the expression of CAT and SOD were upregulated by LPEMFs, which can protect damaged tissue. A previous study also demonstrated the antioxidant protective effects of LPEMFs in neuronal cell lines by elevating endogenous antioxidant properties (36), which was in accordance with the current study.

Heat shock proteins have an important role in transport and folding proteins, which is necessary for numerous biological processes. HSP70 has been considered to protect against cellular stress. HSP70 is also modulates inflammation and oxidative stress (37,38). In an SCI model, HSP70 was demonstrated to promote the survival of motor neurons (39). In the current study, LPEMFs significantly promoted the expression of HSP70 in the ventral horn of the injured spinal cord, where the motor neurons are located. Therefore, the enhanced tolerance to inflammation and oxidative stress may be mediated by upregulation of HSP70 following LPEMF treatment. However, the causal association between HSP70 and inflammation/oxidative stress requires further investigation.

In conclusion, the findings of the present study revealed that the administration of LPEMFs reduces inflammation and oxidative stress to promote functional recovery following SCI, and the potential mechanism involves the activation of

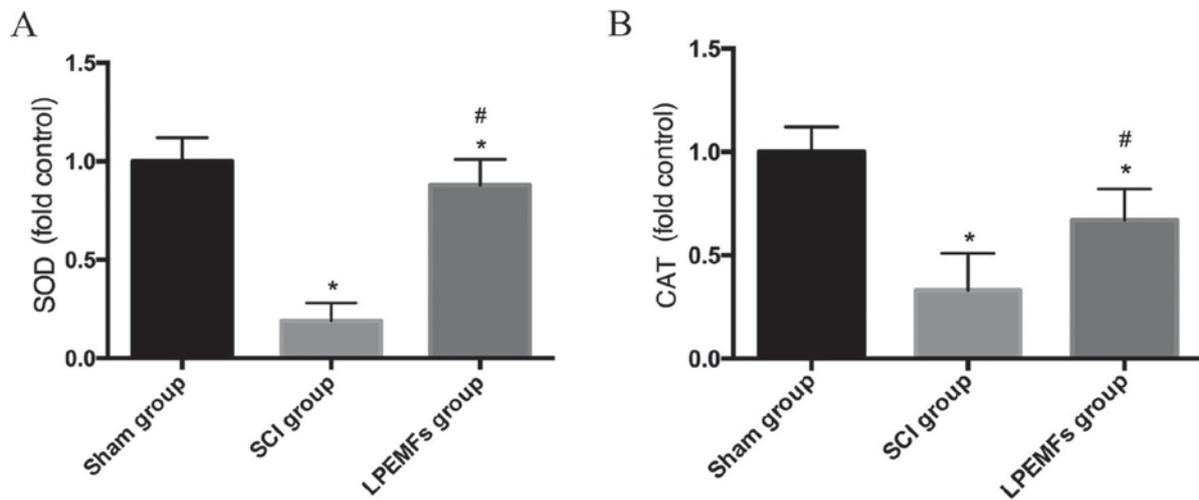


Figure 6. Effect of LPEMFs on the expression of antioxidant enzymes following SCI. SOD and CAT were detected at 14 days post-injury. Expression of (A) SOD and (B) CAT in the spinal cord was measured using ELISA. * $P < 0.01$ vs. Sham group; # $P < 0.05$ vs. SCI group. SOD, superoxide dismutase; CAT, catalase; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

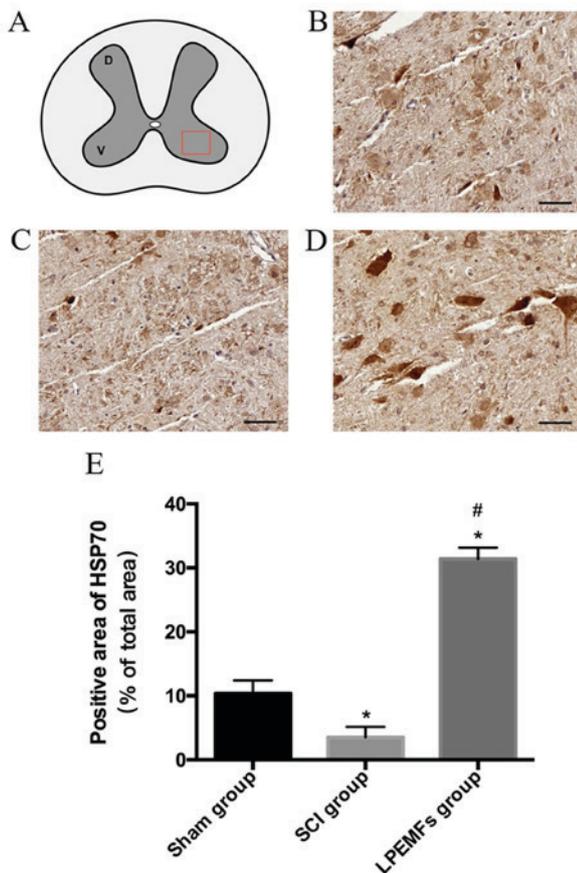


Figure 7. Expression of HSP70 in ventral horn of the spinal cord. HSP70 was detected at 14 days post-injury. (A) Diagram of the spinal cord. Representative image of HSP70 expression in the ventral horn of the spinal cord in the (B) sham group, (C) SCI group and (D) LPEMF group. Scale bar, 50 μ m. (E) Statistical comparison among the three groups. * $P < 0.01$ vs. Sham group; # $P < 0.05$ vs. SCI group. HSP70, heat shock protein 70; SCI, spinal cord injury; LPEMF, low-frequency pulsed electromagnetic fields.

HSP70. The findings provide new perspective for identifying novel noninvasive therapeutic methods for early intervention following SCI.

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Availability of data and materials

The data and materials used or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SF, CXW, CYW and YL conceived and designed the experiments. CYW and YL performed the experiments. YW, ZW and DS provided critical reagents and scientific input. GN and QW maintained the animals. CYW, YL, SF and CXW analyzed data and prepared the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Welfare Committee of Tianjin Medical University (Tianjin, China), which is based on the NIH Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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