

Therapeutic effect of methylprednisolone combined with high frequency electrotherapy on acute spinal cord injury in rats

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Received September 11, 2018; Accepted May 16, 2019

DOI: 10.3892/etm.2019.8147

Abstract. Acute spinal cord injury (SCI) has a high rate of disability and mortality. Although secondary SCI results in local tissue hypoxia and the release of inflammatory mediators, it is both controllable and reversible. Therefore, timely rehabilitation treatment is beneficial for the partial recovery of patients with SCI. The present study aimed to investigate the use of methylprednisolone combined with high-frequency electrotherapy as a method of rehabilitation treatment in rats with SCI. The rat SCI model was prepared using the modified Allen's method with the animals randomly divided into the following 4 groups (n=10 for each group): SCI; methylprednisolone (300 mg/kg); high-frequency electrotherapy; and combination treatment with electrotherapy combined with methylprednisolone (300 mg/kg). The Basso, Beattie and Bresnahan (BBB) score, somatosensory evoked potential (SEP) and motor evoked potential (MEP) were used to assess spinal function. Brain-derived neurotrophic factor (BDNF) and NF- κ B expression levels were detected using reverse transcription-quantitative PCR and western blotting. Tumor necrosis factor (TNF)- α and IL-2 expression levels were determined by ELISA, and caspase 3 activity was also assessed. In all treatment groups, BDNF mRNA and protein expression levels were significantly increased, whilst those of NF- κ B were reduced. Additionally, an elevated BBB score, improved SEPs and MEPs, inhibited caspase 3 activity and downregulated TNF- α and IL-2 expression levels were observed, compared with the SCI group ($P < 0.05$). However, the combination group exhibited more significant effects on SCI. In conclusion, methylprednisolone combined with high frequency electrotherapy may improve the symptoms of SCI by increasing the expression level of BDNF, reducing that of NF- κ B, and suppressing the secretion of inflammatory factors.

Introduction

Due to continuous progress in the construction and mining industries, and the development of the transportation industry, the number of accidental spinal cord injuries (SCIs) caused by crashes or car accidents has increased in recent years (1,2). SCI often results in spasticity and dysfunction under the injured spinal cord segment, with characteristic high morbidity and mortality (3). Furthermore, due to the nature of their occupation, young adults >40 years if age are at high-risk (4). SCI treatment is challenging due to its high cost and invasiveness. SCI not only leads to physical and psychological damage to the patient, but also causes a notable economic burden (5,6). SCI can be classified as primary or secondary in which primary injury can lead to local tissue damage, ischemia and hypoxia, inflammatory mediator release and pathological changes. Secondary lesions are more severe, and result from the cascade-amplification effects of primary injury. Secondary lesions can result in damage to residual neural pathways and further loss of function, but are both controllable and reversible (7,8).

With advancements in medical treatment technology, the emergence of surgical methods and drugs has shown initial success in SCI treatment. Rehabilitation interventions cannot be neglected and are considered to promote spinal cord remodeling (9,10). Different drugs are used to relieve pain in patients with SCI (11); methylprednisolone attenuates the peroxidation of membrane lipids and post-traumatic inflammation, and has consistently been associated with improved neurobehavioral outcome in preclinical studies (12). High-frequency electrotherapy, a non-invasive and inexpensive technique is also widely used for physical therapy to treat pain in SCI patients. Additionally, transcutaneous electrical nerve stimulation is the most commonly used electrotherapy method to relieve pain (13). However, the effect of methylprednisolone combined with high-frequency electrotherapeutic treatment on SCI and its associated mechanisms is yet to be elucidated. Therefore, the present study established a rat SCI model to analyze the impact and possible mechanisms of methylprednisolone treatment combined with high-frequency electrotherapy.

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Key words: spinal cord injury, methylprednisolone, high-frequency electrotherapy, NF- κ B, brain-derived neurotrophic factor, caspase 3

Materials and methods

Experimental animals. Healthy, specific pathogen free (SPF) grade male Wistar rats (2 months old; 250 \pm 20 g) were

purchased from the experimental animal center and maintained in the SPF Xi'an Medical University Animal Experimental Center. The animals were maintained at $21\pm 1^\circ\text{C}$, at a relative humidity of 50-70% and a 12 h day/night cycle. All procedures were approved by the Animal Ethics Committee of The First Affiliated Hospital of Xi'an Medical University.

Reagents and instruments. Pentobarbital sodium was purchased from Zhpharma Ltd. PVDF membranes were purchased from Pall Life Sciences. Western blotting-related chemical reagents were purchased from the Beyotime Institute of Biotechnology and enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare. Rabbit anti-BDNF and rabbit anti-NF- κB antibodies, as well as sheep anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibodies were purchased from Abcam, Inc. Methylprednisolone was purchased from Sigma-Aldrich (Merck KGaA). Tumor necrosis factor (TNF)- α and IL-2 ELISA kits were purchased from R&D Systems, Inc., and the Caspase 3 Activity Assay kit was purchased from Cell Signaling Technology, Inc. Microsurgical instruments were purchased from Suzhou Medical Instrument Factory. The RNA extraction kit and reverse transcription kit were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). The Multi-Parameter Monitor Small Animal Physiology Monitor was purchased from Shanghai Yuken Instrument Company, and the Amp PCR System 2400 DNA Amplification System was purchased from PerkinElmer, Inc. The Imark microplate reader was purchased from BD Biosciences. Key-point evoked potential meters, electromyographs and Magpro magnetic stimulators were purchased from Dantec Dynamics. The UWM-02 Ultrashort Wave Therapy Instrument was purchased from Marubeni Corporation.

Animal grouping and treatment. The rats were randomly divided into four groups: SCI; methylprednisolone (300 mg/kg); high-frequency electrotherapy (treated with microwave irradiation from 7 cm at 10 w for 10 min per day); and combination (treated with electrotherapy combined with 300 mg/kg methylprednisolone). $N=10$ for each group.

SCI modeling. According to current literature, the rat SCI model was established using the modified ALLEN struck method (14). Following anesthetization using an intraperitoneal injection of 30 mg/kg sodium pentobarbital, the rats were immobilized on the operating table. The vertebral plates and spinous processes of the thoracic vertebrae (T9-T11) were removed. The center was set at the T10 spinal cord and a circular area with a diameter of approximately 4 mm was determined as the lesion area. According to the physiological curvature of the dorsal spinal cord of rats, pre-bent plastic flexion pads of 3 mm in length, 2 mm in width, and 1 mm thick were prepared. The pads were placed outside of the spinal cord in the T10 area. Using the modified ALLEN's striking device, the center was set in the median posterior of the spinal cord. The striker rod moves freely through the sleeve at a height of 5 cm and directly hits the plastic gasket. Signs of successful modeling include the body and lower extremities retracting and flapping, in addition to tail swing. The surgical incision was closed and antibiotics (Baytril[®], Bayer AG; 4 mg/kg subcutaneous) were routinely administered.

Collection of spinal cord tissues. Spinal cord tissues were collected as previously described (15). The rats were sacrificed using sodium pentobarbital to effect, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (in PBS). To maintain consistency, each spinal cord was rostrally transected at the T6 spinal root, and a 3 cm segment of spinal cord was immediately dissected and fixed in paraformaldehyde for 2 h at 4°C , followed by the relevant analysis.

Basso, Beattie and Bresnahan (BBB) scoring. On the 20th day after surgery, the BBB score was measured to evaluate the recovery ability of the joints and lower limbs. The score was 0-21 points; 0 points indicated no visible hind limb movement, and 21 points indicated continuous palmar movement, continuous coordination gait, continuous toe grip, the parallel position of the active paw to the body, continued trunk stability and tail tilt. A higher BBB score indicates improved recovery (12).

SEP and MEP measurement. At 20 days post-surgery, evoked potentials and electromyography were used to detect SEP and MEP. The rats were anesthetized and stabilized at the right iliac and the Achilles tendon. A DC square wave pulse current with a wave width of 0.2 msec and a frequency of 2 Hz was selected. The test time was 20 msec, and a superposition range of ± 200 msec was recorded. The current waveform P1 and an incubation period of N1 were recorded. The main electrode was placed in the muscle belly of the right gastrocnemius and the action potential was recorded using a magnetic stimulator with a test time of 0.2 msec.

Sample collection. On the 20th day after surgery, a total of 5 ml blood was collected via the tail vein. The blood was centrifuged at $1,200 \times g$ rpm for 15 min, and the serum was collected and stored at -80°C .

ELISA. TNF- α and IL-2 expression levels were detected by ELISA according to the manufacturer's protocol. A total of 50 μl diluted standard or sample was added in triplicate to a 96-well plate and incubated at 37°C for 1 h. After washing five times, 50 μl enzyme-labeled reagent was added to each well and incubated at 37°C for 30 min. The plate was subsequently treated with 50 μl each of color agent A and B at 37°C for 10 min. Finally, 50 μl stop solution was added and the absorption at 450 nm was recorded using a microplate reader. The concentration of the sample was calculated and linear regression was compared with the absorbance value of the standard.

Caspase 3 activity. Caspase 3 activity in spinal cord tissue was determined using the Caspase 3 Activity Assay kit according to the manufacturer's protocol. Briefly, the cells were enzymatically digested and centrifuged at $600 \times g$ for 5 min (4°C). The cells were subsequently treated with RIPA lysis buffer (Thermo Fisher Scientific, Inc.) on ice for 15 min and centrifuged at $20,000 \times g$ for 5 min (4°C). Absorbance was detected at 405 nm following the addition of 2 mM Ac-DEVD-pNA to each test sample.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the tissue samples using TRIzol[®]

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	GGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC
BDNF	CACTCCGACCCCGCCCGCCG	TCCACTATCTTCCCCTTTTA
NF-κB	AATTGCCCGGCAT	TCCCGTAACCGCGTA

BDNF, brain-derived neurotrophic factor.

reagent (Thermo Fisher Scientific, Inc.) and reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) per the manufacturer's protocol. The primers for qPCR were designed by Primer Premier 6.0 (Premier Biosoft International) and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). qPCR was performed using SYBR Green (Thermo Fisher Scientific, Inc.) and the thermocycling conditions were as follows: 35 cycles at 92°C for 30 sec, 58°C for 40 sec, and 72°C for 35 sec. The 2^{-DDCq} method (16) was used to calculate relative expression levels in reference to GAPDH.

Western blotting. The sample tissues were lysed using RIPA buffer and quantified using a bicinchoninic acid assay. The proteins (40 μg/lane) were separated by SDS-PAGE using a 10% gel, and transferred to a PVDF membrane at 160 mA for 1.5 h. The membrane was blocked with 5% skim milk at room temperature for 2 h, and subsequently incubated with primary antibodies against BDNF (1:1,000; cat. no. ab226843), NF-κB (1:1,500; cat. no. ab16502) and β-actin (1:2,000; cat. no. ab8227) at 4°C overnight. After washing three times with PBS-Tween, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody (1:5,000; cat. no. ab6721) at room temperature for 30 min. The proteins were then visualized using ECL reagent. Each experiment was repeated four times and protein expression was quantified using Quantity One software (version 4.6.8; Bio-Rad Laboratories, Inc.).

Statistical analysis. All data analysis was performed using SPSS 19.0 software (IBM Corp.). The data are presented as the mean ± standard deviation and compared by one-way ANOVA and Newman-Keuls multiple comparisons analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

The impact of methylprednisolone combined with high frequency electrotherapy on SCI rats. BBB score analysis was performed to evaluate the impact of methylprednisolone combined with high frequency electrotherapy on SCI rats. All treatment groups exhibited significantly elevated BBB scores compared with the untreated, SCI group (P<0.05). However, the combination group exhibited more significant effects on SCI (P<0.05; Fig. 1). This suggested that methylprednisolone combined with high frequency electrotherapy improved the pathological process of SCI and promoted recovery.

The influence of methylprednisolone combined with high frequency electrotherapy on the SEPs and MEPs of SCI rats. SEPs and MEPs were analyzed to assess the influence of methylprednisolone combined with high frequency electrotherapy on SCI rats. All treatment groups displayed obviously improved SEPs and MEPs compared with the SCI group (P<0.05), though the combination group exhibited the most significant effects on SCI (P<0.05; Table II). These results indicated that methylprednisolone combined with high frequency electrotherapy alleviated the pathological effects of SCI and promoted recovery.

BDNF mRNA and protein expression levels in spinal cord tissues. RT-qPCR and western blotting were used to determine BDNF mRNA and protein expressions levels in the spinal cord tissues of rats. All treatment groups presented with markedly increased BDNF mRNA and protein expression levels compared with the SCI group (P<0.05). The combination group exhibited more significant effects on SCI (P<0.05) (Fig. 2 and 3).

NF-κB mRNA and protein expression levels in spinal cord tissues. RT-qPCR and western blot analysis were used to evaluate the expression levels of NF-κB mRNA and protein in rat spinal cord tissues. The treatment groups showed considerably reduced expression levels of NF-κB mRNA and protein compared with the untreated, SCI group (P<0.05), and the combination group exhibited the most significant effects on SCI (P<0.05; Fig. 4 and 5).

The effect of methylprednisolone combined with high frequency electrotherapy on serum TNF-α and IL-2 expression levels in SCI rats. The effects of methylprednisolone combined with high frequency electrotherapy on serum TNF-α and IL-2 expression level were assessed by ELISA. The treatment groups revealed a reduction in serum TNF-α and IL-2 expression levels compared with the SCI group (P<0.05); furthermore, the combination group exhibited a more significant effect on SCI (P<0.05; Fig. 6) revealing that methylprednisolone combined with high frequency electrotherapy inhibited cytokine release, alleviating inflammatory damage.

The impact of methylprednisolone combined with high frequency electrotherapy on caspase 3 activity. Caspase 3 activity was detected in the spinal cord tissues. Each treatment group displayed significantly decreased caspase 3 activity compared with the SCI group (P<0.05). The combination group exhibited more significant effects on SCI (P<0.05; Fig. 7), which collectively indicated that methylprednisolone combined with high frequency electrotherapy suppressed caspase 3 activity to regulate apoptosis.

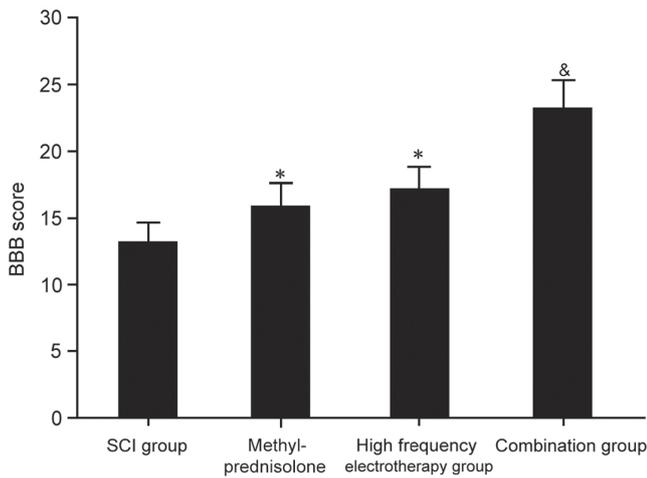


Figure 1. Impact of methylprednisolone combined with high frequency electrotherapy on the BBB scores of SCI rats. *P<0.05 vs. the SCI group; &P<0.05 vs. the methylprednisolone group or high frequency electrotherapy group. BBB, Basso, Beattie and Bresnahan; SCI, spinal cord injury.

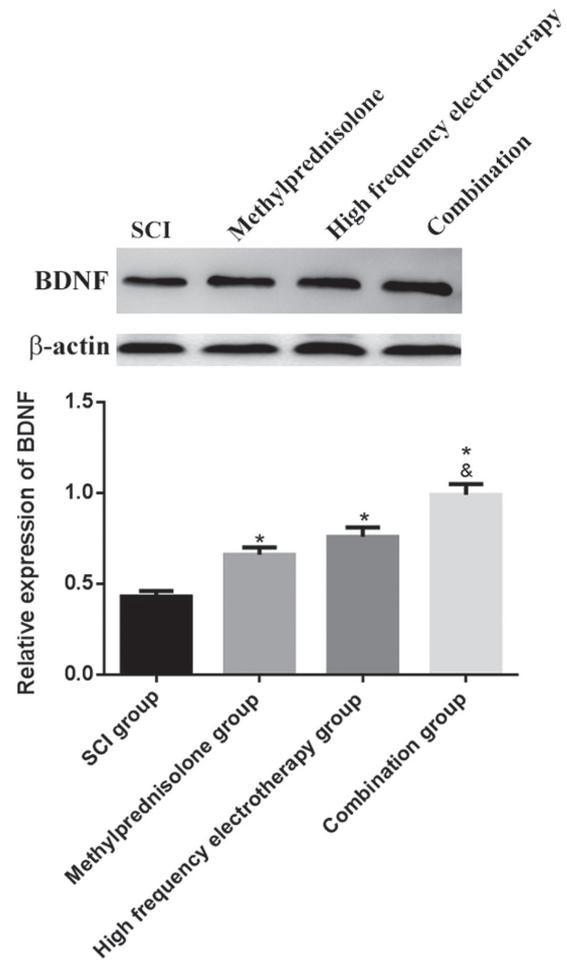


Figure 3. BDNF protein expression levels in the spinal cord tissues of SCI rats following treatment. *P<0.05 vs. the SCI group; &P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. BDNF, brain-derived neurotrophic factor; SCI, spinal cord injury.

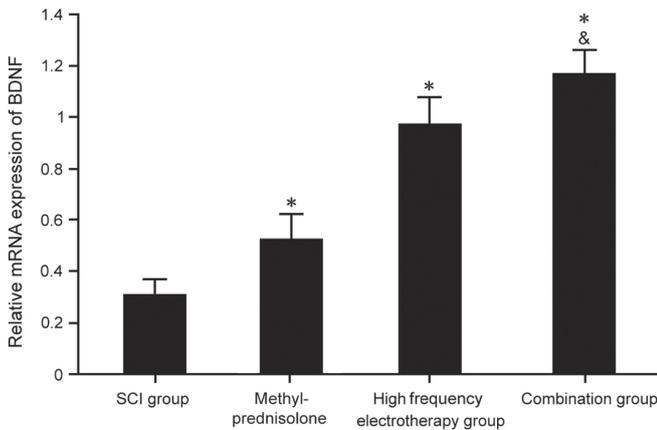


Figure 2. BDNF mRNA expression levels in the spinal cord tissues of SCI rats following treatment. *P<0.05 vs. the SCI group; &P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. BDNF, brain-derived neurotrophic factor; SCI, spinal cord injury.

Discussion

With the rapid development of science and technology, the use of physics at a clinical level is able to significantly promote spinal nerve regeneration and improve the local microenvironment of the spinal cord. It has been confirmed that high-frequency microwave treatment is beneficial to nutritional axons, promoting regeneration at sites of peripheral nerve injury, accelerating tissue recovery and reducing pathological injury (13,14). Conversely, the adrenal cortical hormone methylprednisolone has a strong anti-inflammatory effect that is conducive to recovery in the early stages of SCI (15,17). Therefore, the present study aimed to investigate the effect of combined treatment with methylprednisolone and physical rehabilitation on SCI and its associated mechanisms.

The BBB scoring method is widely used to evaluate the degree of SCI (12), and SEPs and MEPs can effectively analyze the functional recovery degree of SCI. The present

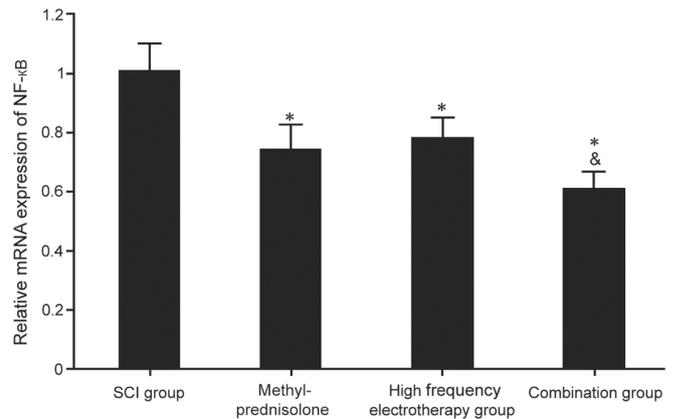


Figure 4. NF-κB mRNA expression levels in the spinal cord tissues of SCI rats following treatment. *P<0.05 vs. the SCI group; &P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. SCI, spinal cord injury.

study revealed significantly increased BBB scores in the methylprednisolone, high-frequency electrotherapy and combination groups, in addition to elevated SEPs and MEPs. However, methylprednisolone combined with high-frequency treatment exhibited a greater significant influence on BBB

Table II. Influence of methylprednisolone combined with high frequency electrotherapy.

Group	SEP		MEP
	P1 incubation period, ms	N1 incubation period, ms	Incubation period, ms
SCI	42.12±2.24	67.92±5.21	14.81±1.47
High-frequency electrotherapy	34.17±3.36 ^a	51.35±3.24 ^a	9.53±0.38 ^a
Combination	21.36±1.47 ^b	43.92±5.48 ^b	6.35±0.28 ^b

^aP<0.05; ^bP<0.01. Combination treatment denotes methylprednisolone (300 mg/kg) combined with high-frequency electrotherapy. SEP, somatosensory evoked potential; MEP, motor evoked potential; SCI, spinal cord injury.

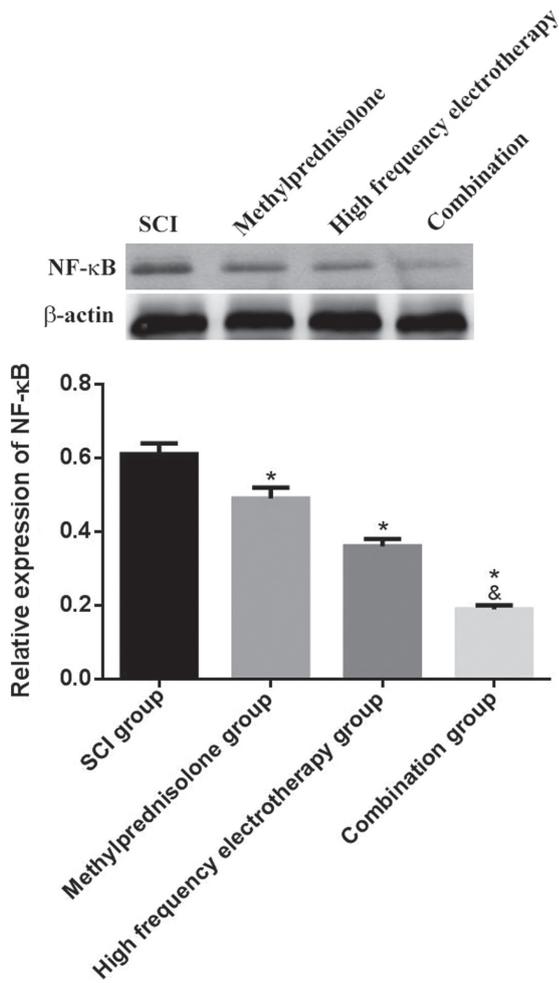


Figure 5. NF-κB protein expression levels in the spinal cord tissues of SCI rats following treatment. *P<0.05 vs. the SCI group; [&]P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. SCI, spinal cord injury.

score, SEPs and MEPs in SCI rats. These results suggested that high-frequency electrotherapy may effectively improve SCI, which may be associated with high-frequency electrotherapy waves promoting the movement, stretching, migration and swinging of membrane lipids and membrane proteins, strengthening metabolism and accelerate neuronal changes in the spinal cord (18,19). Analysis of inflammatory factors revealed that both singular treatments were able to reduce serum TNF-α and IL-2 expression levels in SCI rats, but that

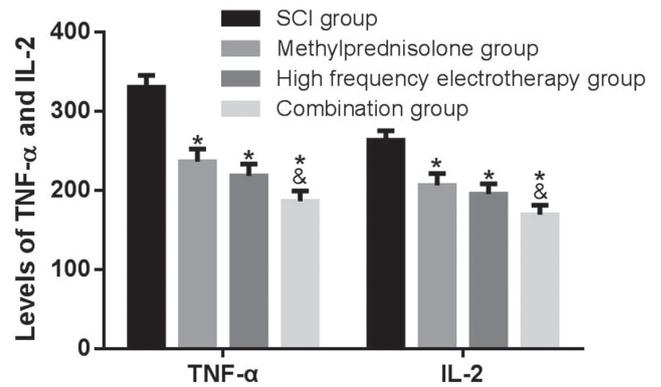


Figure 6. Effects of methylprednisolone combined with high frequency electrotherapy on serum TNF-α and IL-2 expression levels in SCI rats. *P<0.05 vs. the SCI group; [&]P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. SCI, spinal cord injury; TNF, tumor necrosis factor.

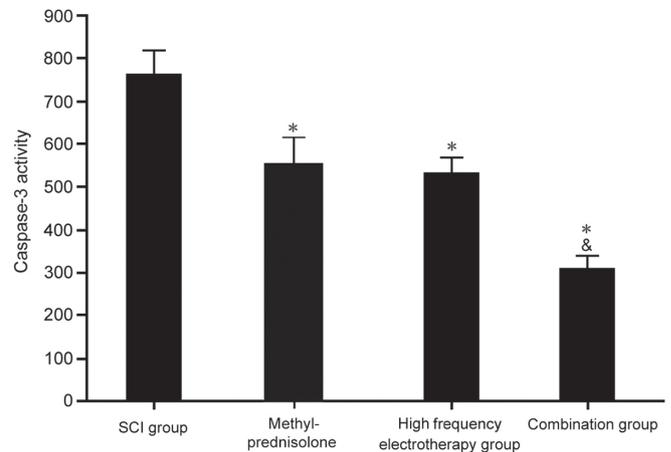


Figure 7. Impact of methylprednisolone combined with high frequency electrotherapy on Caspase 3 activity. *P<0.05 vs. the SCI group; [&]P<0.05 vs. the methylprednisolone or high frequency electrotherapy group. SCI, spinal cord injury.

methylprednisolone treatment combined with high-frequency electrotherapy resulted in a more significant improvement, which may be associated with the marked inhibition of inflammation caused by methylprednisolone (15,17). The combined effects of methylprednisolone effectively boosted the healing effects of high frequency electrotherapy on SCI.

Neurotrophin family member BDNF promotes the growth and survival of sensory and motor nerves, thus serves an important role in nerve regeneration (20,21). Activation of NF- κ B during SCI promotes neutrophil accumulation and macrophage adhesion, leading to the release of a large number of oxygen free radicals; this promotes the secretion of inflammatory cytokines such as TNF- α and IL-2, and subsequently compromises the integrity of spinal cord tissue by inducing vascular endothelial deterioration, increasing edema and necrosis of the spinal cord tissue, and promoting secondary SCI (22,23). Caspase 3 is a critical protease in apoptosis and is also the common downstream target of each apoptotic pathway (24). In the present study, the methylprednisolone, high-frequency electrotherapy and combination groups exhibited significantly increased expression levels of BDNF mRNA and protein, reduced NF- κ B mRNA and protein expression levels, and a reduction in caspase 3 activity. The combination group exhibited the greatest effects on SCI.

The present study aimed to analyze the mechanisms and subsequent effects of methylprednisolone treatment combined with rehabilitation high-frequency electrotherapy on SCI, and to further verify the relevant roles and mechanisms observed in clinical trials. In conclusion, methylprednisolone combined with high frequency electrotherapy was able to improve SCI, possibly by increasing BDNF and decreasing NF- κ B expression levels, in addition to suppressing the release of inflammatory factors.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL, CL, WW, LL and QZ performed the experiments and analyzed the data. YO designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Animal Ethics Committee of The First Affiliated Hospital of Xi'an Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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