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Beneficial Effects of Intrathecal Injection of Methylprednisolone Against Spinal Cord Injury in Rats

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Abstract

Background: High dose intravenous (i.t.) injection of methylprednisolone (MPSS) for spinal cord injury (SCI) is clinically controversial.
Objectives: This study aimed to investigate whether i.t. MPSS would have a beneficial effect on SCI and whether or not it is a safe operation for SCI patients.
Methods: An animal experiment was conducted to explore the safety and feasibility of i.t. Administration of MPSS. Male Sprague-Dawley rats were randomly divided into four groups: (1) sham group, i.t. injection of normal saline (NS) (n = 25); (2) control group, SCI surgery (created using the Infinite Horizon IH-400 impactor) with i.t. injection of NS (n = 25); (3) i.t. MPSS1 group, SCI with i.t. injection of MPSS by a pulse therapy (n = 25); (4) i.t. MPSS2 group, SCI with i.t. injection of MPSS intermittently (n = 25). Malondialdehyde (MDA), superoxide dismutase (SOD), and inflammatory cytokines in serum were measured at 6h, 24h, 48h, 7d, and 14d after surgery with commercial assay kits. Glial fibrillary acidic protein (GFAP) level was observed at 14 days after surgery by immunohistochemistry. Motor evoked potentials (MEP) and somatosensory evoked potential (SEP) were monitored and recorded separately before surgery and 1, 7, and 14 days after surgery. Also, locomotor function was evaluated using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale.
Results: The results showed that the levels of MDA and SOD, and three inflammatory cytokines, including IL-1b, IL-6, and TNF-α were reduced in i.t. MPSS groups than that of the control group (all $P < 0.05$). The expression of GFAP was inhibited after i.t. MPSS treatment. The amplitude was reduced, and the latency period of SEP and MEP recovery was prolonged (all $P < 0.05$) after MPSS administration. In addition, the recovery of limb function (BBB score) was significantly ameliorated ($P < 0.05$) in SCI rats treated with MPSS compared with the control group.
Conclusions: Our results demonstrated that i.t. MPSS was a potential strategy for reducing the secondary damage after SCI, especially the MPSS pulse therapy.

Keywords: Spinal Cord Injury, Injection, Intrathecal, Methylprednisolone, Electrophysiology

1. Background

Spinal cord injury (SCI) is a highly prevalent disease worldwide (approximately 12,500 new cases each year in the United States) (PDF, 2016). Systemic administration of methylprednisolone (MPSS) is administered as a reasonable treatment option for SCI to reduce neurological deficits and improve neurological recovery (1, 2). It had widely been accepted after the Third National Acute Spinal Cord Injury Randomized Controlled Trial of National Acute Spinal Cord Injury study (NASCIS) (3). MPSS is commonly administered by high-dose intravenous drip. However, some studies suggested that high-doses of MPSS would increase the risk of a variety of complications, such as gastrointestinal ulcer, bleeding, and facial flushing, so the application of MPSS is controversial for clinical purposes (4-6).

Intrathecal (i.t.) administration of MPSS has been used in sciatica, subarachnoid hemorrhage, and multiple sclerosis (7, 8). This method of administration is thought to reduce the use of MPSS, reduce complications, and has a positive effect on the disease (9). Wang et al. (10) have indicated that i.t. injection of MPSS can improve the functional recovery of the lower limb and decrease the apoptosis of neuron cells in New Zealand rabbits using HE, TUNEL staining and TARLOV score (10). Other researchers have acquired re-

versed results (11). Therefore, more comprehensible investigation is required on the beneficial effects of i.t. administration of MPSS to SCI.

2. Objectives

The present study aimed to learn whether i.t. MPSS has beneficial effects on SCI and whether or not the administration of MPSS is a safe operation for SCI patients. To this end, we conducted an experiment on rats to explore the way of drug administration and observe the effectiveness and safety of this approach. We measured two oxidative stress parameters, including malondialdehyde (MDA), as an index of the lipid damage involving in the oxidative processes, and superoxide dismutase (SOD) as a kind of antioxidant enzyme protecting cells from oxidative damage by eliminating oxygen free radicals (12). In addition, we analyzed inflammation cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, as well as glial fibrillary acidic protein (GFAP), motor evoked potentials (MEP), and somatosensory evoked potential (SEP), and locomotor function. The results may help illustrate the effects of the administration method of MPSS for SCI.

3. Methods

3.1. Animals

Male Sprague-Dawley rats (220 - 240 g) were obtained from the Experimental Animal Center of the Second Military Medical University, Shanghai, China. The animals were in-house bred under standard laboratory conditions (temperature, 25°C; humidity, 60%; 12 h light/dark cycle). All animals were given free access to food and water. The international, national, and/or institutional guidelines were strictly followed regarding the animal's welfare. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Second Naval Military Medical University, Shanghai, China (ethical number: DWLL-103).

3.2. Experimental Groups and Treatment

Rats were randomly divided into four groups as follows: (1) sham group (n = 25): i.t. injection of 10ul of normal saline with a 5 mL syringe (Shanghai Chuding Instrument Company, Shanghai, China); (2) control group (n = 25): SCI with given i.t. injection of 10 μL of NS; (3) i.t. MPSS1 group (n = 25): SCI with i.t. injections of MPSS (intravenous MPSS pulse therapy: repeat bolus i.t. injection of 1.15 mg/kg of MPSS within 15min, followed by a 45 min period of rest, the rats received an MPSS infusion of 0.207 mg/kg/h for 23 h); (4) i.t. MPSS2 group (n = 25): SCI with i.t. injection of 10 μL MPSS at dose of 1.15 mg/kg on 0, 3, 6, 9, and 12-day after surgery. All the animals in the groups received treatments immediately after surgery. In the control and the i.t. MPSS2 group, the rats keep prone trendelenburg position for 30 minutes.

3.3. Contusion Model of SCI

The rats were briefly anaesthetized with 10% chloral hydrate (0.3 mL/100 g, intraperitoneally). The spinous process and the vertebrae lamina were removed to expose the region of dura matter at the T9-T11 level. A standardized model of contused SCI was created using the Infinite Horizon IH-400 impactor (IH-400; Precision Systems and Instrumentation, LLC, Versailles, KY, USA) (13) under stereotaxic control (175 kdynes). A successful contusion of the spinal cord was indicated by wagging the lower limbs and body torso and tail flapping in a spastic manner. A polyethylene catheter (P.E.; i.d. 0.28 mm ve o.d. 061 mm, Becton Dickinson, Philadelphia, USA) was i.t. pushed 1.5 cm into the caudal (14). After the rats recovered from anesthesia, both lower extremities had become paralyzed. The sham-operated rats had only their spinous process, and vertebral lamina was removed. All rats received a single dose of preventive 20 IU benzylpenicillin sodium antibiotic every day for three consecutive daysa and their bladder was evacuated. The rats in i.t. MPSS 1 were injected with a micro-injection pump (The Pump 11 Elite Series, Harvard Apparatus, US).

3.4. Measurement of Oxidative Stress and Antioxidant Enzymatic Activities

MDA and SOD were measured by collecting 2.0 mL blood from the orbital vein of five rats in each group at 6, 24, and 48 h and 7 and 14 d. The blood samples were immediately stored for 1.0 h in 4.0°C, followed by centrifugation at 3000 g for 15 min at 4.0°C, then were aliquoted, and stored at -80°C until assayed. The detection methods were performed using commercial assay kits (Sigma-Aldrich, USA).

3.5. Measurement of Inflammatory Cytokines

The expression levels of TNF-α, IL-1β, and IL-6 were measured with ELISA kits (R & D Systems, Inc., Minneapolis, MN, USA). The optical density (OD) was measured at 450 nm within 10 min. All assays were performed in duplicate.

3.6. Tissue Processing

At 6, 24, and 48 h and 7 and 14 d after surgery, five rats from each group were sacrificed for tissue samples. The rats were deeply anesthetized with 10% chloral hydrate (0.4 mL/100 g, i.p.) and transcardially perfused with 50 mL

of phosphate-buffered saline (PBS), followed by 150 mL of fixative containing 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4; 4.0°C) over 30 min. A 10-mm segment of the spinal cord containing the T10 impact epicenter was removed from each rat and was fixed in the same fixative overnight (4.0°C). The samples were transferred to 20% sucrose buffer overnight, after which they were transferred into 30% sucrose buffer for dehydration and cryoprotection at 4.0°C overnight. Finally, the samples were sagittally cut into 4.0-um-thick sections.

3.7. GFAP Staining with Immunohistochemistry

Tissue sections were deparaffinized, and the antigen retrieval was performed with EDTA. Sections were then rinsed in 0. 01 mol/L PBS and immersed in 3% hydrogen peroxide to block endogenous peroxidase at room temperature (RT) for 25 min, followed by incubation with BSA for 30 min. The sections were placed on a solution of the rabbit anti-GFAP antibody (Sigma-Aldrich, USA, dilution 1:300) at 4°C overnight, followed by the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, USA, dilution 1:1000) at RT for 50 min. Diaminobenzidine (DAB) color developed rapidly for 5 min (under a microscope control staining).

3.8. SEP Recording

After anaesthetized with 10% chloral hydrate (0.3 mL/100 g), a reference electrode needle was placed on the lateral malleolus tendon; a stimulating electrode needle was inserted into the gastrocnemius; a ground needle electrode was placed in the tail of rats. At the midpoint between ears, recording electrode was placed; the reference electrode was placed at the subcutaneously across the forehead. When the lower limb shake obviously, the stimulus intensity would be appropriate. The stimulation intensity was administered at 3.0 mA and a frequency of 1.3 Hz. Each curve was created after an average of 100 repetitions to improve the signal-to-noise ratio. The waveform was recorded by a monitor for detection of a neuroelectrophysiological parameter (Nuocheng electric Co. Ltd, Shanghai, China).

3.9. MEP Recording

The magnetic stimulation coil was focused on the rat head (Transcranial Magnetic Stimulator, Yiruide Co., Wuhan, China). The coil position was adjusted according to the response of rats to achieve the best stimulating effect. A stimulating electrode needle was placed at the midpoint between ears; a reference electrode was placed subcutaneously at the forehead; a recording electrode needle was inserted into the gastrocnemius to record MEP; a ground needle electrode was placed in the tail of rats; a reference electrode needle was placed at the lateral malleolus tendon. Each result was repeated at least twice to obtain a stable waveform. The lower limb was obviously shaken under the stimulation of the magnetic field. Also, the waveform was recorded by the monitor for neuroelectrophysiological parameter.

3.10. Functional Test

Neurological assessment of hind limbs was evaluated by the observer blinded to the group assignments using motor function assay before surgery and at 1, 4, 7, 10, and 14 d after surgery. The Basso, Beattie, and Bresnahan (BBB) locomotor scale was used as described previously (15) and all surviving rats were given the functional test.

3.11. Statistical Analyses

The statistical analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as the means ± standard deviation (x ± S) for each group. The differences among the experimental groups were detected by one-way analysis of the variance (ANOVA) and the independent samples *t*-test. The variance was determined using the Student-Newman-Keuls test. Two-way repeated-measure ANOVA was used to compare matched data at multiple time points. A P value < 0.05 was considered to be statically significant.

4. Results

4.1. MDA and SOD

The serum levels of MDA and SOD at 6 h, 24 h, 48 h, 7 d, and 14 d were measured. The results showed that the serum MDA and SOD levels of the other four groups were significantly increased than the sham group ($P < 0.05$, Figure 1A and B). Unlike the control group, the SOD level in i.t. MPSS1 significantly maintained their initial quality after surgery ($P < 0.05$). The MDA levels of i.t. MPSS1 and i.t. MPSS2 were lower than that of the control group, and that of i.t. MPSS1 was lowest ($P < 0.05$).

4.2. Inflammatory Cytokines

The ELISA results showed that at 6, 24, and 48 h and at 7 and 14 d after surgery, the levels of proinflammatory cytokines, including IL-1b, IL-6, and TNF-α were remarkably increased in the control group compared to that in the sham group ($P < 0.05$), and attenuated notably in the i.t. MPSS groups (i.t. MPSS1 and i.t. MPSS2) at 6 h, 24 h, and 48 h (vs. sham, $P < 0.05$, Figure 1C-E). The levels of IL-1b, IL-6, and TNF-α in i.t. MPSS1 was continued to decline at 7 d and 14 d after surgery, but those in i.t. MPSS2 were raised higher than that in the control group.

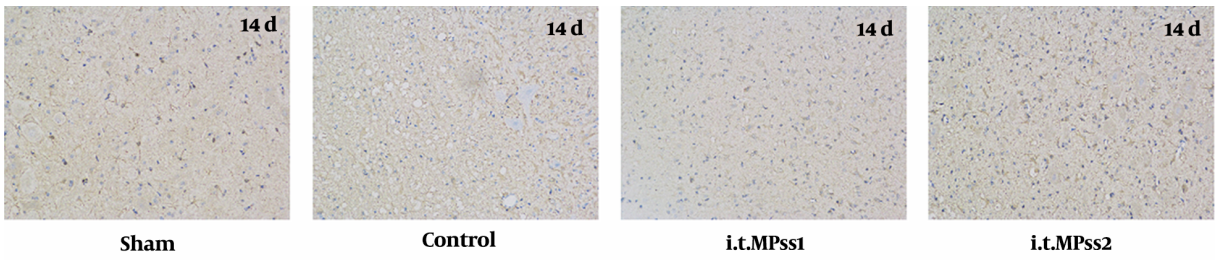

Figure 1. A, The serum levels of superoxide dismutase (SOD); B, malondialdehyde (MDA); C, tumor necrosis factor alpha (TNF-α); D, interleukin (IL)-1β; E, and IL-6 were measured at 6, 24, and 48 h and at 7 and 14 d after spinal cord injury (SCI). The content of MDA was increased in the control group ($P < 0.05$ vs. Sham) and was significantly reduced by intrathecal (i.t.) methylprednisolone (MPSS) ($P < 0.05$ vs control). The levels of TNF-α, IL-1β, IL-6 were increased in the control group after SCI ($P < 0.05$ vs. Sham) and were significantly reduced by MPSS administration until 7 days after surgery ($P < 0.05$ vs Control). Seven days after surgery, that of i.t. MPSS1 group were continued to maintain low levels, but that of i.t. MPSS2 group was higher than the control group. Data are expressed as means $\pm$ SD (n = 5 per group). #, $P < 0.05$ vs. Sham group; *, $P < 0.05$ vs. control group.

4.3. GFAP Staining

The GFAP expression levels of rats at 14 days after SCI were observed to evaluate the astrocyte proliferation. The results indicated that the GFAP level in the control group was increased significantly compared to the sham group; the expression level of GFAP in i.t. MPSS1 was lower than that in i.t. MPSS2 (Figure 2).

4.4. SEP and MEP

SEP and MEP were recorded before the surgery and at 1, 7, and 14 days after the surgery (Figure 3A and B). The peak-to-peak amplitude and latency of SEP could not be recorded at 1 day after SCI, a gradual recovery was observed at 7 and 14 days of the waveform. The latent period was prolonged, and the amplitude was reduced in i.t. MPSS1 and i.t. MPSS2 compared with those in sham group ($P < 0.05$). Unlike the control, the recovery of the latent period was significantly promoted and the amplitude was increased after i.t. MPSS treatment ($P < 0.05$). The i.t. MPSS1 group seemed to be better recovered than the i.t. MPSS2 group. At 1 day after the injury, the amplitude of MEP in SCI groups was significantly lower than the sham group ($P < 0.05$), the latency was not significantly changed ($P > 0.05$). The MEP amplitude was further recovered, but there was no significant difference among the SCI groups ($P > 0.05$) (Table 1).

4.5. Functional Test

Locomotor function was evaluated before the surgery and at 1, 4, 7, 10, and 14 d after the surgery using the BBB scale (Figure 4). The BBB score of all rats were significantly decreased in the control and i.t. MPSS groups on day 1 after surgery. Rats in the i.t. MPSS groups had higher BBB scores than the control group after surgery, until day 14 ($P < 0.05$). It is important to mention that the BBB score of i.t. MPSS2 was raised until day 7, while that of i.t. MPSS1 was continued to rise, and reached a peak on day 14.

5. Discussion

Previous studies suggested that MPSS could exert a neuroprotective effect on SCI treatment through improving blood flow, reduce inflammatory responses and inhibit the cell apoptosis (16, 17). High-dose MPSS was widely used in experimental models and clinical treatment. However, they often had high complication rates (18). The i.t. drug delivery of MPSS can reduce the dose and interact directly with the spinal cord. We designed this experiment to learn the effects of i.t. MPSS injection on SCI, and verify the feasibility of the i.t. MPSS pulse therapy. The results demonstrated that the MDA levels of rats, the inflammatory cytokine levels were reduced, and the expression of GFAP was inhibited after i.t. MPSS. In addition, the amplitude of SEP and MEP, and the latency of SEP would be promoted after i.t. MPSS in SCI rats. Also, this method of administration of MPSS would improve recovery of the limb function significantly.

The serum level of SOD was increased after SCI which may be due to the stimulation of antioxidant defense system after injury (19). Unlike the control rats, the serum SOD in SCI rats was significantly raised after i.t. MPSS1 treatment. MPSS may play a protective role by inhibiting lipid peroxidation and keeping SOD activity (20). MDA is an end product of lipid peroxidation, used as an index of the lipid damage to reflect the serious course of the body cells being attacked by free radicals (21). It had deleterious effects and could accelerate the oxidative processes (22). Previous studies suggested that the anti-lipid peroxidation of MPSS was the main mechanism of neuroprotective effects (23). The i.t. MPSS treatment significantly decreased the serum levels of MDA which indicated the low of MPSS dosing enhance harming treatment efficacy.

The inflammation cytokines, including TNF-α, IL-1β, and IL-6 could directly or indirectly involve in the sec-

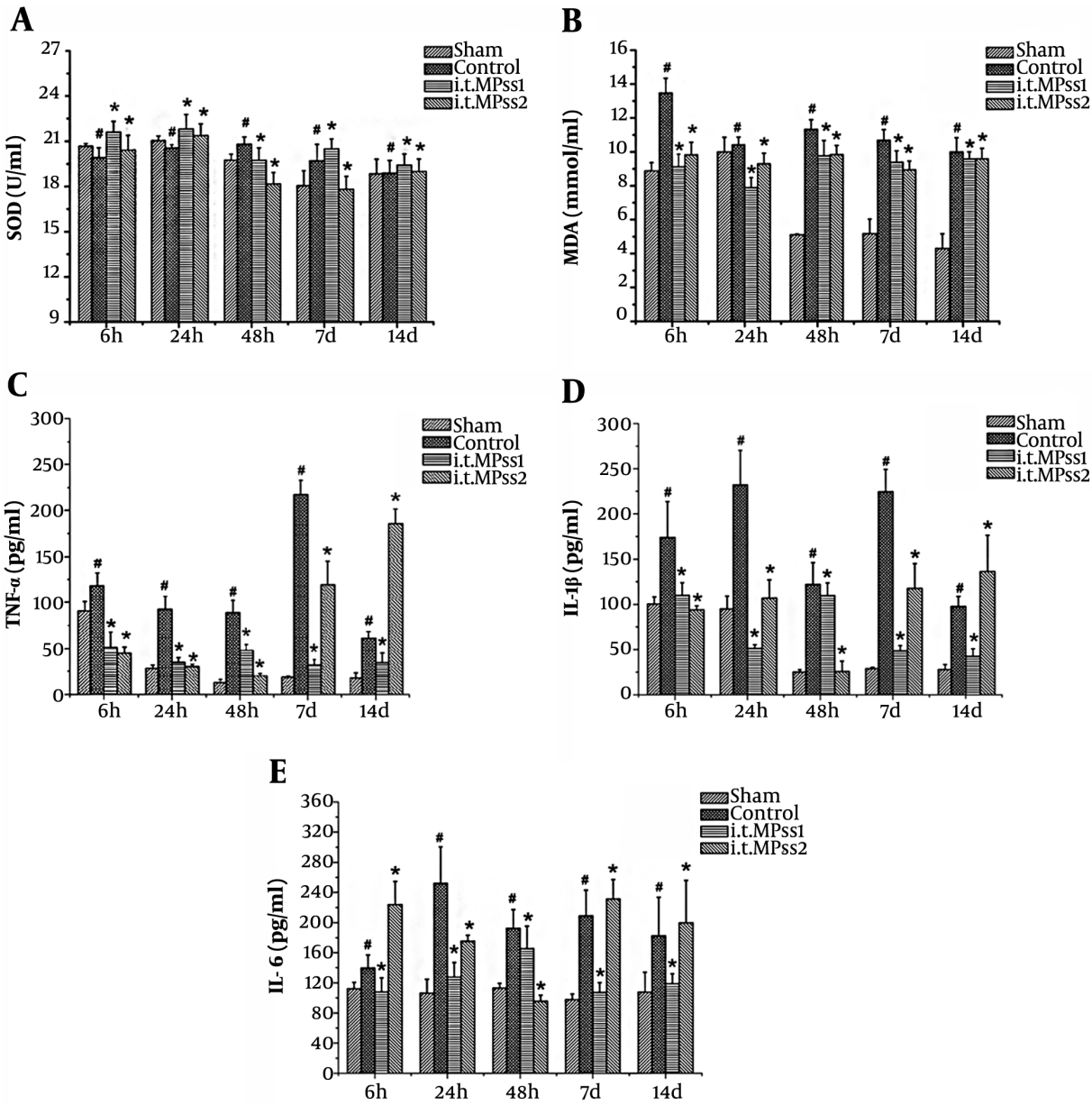

Figure 2. Immunohistochemistry assay of glial fibrillary acidic protein (GFAP) at 14 d after surgery in each group. The number and the size of GFAP-immunoreactive astrocytes were increased in rats of control group. After intrathecal (i.t.) injection of methylprednisolone (MPSS), the GFAP of astrocytes was inhibited, especially in i.t. MPSS1 group.

ondary trauma after SCI (24). Our results demonstrated that the inflammatory cytokine levels were decreased after MPSS treatment. However, the inflammatory factor indexes were increased after repeated injections, which may be a side effect of repeated injection. These results are consistent with some previous reports (8, 23). Wang et al. (10) proved that benzene, formaldehyde, and some other auxiliary drugs in MPSS may cause local inflammatory cell infiltration. There was also one study suggesting that MPSS itself may play a role in the inflammatory response (8). Therefore, a further purification of MPSS is needed. The results also indicated that i.t. MPSS pulse group (i.t. MPSS1) has a better effect and leads to fewer complications.

In the damage local of SCI and the adjacent areas, glial cell proliferation is significantly increased, which is typically characterized by increased amounts of intermediate

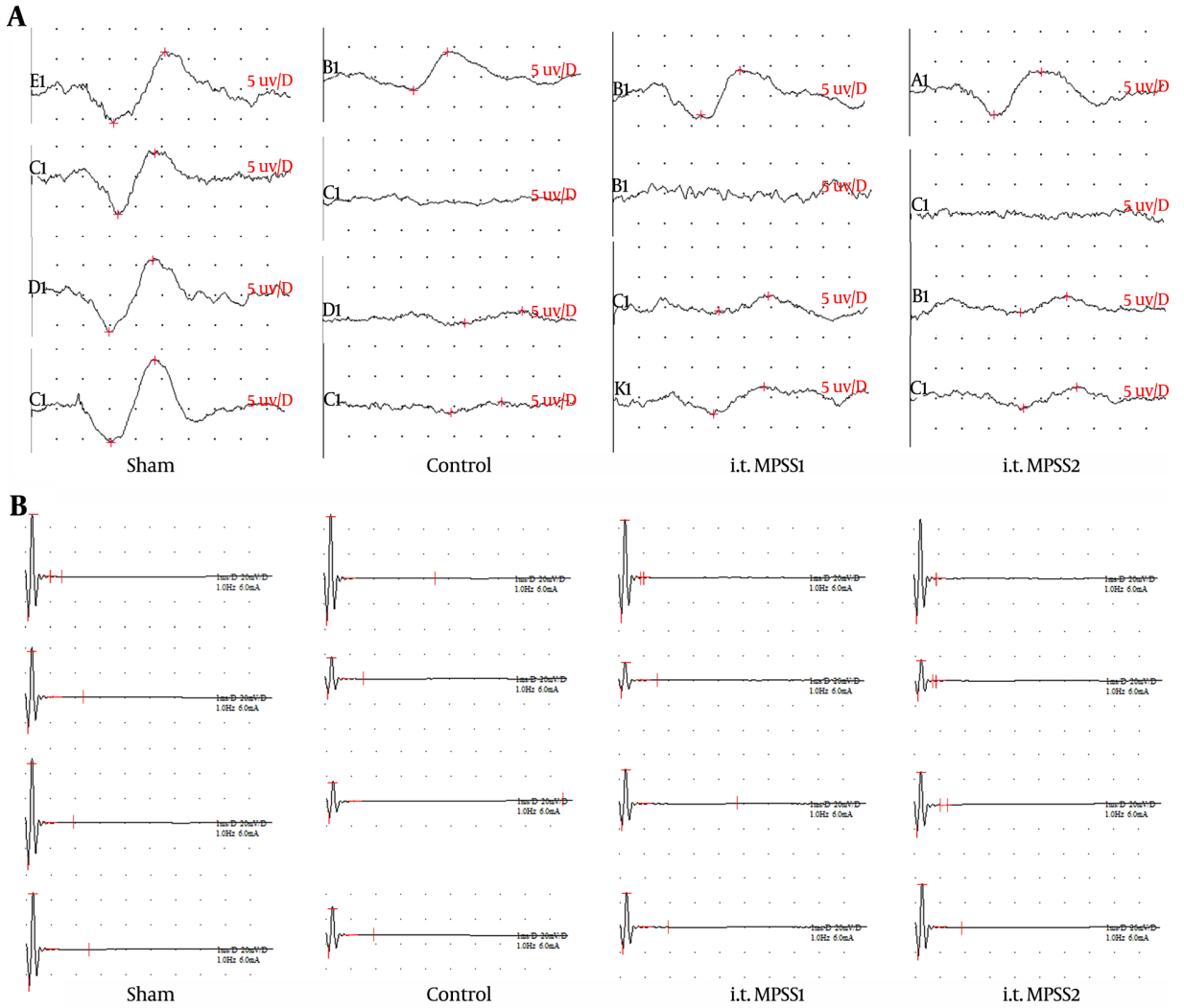

Figure 3. A, Somatosensory evoked potential (SEP); B, and motor evoked potentials (MEP) were recorded before surgery and at 1, 7, and 14 days after spinal cord injury (SCI) surgery. A, The amplitude and latency of SEP could not be recorded at day 1 after SCI, and the waveform was gradually recovered at 7 and 14 days ($P < 0.05$, vs. control group); B, one day after the injury, the amplitude was significantly decreased and the latency was not significantly changed. Amplitude was further recovered, but there was no significant difference among the SCI groups ($P > 0.05$).

filament protein GFAP (25). In SCI rats, the reactive astrocytes were clustered at the border of a lesion in 1 - 2 weeks after injury, and the glial scar became matured at 2 - 3 weeks after surgery (26). As shown in our results, i.t. MPSS reduced the expression of GFAP which may be a reason for MPSS to facilitate the recovery following SCI.

The evoked potential is considered as an important index of the recovery of SCI. MEP and SEP have been used to evaluate the motor and sensory function of injury after SCI (27, 28), their accuracy were 92.8% and 91.3% (29), respectively. Our results showed that the amplitude and latency of SEP could not be recorded at day 1 after SCI, and the waveform was gradually recovered at day 7 and 14. The amplitude and latency were positively correlated with the degree of injury. In MEP, the amplitude was significantly decreased at day 1 after the injury. The amplitude was further recovered, but there was no significant difference among the groups because the MEP is less sensitive to post damage than SEP; it may be the result from that almost all of the anesthetics could reduce the volatility of MEP in varying degrees (30). The combined application of SEP and MEP would more comprehensively evaluate the spinal cord function.

The BBB locomotor score was a universal measure of

Table 1. Somatosensory Evoked Potential (SEP) (A) and Motor Evoked Potentials (MEP) (B) were Recorded Before Surgery and at 1, 7, 14 Days After Spinal Cord Injury (SCI) Surgery[a]

	Sham Group		Control Group		i.t.MPss1 Group		i.t.MPss2 Group	
	Latency, ms	Amplitude, μV	Latency, ms	Amplitude, μV	Latency, ms	Amplitude, μV	Latency, ms	Amplitude, μV
A				SEP				
0	31.0 ± 1.4	14.1 ± 0.9	31.2 ± 6.1	10.8 ± 2.0	32.5 ± 2.1	9.9 ± 3.8	33.8 ± 3.3	9. 3 ± 1.9
1 d	32.1 ± 1.6	13.7 ± 1.3	-	-	-	-	-	-
7 d	31.4 ± 3.3	12.7 ± 1.1	54.6 ± 3.7[b]	3.6 ± 1.5[b]	39.3 ± 2.3[c]	4.0 ± 1.1[c]	40.8 ± 1.8[c, d]	3.6 ± 0.7[c, d]
14 d	30.1 ± 2.1	14.2 ± 1.6	43.3 ± 2.3[b]	3.0 ± 1.4[b]	35.0 ± 2.1[c]	4.8 ± 0.8[c]	43.9 ± 5.9[c, d]	3.3 ± 1.1[c, d]
B				MEP				
0	0.1	82.1 ± 5.9	0.1	79.8 ± 6.52.0	0.1	89.9 ± 5.63.8	0.1	89.3 ± 1.91.9
1 d	0.1	77.7 ± 6.3	0.1	21.3 ± 2.5[b]	0.1	18.5 ± 3.3	0.1	20.5 ± 3.2
7 d	0.1	83.8 ± 8.1	0.1	18.6 ± 1.5[b]	0.1	33.0 ± 3.1	0.1	33.6 ± 0.7
14 d	0.1	79.2 ± 4.6	0.1	23.0 ± 1.4[b]	0.1	34.8 ± 5.8	0.1	33.3 ± 1.1

Abbreviations: i.t., intrathecal; MPSS, methylprednisolone.
[a]Values are expressed as mean ± SD.
[b]P < 0.05 vs. Sham group.
[c]P < 0.05 vs. control group.
[d]P < 0.05 vs. i.t. MPss1 group.

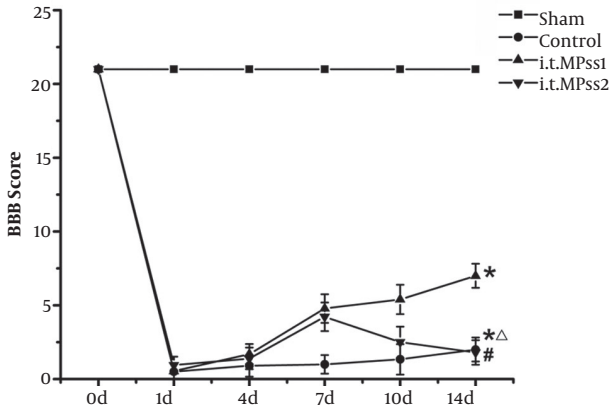

Figure 4. Using the Basso, Beattie, and Bresnahan locomotor scale (BBB scale) to assess the hindlimb function before surgery and at 1, 4, 7, 10, and 14 day after spinal cord injury (SCI) surgery. The BBB scores were significantly decreased at day 1 after SCI surgery. The BBB scores of rats receiving MPSS treatment were higher than that of control at 4 d and 7 d (P < 0.05). Seven days after surgery, the BBB score of i.t. MPSS1 continued to rise, but that of the i.t. MPSS2 group was decreased. Data are expressed as means ± SD. #, P < 0.05 vs. Sham group; *, P < 0.05 vs. Control group; [d], P < 0.05 vs. i.t. MPSS1 group.

functionality to follow the induction and treatment of SCI in rats for hind limb motility during walking (15), an important functional performance. Our results indicated that in the first 7 days, rats in the i.t. MPSS groups had higher BBB scores than the control group. After 7 days, the BBB score of i.t. MPSS1 group continued to rise, but the BBB score of the i.t. MPSS2 group was decreased. This result was consistent with the changes of serological indicators and electrophysiological indices of SEP.

5.1. Conclusions

MPSS administration could reduce the release of proinflammatory cytokines and suppress reactive astrogliosis after SCI. It could promote the recovery of the amplitude and latency of SEP and MEP after SCI, and significantly ameliorate the recovery of limb function. Our results demonstrated that i.t. MPSS was a potential strategy for reducing the secondary damage after SCI, especially the pulse therapy. However, there were some limitations in the study; the results should be verified in clinical trials.

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Footnotes

Authors' Contribution: Conception and design of the research: Hui Ma and Zhe Zhang. Acquisition of data: Zhe Zhang and Cong Wang. Analysis and interpretation of data: Zhe Zhang, Cong Wang, and Zhengmao Guan. Statistical analysis: Cong Wang and Zhengmao Guan. Drafting the manuscript: Zhe Zhang. Revision of manuscript for important intellectual content: Hui Ma.

Conflict of Interests: The authors declare that they have no competing interests.

Ethical Approval: The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Second Naval Military Medical University, Shanghai, China (code: DWLL-103).

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Informed Consent: Not applicaple.

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