

Effects of decompression joint Governor Vessel electro-acupuncture on rats with acute upper cervical spinal cord injury

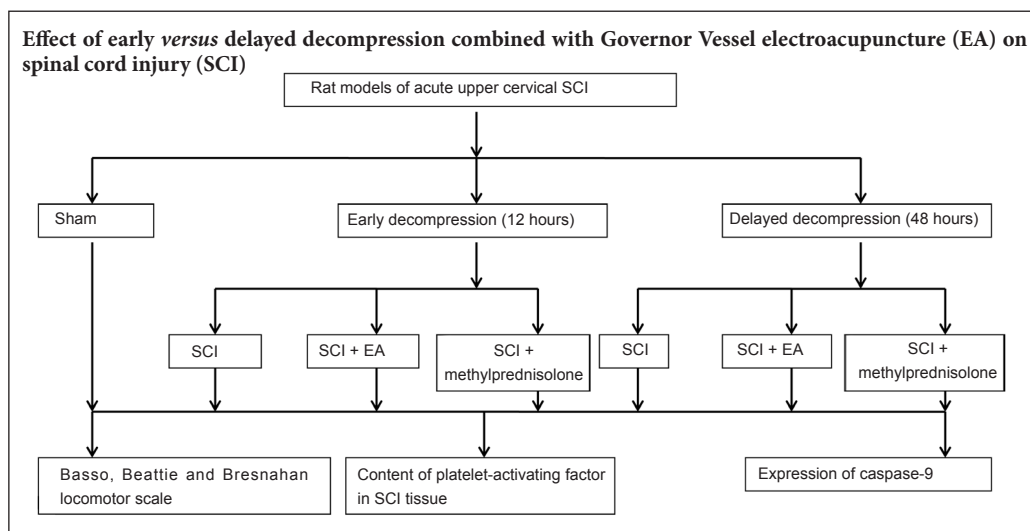
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Graphical Abstract



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Abstract

Decompression is the major therapeutic strategy for acute spinal cord injury, but there is some debate about the time window for decompression following spinal cord injury. An important goal and challenge in the treatment of spinal cord injury is inhibiting or reversing secondary injury. Governor Vessel electroacupuncture can improve symptoms of spinal cord injury by inhibiting cell apoptosis and improving the microenvironment of the injured spinal cord. In this study, Governor Vessel electroacupuncture combined with decompression at different time points was used to treat acute spinal cord injury. The rat models were established by inserting a balloon catheter into the atlanto-occipital space. The upper cervical spinal cord was compressed for 12 or 48 hours prior to decompression. Electroacupuncture was conducted at the acupoints *Dazhui* (GV14) and *Baihui* (GV 20) (2 Hz, 15 minutes) once a day for 14 consecutive days. Compared with decompression alone, hind limb motor function recovery was superior after decompression for 12 and 48 hours combined with electroacupuncture. However, the recovery of motor function was not significantly different at 14 days after treatment in rats receiving decompression for 12 hours. Platelet-activating factor levels and caspase-9 protein expression were significantly reduced in rats receiving electroacupuncture compared with decompression alone. These findings indicate that compared with decompression alone, Governor Vessel electroacupuncture combined with delayed decompression (48 hours) is more effective in the treatment of upper cervical spinal cord injury. Governor Vessel electroacupuncture combined with early decompression (12 hours) can accelerate the recovery of nerve movement in rats with upper cervical spinal cord injury. Nevertheless, further studies are necessary to confirm whether it is possible to obtain additional benefit compared with early decompression alone.

Key Words: nerve regeneration; acute spinal cord injury; decompression; Governor Vessel electroacupuncture; platelet-activating factor; apoptosis; methylprednisolone; caspase family; upper cervical spine; animal model; Basso, Beattie and Bresnahan locomotor scale; neural regeneration

Introduction

Traumatic spinal cord injury (SCI) occurs in 3.6 to 195.4 patients per million worldwide (Jazayeri et al., 2015). Many cases of SCI result in tetraplegia and although this complication leads to tremendous personal loss and societal costs, treatments for SCI are not standardized and are highly variable.

The pathophysiology of SCI involves the primary injury

and the subsequent secondary injury, which results from a progressive local cascade of tissue destruction, including the change in the microenvironment of the injured spinal cord (ischemia of the microcirculation, edema, inflammation, glutamatergic excitotoxicity) and apoptosis. The change in the microenvironment is the pathological basis of the functional deficits after SCI. The increased concentration

of platelet-activating factor in the blood and tissue is an important factor that promotes the changes in the microenvironment (Faden et al., 1992; Wang et al., 2016). The long-term neurological deficits after SCI may be due in part to widespread apoptosis of the neurons and oligodendroglia in distant regions (Crowe et al., 1997; Emery et al., 1998). Many studies have shown that an important apoptotic pathway that mediates apoptosis after SCI is the family of caspases, including caspase-3 and caspase-9 (Springer et al., 1999; Nakagawa et al., 2000; Dong et al., 2015).

Decompression has been proven to be effective (Rabinowitz et al., 2008; Fehlings et al., 2012; Jones et al., 2012; Wilson et al., 2012). The focus of debate is on outlining the optimal timing of decompression for patients with acute SCI. Many scholars agree that early decompression (< 24 hours) leads to a clinical improvement in neurological status, but delayed decompression (> 24 hours) for acute SCI has not resulted in optimal outcomes in neurological status (Wilson et al., 2012; Dahdaleh et al., 2013). SCI cannot be cured by decompression, so performing decompression along with effective adjunctive therapies is an appropriate approach to enhance the treatment of acute SCI.

Adjunctive therapies are a key factor in continuously promoting optimal treatment of acute SCI. The adjunctive therapies, such as corticosteroids (Fehlings et al., 2014; Schroeder et al., 2014) and neuroprotectant agents (Wilson et al., 2013; Grossman et al., 2014), have some protective effect on the spinal cord and nerve roots, but the overall effects are not ideal.

Regarding traditional Chinese medicine, the spine has a close relationship with the Governor Vessels. SCI is regarded as a stasis in the meridian of the Governor Vessels. The cardiovascular complications and the change in hemorheology after SCI are evidence of the relationship between SCI and stasis in the Governor Vessel (Berlly et al., 2007; Furlan et al., 2008). Also, Governor Vessel electroacupuncture (EA) has been proven to prevent secondary damage and to improve neuroprotective effects after SCI (Liu et al., 2011; Juarez Becerril et al., 2015; Wei et al., 2017). A recent meta-analysis showed that combining acupuncture with other therapies had a higher cure rate and effectiveness than acupuncture alone (Deng et al., 2017). Thus, Governor Vessel EA may be an effective adjunctive therapy for SCI.

The upper cervical spine is adjacent to the medulla oblongata, so when the cervical spine is injured, it can affect breathing and be life-threatening. So far, no mature upper cervical SCI animal model has been developed. Thus, there have been fewer studies of upper cervical SCI (Sharifalhosseini et al., 2017).

To identify effective treatment strategies for acute upper SCI, we attempted to combine decompression with EA to treat acute upper SCI. Given the different effects of early and delayed decompression, we investigated the effect of EA combined with early or delayed decompression in rats with acute upper cervical SCI.

Materials and Methods

Animals

A total of 42 female Wistar rats aged 6 months and weighing

280 ± 20 g were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences in Beijing of China (animal license No. SCXK (Jun) 2017-0004). The rats were housed in individual cages at 23 ± 2°C, and allowed free access to food and water. The rats ($n = 42$) were equally and randomly divided into seven groups ($n = 6$ per group): sham, 12-hour SCI, 12-hour EA (SCI + EA), 12-hour methylprednisolone (MP) (SCI + MP), 48-hour SCI, 48-hour EA (SCI + EA), and 48-hour MP (SCI + MP). All experiments were approved by the Institutional Animal Care and Use Committee of the China-Japan Friendship Hospital of China (No. 170102).

Establishment of SCI models

The establishment of an acute upper cervical SCI animal model was based on a previous study of ours (Tan et al., 2016). All rats were anesthetized with pentobarbital (Sigma-Aldrich, St. Louis, MO, USA). The external occipital protuberance was cut longitudinally to expose the atlanto-axial space and the atlanto-occipital space on the right side (Figure 1A). After removal of soft tissue and ligaments above the atlanto-axial space and the atlanto-occipital space, a balloon catheter (SPL25012X, 2.5 mm × 12.0 mm; Medtronic, Inc., Minneapolis, MN, USA) was inserted into the atlanto-occipital space, and the top of a balloon catheter was pulled out from the atlanto-occipital space (Figure 1B) by a balloon catheter compression system (Figure 1C). Finally, the balloon catheter was fixed on the back and head of the rats (Figure 1D). When the rat models were successfully generated 24 hours later, the sham group was non-pressurized; in the other groups, the end of the balloon catheters was connected with manumatic force-pumps (30 atm, Medtronic, Inc.). Iohexol (General Electric Pharmaceutical Co., Ltd., Shanghai, China) was injected into the balloon catheter continuously until the pressure reached 3 bar (1 bar = 100 kPa). Half of the rats maintained the compression for 48 hours and the other half maintained the compression for 12 hours. The balloon catheters were then decompressed and removed. During the model development, all rats were kept in separate cages with free access to food and water at 25 ± 3°C. The standard of model evaluation was spasmodic oscillation of the tail, unilateral limb and body retraction, and unilateral or bilateral paralysis. If there were rat deaths or serious complications during the experiment, we would fill in the rats and make sure that each group had six rats.

EA and decompression

The rats in the sham group, the 12-hour SCI group, and the 48-hour SCI group were not handled again. The rats in the 12-hour EA (SCI + EA) group and the 48-hour EA (SCI + EA) groups were given the EA treatment 2 hours after decompression. The stainless steel acupuncture needles (0.3 mm in diameter, Jiajian Medical Equipment Co., Ltd., Wuxi, China) were inserted at the acupoints *Dazhui* (GV14; between the seventh cervical vertebra and the first thoracic vertebra at the middle of the back) and *Baihui* (GV 20; the center of the parietal bone) to a depth of 3–4 mm (Figure 2). The EA treatment (Hans-200A, Jisheng Medical Technology

Table 1 BBB scores of the groups compressed for 12 and 48 hours

Group	Time after SCI					
	0 hours	24 hours	3 days	7 days	14 days	
Compressed for 48 hours	Sham	21.00±0.00	21.00±0.00	21.00±0.00	21.00±0.00	21.00±0.00
	EA	1.33±0.26	3.33±0.41*	6.583±0.38*	16.67±0.61*	17.17±0.93*
	MP	1.25±0.27	3.00±0.45*	6.17±0.41*	16.25±0.27*	17.33±0.41*
	SCI	1.17±0.26	1.50±0.32**	4.25±0.27**	9.00±0.55**	11.92±0.58**
Compressed for 12 hours	Sham	21.00±0.00	21.00±0.00	21.00±0.00	21.00±0.00	21.00±0.00
	EA	1.33±0.26	3.92±0.38*	10.67±0.52*	17.75±0.52*	17.92±0.49*
	MP	1.25±0.27	3.83±0.61*	10.50±0.63*	17.92±0.59*	18.00±0.45*
	SCI	1.17±0.26	2.42±0.38**	7.00±0.45**	11.00±0.45**	17.33±0.52*

Compressed for 48 hours: $F_{\text{time}} = 3200.61$, $P = 0.00 < 0.05$; $F_{\text{group}} = 3656.30$, $P = 0.00 < 0.05$; $F_{\text{interaction}} = 416.78$, $P = 0.00 < 0.05$. Compressed for 12 hours: $F_{\text{time}} = 4182.29$, $P = 0.00 < 0.05$; $F_{\text{group}} = 7752.59$, $P = 0.00 < 0.05$; $F_{\text{interaction}} = 524.15$, $P = 0.00 < 0.05$. All data are expressed as the mean \pm SD ($n = 6$ per group; repeated measures analysis of variance). Lower BBB scores indicate poorer locomotor function. The sham group was constant and the other three groups had an improved trend at various time points. * $P < 0.05$, vs. sham group, # $P < 0.05$, vs. EA and MP group. EA: Electroacupuncture; MP: methylprednisolone; SCI: spinal cord injury; BBB: Basso, Beattie, and Bresnahan locomotor scale.

Co., Ltd., Nanjing, China) was administered at 2 Hz for 15 minutes each day for 14 consecutive days. The current intensity ranged from 0.6 mA to 1.0 mA. The 12-hour and 48-hour MP groups received 30 mg/kg MP (Pfizer Pharmaceuticals Ltd., New York, NY, USA) *via* the tail vein within 1 hour after decompression, and then at a dose of 5.4 mg/kg per hour once every four hours; all doses were given within 24 hours. All rats were sacrificed after 14 days of treatment. Afterwards, the damaged spinal cord segments were extracted.

Hind limb locomotor function score

Evaluation of motor ability was graded using a 0-21 point scoring system called the Basso, Beattie, and Bresnahan locomotor scale (BBB) (Bhimani et al., 2017), which assesses a combination of rat hind limb movements, trunk position, stability, stepping, coordination, paw placement, toe clearance, and tail position. The score was recorded at 0 and 24 hours, as well as at 3, 7, and 14 days after decompression independently by two outside researchers, and the results were averaged.

Enzyme linked immunosorbent assay (ELISA) for platelet-activating factor concentration in the damaged spinal cords

The platelet-activating factor concentration of damaged spinal cords was measured using a rat ELISA kit (Huamei Biological Engineering Co., Ltd., Wuhan, China). Optical density (OD) values were measured at 450 nm. Platelet-activating factor concentration was calculated using OD values in each group.

Western blot assay for the expression of caspase-9

After caspase-9 protein extraction, protein quantification, and the adjustment of protein concentration, the proteins were subjected to electrophoresis (Mini P-4 electrophoresis chamber; Kaiyuan Xinrui Instrument Co., Ltd., Beijing, China) and then transferred on the membrane. The membrane was incubated with primary GAPDH mouse monoclonal antibodies (ImmunoWay Biotechnology, Newark, DE, USA) for 10 minutes at 4°C overnight, and with secondary IgG/FITC (H + L) horseradish peroxidase antibodies (1:10,000; Kangwei Century Biotechnology Co., Ltd., Beijing, China),

and IgG/TRITC (H + L) horseradish peroxidase antibodies (1:10,000; Kangwei Century Biotechnology Co., Ltd.), with oscillation for 40 minutes at room temperature. Reaction products were visualized using ECL Gel Pro analysis software (Image J, National Institutes of health, Bethesda, MD, USA) to quantify the amount of protein. OD values were normalized to those of β -actin (1:400; Sigma, St. Louis, MO, USA).

Statistical analysis

The data were recorded as the mean \pm SD and analyzed using SPSS 20.0 software (IBM, Armonk, NY, USA). The BBB scores among the groups were compared using repeated measures analysis of variance, while the other data were analyzed using one-way analysis of variance followed by the least significant difference *post hoc* test. A P value of < 0.05 was considered statistically significant.

Results

Effects of decompression combined with EA on hind limb locomotor function in rats with acute upper cervical SCI

BBB scores were significantly lower in the SCI groups compared with the sham group. There was no significant difference in BBB scores among the SCI groups at 0 hours after decompression ($P > 0.05$). For the groups compressed for 12 hours, there were no significant differences in BBB scores at any time point between the 12-hour EA and 12-hour MP groups. BBB scores were lower in the 12-hour SCI group than in the 12-hour EA and 12-hour MP groups at 24 hours, 3 days, and 7 days ($P < 0.05$). Also, BBB scores were similar at 0 and 48 hours between the 12-hour EA and MP groups ($P > 0.05$). For the groups compressed for 48 hours, there were no significant differences in BBB scores at any time point between the 48-hour EA and MP groups. BBB scores at all time points were lower in the 48-hour SCI group than in the 48-hour EA and MP groups ($P < 0.05$; Table 1).

Effects of decompression combined with EA on platelet-activating factor contents in injured tissues of rats with acute upper cervical SCI

ELISA results showed that the trends for changes in platelet-ac-

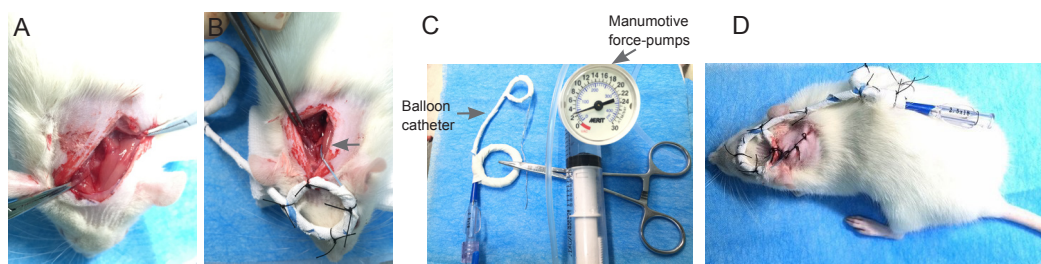


Figure 1 Establishment of a rat model of acute upper cervical spinal cord injury. (A) Exposure of the atlanto-occipital and atlanto-axial space; (B) insertion of balloon catheter (arrow) into the space; (C) balloon catheter compression system; (D) completion of the model.

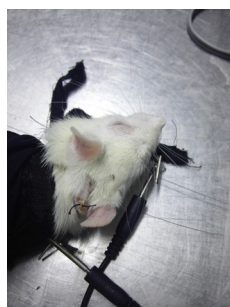


Figure 2 Treatment by Governor Vessel electroacupuncture.

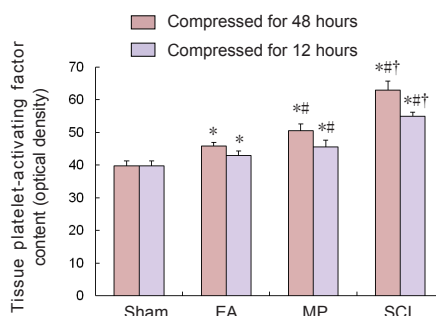


Figure 3 Comparison of tissue platelet-activating factor content in the groups compressed for 12 and 48 hours.

All data are expressed as the mean \pm SD ($n = 6$ per group). * $P < 0.05$, vs. sham group; # $P < 0.05$, vs. EA group; † $P < 0.05$, vs. MP group (one-way analysis of variance followed by the least significant difference *post hoc* test). EA: Electroacupuncture; MP: methylprednisolone; SCI: spinal cord injury.

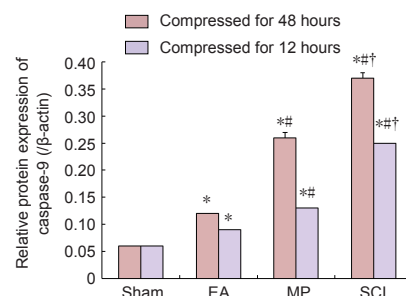
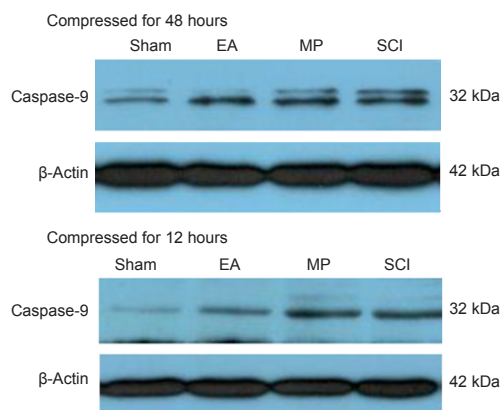


Figure 4 Comparison of caspase-9 protein expression in the groups compressed for 12 and 48 hours (western blot assay).

The caspase-9 expression was lower in the 12-hour and the 48-hour EA groups than in the 12-hour MP and SCI groups, and the 48-hour MP and SCI groups. The caspase-9 expression was lower in the 12-hour and the 48-hour MP groups than in the 12-hour and the 48-hour SCI groups. All data are expressed as the mean \pm SD ($n = 6$ per group). * $P < 0.05$, vs. sham group; # $P < 0.05$, vs. EA group; † $P < 0.05$, vs. MP group (one-way analysis of variance followed by the least significant difference *post hoc* test). EA: Electroacupuncture; MP: methylprednisolone; SCI: spinal cord injury.

tivating factor among groups were similar. The platelet-activating factor contents for the groups were as follows: sham group < EA group < MP group < SCI group ($P < 0.05$; **Figure 3**).

Effects of decompression combined with EA on caspase-9 protein expression in injured spinal cord tissues of rats with acute upper cervical SCI

Western blot assay results showed that the trends for changes in caspase-9 expression among groups were similar. The caspase-9 protein expression in the groups were as follows: sham group < EA group < MP group < SCI group ($P < 0.05$; **Figure 4**).

Discussion

Early decompression has been proven to be more effective in neural functional recovery than delayed decompression following acute SCI. Regarding the pathophysiology, early decompression can more effectively improve neurological function after SCI by inhibiting the expression of tumor necrosis factor α compared with delayed decompression (Xie et al., 2015). Additionally, delayed decompression can exac-

erbate reperfusion injury and is associated with ongoing enhanced levels of cytokine expression, microglial activation, and astrogliosis (Vidal et al., 2017). Decompression can only remove the insult above the spinal cord. The pathological lesion cannot be completely cured, and thus adjuvant treatment is necessary. In light of the different therapeutic effects of early and delayed decompression, different adjuvant treatments are necessary.

In the traditional Chinese medicine approach, SCI is thought to be strongly associated with stasis of the Governor Vessels. The disturbance of the microcirculation after SCI is an important link between neurological injury, cardiovascular complications, and the change in hemodynamics after SCI (Sezer et al., 2015), which are consistent with stasis of the Governor Vessels in traditional Chinese medicine. Governor Vessel EA can be used at the lesion site to facilitate the recovery of nerve function. This study investigated the effect of early and delayed decompression combined with EA after acute SCI from the standpoint of recovering neural function, improving spinal microenvironment, and inhibiting apoptosis.

In this study, in the groups compressed for 12 hours, BBB

scores showed that the recovery of neural function was fast in the EA and MP groups. However, the final recovery of the three groups was similar. For the groups compressed for 48 hours, BBB scores showed that the recovery of neural function was fast and the final recovery was good in the EA and MP groups; moreover, the final recovery was similar among the three groups. Therefore, for decompression at 12 hours, EA and MP treatment can promote the recovery of motor function, but may not make a difference in final functional recovery. For decompression at 48 hours, EA and MP treatment can promote the recovery of motor function and improve the final functional recovery.

Platelet-activating factor is a soluble phospholipid metabolite that can cause the platelet to be activated, released, and aggregated. Platelet-activating factor has been proven to be an important pathological factor for secondary damage after SCI. Once SCI occurs, the platelet-activating factor content will rise rapidly in the SCI tissue, and prompt microvascular thrombosis, vascular endothelial cell damage, increased permeability of blood vessel walls, and blood-spinal cord barrier disruption, thereby altering the microenvironment of the injured spinal cord (Lindsberg et al., 1990; Faden et al., 1992; Xiao et al., 1996). Platelet-activating factor can promote platelet activation by strengthening the expression of adhesion molecule on the surface of platelets. The activation of platelets can combine and activate leukocytes, releasing a large number of oxygen free radicals, interleukins, and tumor necrosis factor- α , with inflammatory infiltration occurring around the injured spinal cord tissue (Guo et al., 2005). In this study, for the groups compressed for 12 and 48 hours, the change was similar; in other words, platelet-activating factor content was decreased in the EA group compared with the MP and SCI groups. These results confirm that EA can strongly improve the microenvironment of the injured spinal cord, and the EA group had a better long-term outcome than the MP and SCI groups.

Previous studies have found that the death of the spinal cord neurons after SCI occurs mainly *via* apoptosis (Springer et al., 1999; Zhang et al., 2012). Permanent or long-term loss of neural function caused by SCI is associated with spinal cord edema, degeneration, necrosis, and apoptosis of neurons and oligodendrocytes. SCI-induced apoptotic cell death of neurons and oligodendrocytes has been shown to cause progressive degeneration of the spinal cord, leading to permanent functional deficits (Moon et al., 2012). The key link in cell apoptosis is the cleavage of related cysteine protease proteins (Park et al., 2012). Among them, caspase-9 is an initiator of the caspase apoptotic protease. When caspase-9 is activated, it can activate the downstream protease cascade, including caspase-3, which can act on the poly (ADP-ribose) polymerase and lead to apoptosis, finally causing cell death (Eldadah et al., 2000). Therefore, caspase-9 is an important mediator of cell apoptosis. Inactivating caspase-9 can inhibit apoptosis and promote the recovery of nerve function. In the present study, for the groups compressed for 12 and 48 hours, there were similar changes; in other words, there was greater inactivation of caspase-9 in the EA group than in the MP and

SCI groups. This result proves that EA can more strongly promote the recovery of neural function and may lead to a better long-term outcome than observed in the MP and SCI groups.

Our results showed that the platelet-activating factor contents of the 12-hour EA group were lower than in the 12-hour SCI group, and the caspase-9 expression of the 12-hour EA group was lower than in the 12-hour SCI group. Thus, why were there no significant differences in BBB scores at 14 days between the 12-hour EA and the 12-hour SCI groups? The authors believe that the reasons are as follows. First, we can conclude that the EA group had a faster recovery than the 12-hour SCI group, which was the effect of EA, but the short duration of compression and the strong ability of the rats to recover may have been the primary reasons for this. Second, spinal cord decompression may have some beneficial effects that have not yet been identified. For example, over time, the levels of substance P, a potential anti-inflammatory modulator used to treat injury-induced inflammatory central nervous system disorders (Jiang et al., 2012), constantly change after decompression in SCI patients (Da et al., 2017), which may influence recovery. Third, acute SCI is an extremely complex physiological process, so we cannot exclude factors that we have not studied yet that may influence recovery.

Our study focused on acute upper cervical SCI. Given the high mortality and instability of the previous upper cervical SCI animal models, therapeutic and mechanistic studies of acute upper cervical SCI have been limited, and our model solved the problem of high mortality and instability and enriched this academic field. Early and late decompression leads to different outcomes following acute upper cervical SCI. We chose two time points to intervene with Governor Vessel EA, which made the study systematic and integrated. Meanwhile, the syndrome differentiation and treatment theory of traditional Chinese medicine was reflected.

The limitation of this study was primarily the small sample size, which may have increased error bias and influenced the results. Besides, the mechanism of secondary injury includes not only changes in the microenvironment of the spinal cord and apoptosis, but also the axonal regeneration barrier (Tran, et al., 2018). Our follow-up studies will assess different factors associated with acute upper cervical SCI.

In summary, EA was effective for SCI, and delayed decompression combined with EA was a more effective treatment than decompression alone. Early decompression combined with EA promoted the recovery of motor function more effectively than decompression alone. However, whether it has a favorable final effect requires additional study.

Author contributions: YLW and YNQ performed the experiments and wrote the manuscript. WW and PY designed and supervised the study. FY and XST analyzed the data and provided technical support. MST planned the overall and provided critical revision of the manuscript for intellectual content. All authors approved the final version of the paper.

Conflicts of interest: The authors have no conflicts of interest to declare.

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