● RESEARCH ARTICLE

"Three Methods and Three Points" regulates p38 mitogen-activated protein kinase in the dorsal horn of the spinal cord in a rat model of sciatic nerve injury

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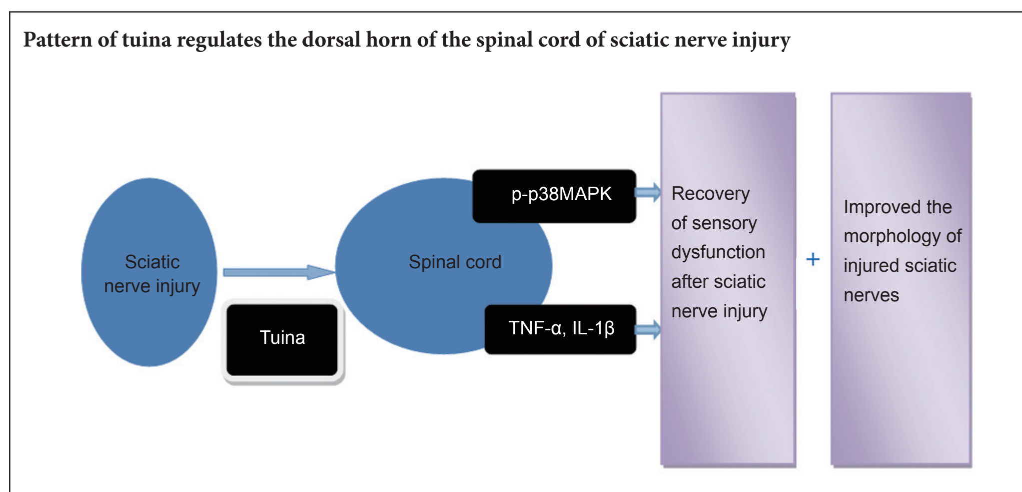
Graphical Abstract



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Abstract

Tuina is a traditional Chinese treatment for sensory disturbances caused by peripheral nerve injury and related diseases. Our previous studies showed that tuina regulates relevant regions and indices of the spinal dorsal horn using the *Dian*, *Bo*, and *Rou* method in *Yinmen* (BL37), *Yanglingquan* (GB34), and *Weizhong* (BL40). Treatment prevents muscle atrophy, protects spinal cord neurons, and promotes sciatic nerve repair. The mechanisms of action of tuina for treating peripheral nerve injury remain poorly understood. This study established rat models of sciatic nerve injury using the crushing method. Rats received Chinese tuina in accordance with the principle of "Three Methods and Three Points," once daily for 20 days. Tuina intervention reduced paw withdrawal latency and improved wet weight of the gastrocnemius muscle, as well as promoting morphological recovery of sciatic nerve fibers, Schwann cells, and axons. The protein expression levels of phospho-p38 mitogen-activated protein kinase, tumor necrosis factor-α, and interleukin-1β also decreased. These findings indicate that "Three Methods and Three Points" promoted morphological recovery and improved behavior of rats with peripheral nerve injury.

Key Words: *nerve regeneration; tuina; Three Methods and Three Points; phospho-p38 mitogen-activated protein kinase; sciatic nerve injury; tumor necrosis factor-α; interleukin-1β; dorsal horn of the spinal cord; neural regeneration*

Introduction

Peripheral nerve injury, a common clinical disease, is one of the leading causes of disability. Sciatic nerve injury is a typical form of peripheral nerve injury. When peripheral nerve injury occurs, the muscle (Tuffaha et al., 2015, 2016) and tissue (Nishihara et al., 2015; Bozkurt et al., 2016) innervated by the injured nerve may suffer from pain (Goswami et al., 2016), numbness (Chowdhry et al., 2015; Jang et al., 2016), and other sensory dysfunctions. New research shows that sciatic nerve injury can lead to increased levels of p38 mito-

gen-activated protein kinase (p38MAPK) phosphorylation in the dorsal horn of the spinal cord (Xu et al., 2012; Zhou et al., 2014a), thus promoting synthesis of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the spinal cord, which is closely related to sciatic nerve injury (Zhong et al., 2012; Zhao, 2014). p38MAPK phosphorylation in the dorsal horn of the spinal cord is a key factor in pathogenesis of peripheral nerve injury and sensory dysfunction. Therefore, regulation of p38MAPK phosphorylation in microglia using external interventions is of great significance for recovery of sensory dysfunction in peripheral nerve injury.

As a representative form of both naturopathy and physical therapy, Chinese tuina, which was originally termed massage or anqiao at the time of its inception 2000 years ago (Xu, 2013), is one of the earliest treatment methods found in the practice of clinical Chinese medicine. Tuina is a therapeutic modality guided by the theory of Chinese medicine and utilizes massage manipulations applied to certain parts or points on the patient's body *via* either hand manipulations or massage implements (Yu, 2015). This treatment method is widely used to treat many diseases (Hu et al., 2012). In China, tuina has been and continues to be a common method used to treat sensory dysfunction and related diseases caused by peripheral nerve injury, including cervical spondylosis (Wen et al., 2015; Hu and Wang, 2016) and prolapse of the lumbar intervertebral disc (Wang and Yang, 2015; Chen et al., 2016). The treatment method is well established and has been applied widely to treat many clinical conditions (Shen et al., 2015; Xu et al., 2016). Our previous studies found that tuina improved behavioral indicators in rats with injured sciatic nerves and prevented muscle atrophy, thus protecting spinal cord neurons promoting sciatic nerve repair (Gao et al., 2013; Yao et al., 2013). "Three Methods and Three Points," which was invented by Professor Yu at the College of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, China, utilizes the most commonly used methods of tuina, *Dian*, *Bo*, and *Rou*, which act on the three most commonly used acupuncture points—*Yinmen* (BL37), *Weizhong* (BL40), and *Yanglingquan* (GB34)—to treat peripheral nerve injury (Lu, 2016). Results showed that tuina usage of the *Dian*, *Bo*, and *Rou* method in *Yinmen* and *Yanglingquan* regulated nerve growth factor, p75 neurotrophin receptor ($p75^{NTR}$), TrkA (Mei et al., 2013b), TrkC, NT-3, MAP-2, and NF-M (Gao et al., 2014) in the dorsal horn of the spinal cord, and peripheral nerve injury repair was strongly associated with the spinal cord. Quantitative research on the effect of tuina on p38MAPK phosphorylation in the dorsal horn has not yet been reported, and this is likely because most studies report on how tuina therapy affects peripheral nerve injury in a clinical setting. Hence, the aim of this study was to determine whether p38MAPK in the dorsal horn was affected by tuina therapy using "Three Methods and Three Points" after nerve crush injury.

Materials and Methods

Group assignment

The protocols were conducted in compliance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the National Institute of Health. All experimental procedures were approved by the Medical and Experimental Animal Ethics Committee at Beijing University of Chinese Medicine (BUCM-3-20151202-4001). Sixty-four male specific pathogen-free Sprague-Dawley rats (Adamas Beifu, Beijing, China; SCXK (Jing) 2011-0004) aged 6–7 weeks and weighing 200 ± 10 g were raised at 23 ± 2°C and 45% humidity, with a 12-hour light/dark cycle (lights were turned on at 8:00 a.m.) and allowed free access to food and water. All interventions on the various groups were performed between 8:00 a.m. and 12:00 a.m. The number of rats used and their discomfort were minimized as much as possible.

The rats were randomly divided into four groups: 16 rats for the sham-operated group, and 48 rats for sciatic nerve crush injury intervention, including: (1) 16 model rats requiring no intervention; (2) 16 model rats as control group given bound control (Tie the small board to the right lower limb of the rat with a rope) once a day (9 minutes/day) for 20 days at 7 days post-surgery; and (3) 16 rats in the tuina group that received tuina therapy of "Three Methods and Three Points" once daily (9 min/d) for 20 days at 7 days post-surgery.

Establishment of sciatic nerve injury models

Fasting and water deprivation were conducted for 24 hours prior to surgery. The rats were intraperitoneally anesthetized using a premixed solution containing 10% chloral hydrate (350 mg/kg body weight). The right lateral thigh was shaved and the skin was disinfected with 10% povidone iodine. The right sciatic nerve was exposed using the gluteal-splitting approach (Lu et al., 2015). According to Sunderland's classification (Sunderland, 1951) of peripheral nerve injury and calculations of pressure intensity, sciatic nerves at the mid-thigh level were exposed and crushed using a pair of non-serrated forceps for 30 seconds. Subsequently, the skin was sutured with four stitches. Thus, grade III nerve injury was established (Wu, 2014). The rats fasted for 24 hours postoperatively, but were allowed free access to water. Gluteal-splitting without damaging the sciatic nerve was performed in the sham-operated group.

Tuina treatment

In 2007, a tuina manipulation emulator (patent No. ZL200710187403.1) (**Figure 1**) was designed by our team. The manipulator was designed to stimulate tuina techniques, yet at the same time to also maintain qualitative and quantitative control. The essential structural elements of the emulator include a contact point, disc, and stepper motor. A metallic strip connects the pressure sensor and the contact point to ensure sensor sensitivity and avoid abrasion due to long-term use. A lead screw was used to adjust the amount of pressure applied at the contact point, which was displayed on the control screen. The contact point was a 10-mm diameter cylinder. According to the "Three Methods and Three Points," the emulator was used to perform the *Dian*, *Bo*, and *Rou* method on *Yinmen*, *Weizhong*, and *Yanglingquan* (Wu et al., 2013; Deng et al., 2016) sequentially on the affected side, thereby using three different tuina

methods at three acupuncture points. A frequency of 30 times/minute and a force of 0.98 N were selected. Each tuina method was applied for 1 minute on each acupuncture point, respectively. Tuina treatment was administered once daily for 20 days. Based on the principle of comparative anatomy, *Yinmen* is located on the back of the thigh, 3/7 down the line connecting the midpoint of the buttocks fold and center of the popliteal fossa. *Weizhong* is located in the center of the crease of the popliteal fossa. *Yanglingquan* is located in the depression anterior and inferior to the fibular capitulum.

Solar-thermal pain threshold

According to the preliminary study, 7 days after model establishment, and 20 days after massage, thermal pain tolerance began to recover. Therefore, treatment was performed for 7 days after modeling, with an intervention period of 20 days. In other words, 27 days after modeling, the tests were conducted. At 7 and 27 days after sciatic nerve injury, eight rats from each group were evaluated for pain recovery and temperature sensations. The test was individually administered using the following steps: (1) the rat was placed into the detection box of the PL-200 thermal sensitivity apparatus (Chengdu Taimeng Technology Co., Ltd., Chengdu, Sichuan Province, China); (2) timing began: the rats were allowed to adapt to the environment, then the infrared light source was placed on the lower 1/3 of the rat's rear paw, and the start button was pressed; (3) timing stopped: the timer automatically stopped when the rat spontaneously lifted its paw, and the time was recorded as the paw withdrawal latency. The laboratory surroundings were kept quiet throughout testing, and the room temperature remained stable, between 20°C and 25°C. The cut-off time was set to 21 seconds to prevent thermal burns.

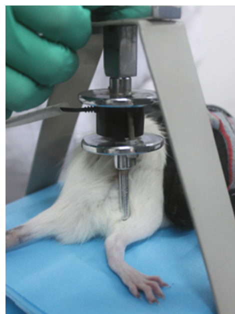

Figure 1 Tuina manipulation emulator.

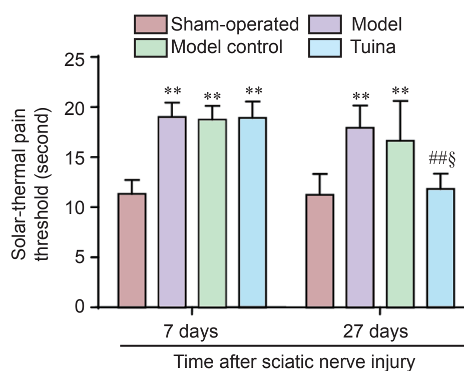

Figure 2 Effects of tuina treatment on the solar-thermal pain threshold in a rat model of sciatic nerve injury.
The solar-thermal pain threshold was expressed as paw withdrawal latency. Data are expressed as the mean ± SEM (n = 8; one-way analysis of variance and *post hoc* least significant difference test). $**P < 0.01$, *vs.* sham-operated group; $\#\#P < 0.01$, *vs.* model group; $\S P < 0.05$, *vs.* model control group.

Quantification of muscle atrophy

Gastrocnemius atrophy is a common symptom of sciatic nerve injury due to muscle denervation; the degree of muscle atrophy depends on injury severity and speed of nerve recovery (Wu et al., 2015; Tuffaha et al., 2016). At 7 and 27 days after sciatic nerve injury, the recovery rate of muscle wet weight was evaluated in eight rats from each group. Briefly, fasting and water deprivation were performed for 24 hours prior to surgery. The rats were intraperitoneally anesthetized with a premixed solution containing 10% chloral hydrate (350 mg/kg body weight). The right lateral thigh area was shaved and the skin was disinfected with 10% povidone iodine. The right and left gastrocnemius muscle was exposed through the crural-splitting approach. The recovery rate of muscle wet weight was defined by muscle weight of the experimental side divided by muscle weight of the control side (Jiang et al., 2016).

Morphological observation

At 7 and 27 days after sciatic nerve injury, morphology of the sciatic nerve was observed in eight rats from each group. After anesthesia with chloral hydrate and intracardial perfusion with normal saline, sciatic nerves were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 hours. The sciatic nerves were washed fully with water, dehydrated through a graded alcohol series, permeabilized with xylene, and embedded in paraffin. The sciatic nerve was perpendicular to the long axis of the nerve transverse section, and then stained with hematoxylin and eosin. Morphology of the sciatic nerve was observed using a light microscope (Motic, Xiamen, Fujian Province, China).

Western blot assay

At 7 and 27 days after sciatic nerve injury, four rats from

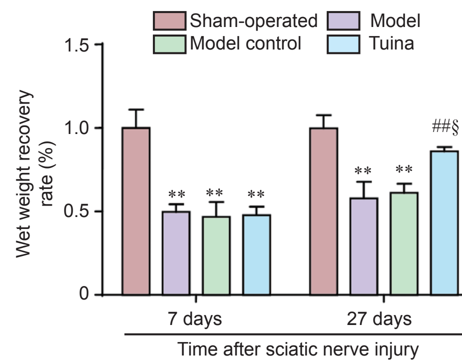

Figure 3 Effects of tuina treatment on the recovery rate of wet weight of gastrocnemius muscle in a rat model of sciatic nerve injury.
The recovery rate of muscle wet weight is defined by muscle weight on the experimental side divided by muscle weight on the control side. Data are expressed as the mean ± SEM (n = 8; one-way analysis of variance and *post hoc* least significant difference test). $**P < 0.01$, *vs.* sham-operated group; $\#\#P < 0.01$, *vs.* model group; $\S P < 0.05$, *vs.* model control group.

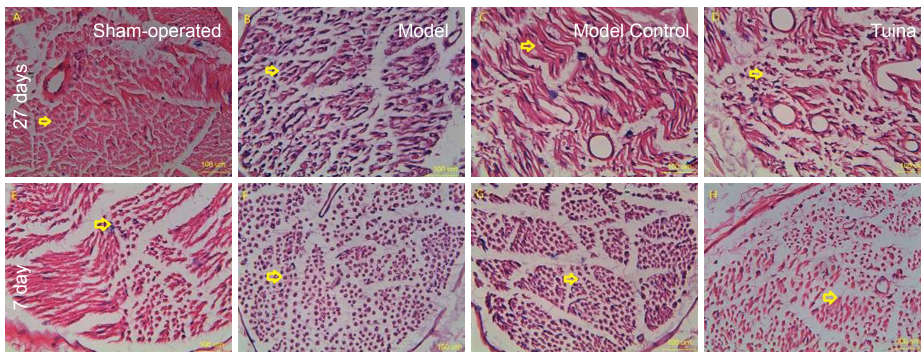

Figure 4 Effects of tuina treatment on morphology of injured sciatic nerves of rats at 7 days and 27 days after sciatic nerve injury (hematoxylin-eosin staining, × 400).
Arrows show the myelin sheath and axon. Scale bars: 100 μm.

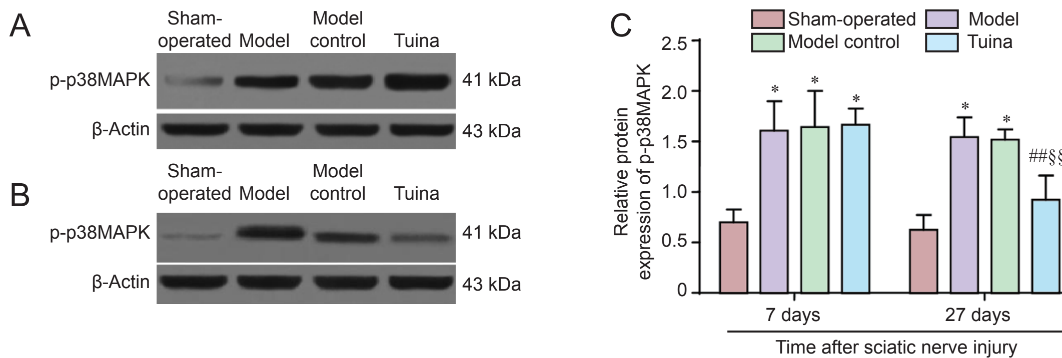

Figure 5 Effects of tuina treatment on p-p38MAPK in the dorsal horn of the spinal cord in a rat model of sciatic nerve injury.
(A, B) Western blots of p-p38MAPK on day 0 (A) and day 20 (B). (C) Quantification of p-p38MAPK protein expressions (western blot assay). The p-p38MAPK protein expressions were expressed as a ratio of integrated optical density to β-actin. Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance and *post hoc* least significant difference test). *P < 0.05, *vs*. sham-operated group; ##P < 0.01, *vs*. model group; §§P < 0.01, *vs*. model control group. p38MAPK: p38 mitogen activated protein kinase; p-p38MAPK: phospho-p38MAPK.

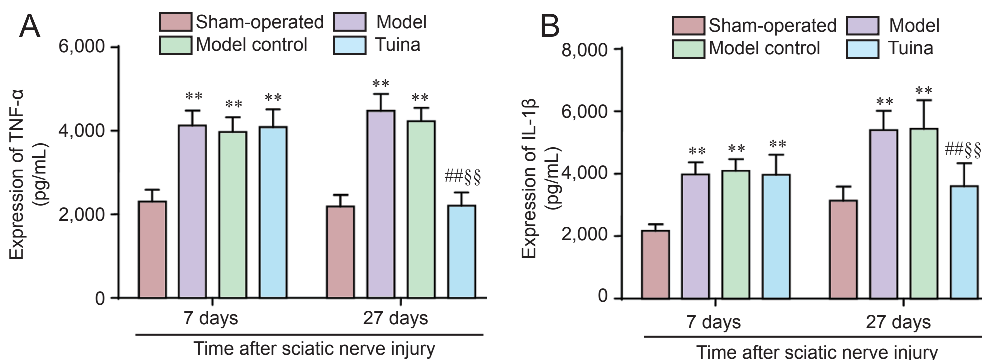

Figure 6 Effects of tuina treatment on TNF-α (A) and IL-1β (B) in the spinal cord of rats with injured sciatic nerves (enzyme-linked immunosorbent assay).
Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance and *post hoc* least significant difference test). **P < 0.01, *vs*. sham-operated group; ##P < 0.01, *vs*. model group; §§P < 0.01, *vs*. model control group. TNF-α: Tumor necrosis factor-α; IL-1β: interleukin-1β.

each group were used for western blot analysis of phospho-p38MAPK (p-p38MAPK) expression. The rats were anesthetized with chloral hydrate. After taking a blood sample from the abdominal aorta, the injured side of the lumbar spinal cord was extracted ($L_{4–6}$) on an iced tray. The specimen was then placed in a tub of ice. The spinal cord was equally divided into two coronal parts. The inferior half of the spinal cord, namely the dorsal horn, was placed in liquid nitrogen for preservation (Shao et al., 2014). The different groups of cellular proteins were extracted, and the protein concentration was determined using the Coomassie brilliant blue assay. Each well was filled with 50 μg of protein sample. The samples were then separated on a sodium dodecyl sulfate-polyacrylamide gel by electrophoresis. Separated proteins in the gels were electrophoretically transferred onto polyvinylidene fluoride membranes at a constant voltage of 100 V at 4°C. The membranes were blocked in 6% skimmed milk powder for 2 hours, and then incubated with goat anti-rat p-p38MAPK monoclonal antibody (1:1,000; No. ab38238, Abcam, Cambridge, UK) and goat anti-rat β-actin monoclonal antibody (1:2,000; No. AC001-M, Santa Cruz Biotechnology, Santa, Cruz, CA, USA) at 4°C overnight. Afterwards, the membranes were washed with phosphate-buffered saline, and incubated with rabbit anti-goat horseradish peroxidase-IgG (1:5,000; No. SH-0031, Beijing, China) at room temperature for 1 hour. The membranes were then detected using enhanced chemiluminescence (ECL-0012, Pierce, FL, USA), X-ray exposure imaging was performed using a scanning analysis software system (Labworks™ Analysis Software, ProteinSimple, Silicon Valley, CA, USA). β-Actin served as the standard reference.

Relative protein expression levels were expressed as the integrated optical density ratio of each target protein to β-actin.

Enzyme-linked immunosorbent assay (ELISA)

At 7 and 27 days after sciatic nerve injury, four rats from each group were used for ELISA analysis of TNF-α and IL-1β protein expression in the spinal cord. After sacrifice by blood-letting through the abdominal aorta, the injured side of the lumbar spinal cord (L_{4-6}) was extracted on an iced tray. The ELISA kit was used to detect expression levels of TNF-α (No. CSB-E11987r, CUSABIO, Wuhan, Hubei Province, China) and IL-1β (No. CSB-E08055r, CUSABIO) in the L_{4-6} spinal segment.

Statistical analysis

Data, expressed as the mean ± standard error of mean, were analyzed using SPSS 23.0 software (SPSS Inc, Chicago, IL, USA). One-way analysis of variance and *post hoc* least significant difference test were used to analyze data that obeyed normal distribution and homogeneity of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of tuina treatment on the solar-thermal pain threshold in a rat model of sciatic nerve injury

As shown in **Figure 2**, prior to intervention, a significant increase in paw withdrawal latency was detected in the sciatic nerves ($P < 0.01$, *vs*. sham-operated group). On day 20 post-intervention, the paw withdrawal latency of the tuina group was significantly decreased compared with the model ($P < 0.01$) and model control group ($P < 0.05$). Paw withdrawal latency of the tuina group was similar to the sham-operated group ($P > 0.05$).

Effects of tuina treatment on the gastrocnemius muscle atrophy in a rat model of sciatic nerve injury

As shown in **Figure 3**, prior to intervention, a significant decrease was found in the recovery rate of wet weight of gastrocnemius muscle subjected to sciatic nerve crush injury ($P < 0.01$, *vs*. sham-operated group). On day 20 post-intervention, the recovery rate of wet weight of gastrocnemius muscle was significantly increased in the tuina group compared with the model and model control groups ($P < 0.01$).

Effects of tuina treatment on the morphology in a rat model of sciatic nerve injury

As shown in **Figure 4**, prior to intervention, the myelin sheath and axon were observed under a microscope in the sham-operated group; scattered myelin and axonal collapse were visible in the model, model control, and tuina groups. On day 20 post-intervention, the integrated structures of nerve fibers, axons, and Schwann cells were observed under a microscope in the sham-operated group. Disintegrated axis, broken myelin sheaths, and many Schwann cells were observed in the model and model control groups. The sciatic nerves were essentially normal, orderly, clear, and complete; nerve fiber axons and recovery of Schwann cells were observed in the tuina group.

Effects of tuina treatment on p-p38MAPK, TNF-α and IL-1β in in a rat model of sciatic nerve injury

The results of western blot assay showed a significant increase in p-p38MAPK in the model, model control, and tuina groups on day 0 post-intervention ($P < 0.05$, *vs*. sham-operated group). On day 20 post-intervention, p-p38MAPK values in the tuina group were significantly decreased compared with the model and model control groups ($P < 0.01$), whereas values in the tuina group were similar to the sham-operated group ($P > 0.05$; **Figure 5**).

As shown in **Figure 6**, prior to intervention, a significant increase in TNF-α and IL-1β levels was detected in the sciatic nerves with sciatic nerve crush injury ($P < 0.01$, *vs*. sham-operated group). On day 20 post-intervention, TNF-α and IL-1β levels were significantly decreased in the tuina group compared with the model and model control groups ($P < 0.01$). These values were similar between the tuina group and the sham-operated group ($P > 0.05$).

Discussion

Recent studies have found that a phenotypic change in glial cells plays a key role in transmission of information along the entire nociceptive pathway. p38MAPK is mainly expressed in microglia in the dorsal horn of the spinal cord, and peripheral nerve injury can result in increased p38MAPK phosphorylation in the dorsal horn (Zhou et al., 2014b; Tatsumi et al., 2015). Previous studies of peripheral nerve injury treatment with tuina have shown that tuina regulates nerve growth factor and its related receptors in dorsal horn neurons and C fibers in the superficial layers of the spinal cord to promote repair of peripheral nerve injury (Mei et al., 2013a). These findings provide evidence that tuina regulates relevant regions and indices of the dorsal horn, as well as promotes recovery of sensory dysfunction in rats with injured sciatic nerve.

Sciatic nerve injury is a common standard and well-established peripheral nerve injury model to investigate the impact of different treatments in neural injury repair (Ma et al., 2013). This method is relatively inexpensive and easy to perform; the capacity for repairing this type of injury is equivalent in rats and subhuman primates (Marcolino et al., 2013). According to Chinese medicine, sciatic nerve injury is classified as a form of arthromyodynia, and is usually ascribed to meridian obstruction. Professor Yu innovated the "acupoint-nerve-muscle" theory based on many years of clinical practice and laboratory research, and this theory was used to select acupoints for the present study (Pan et al., 2015). Based on the "Three Methods and Three Points," *Yinmen*, *Weizhong*, and *Yanglingquan* are located along three nerves: the sciatic, common peroneal nerve, and tibial nerve, respectively. The muscular locations of the three acupoints are the biceps femoris, semitendinosus, and anterior tibial muscle, respectively. Based on channel theory of traditional Chinese medicine, *Yinmen* and *Weizhong* belong to the *Taiyang* Bladder Foot Channel. *Yanglingquan* is located on the *Shaoyang* Gallbladder Foot Meridian. Therefore, the tuina manipulations based on the "Three Methods and Three

Points" were designed to stimulate the anatomical structures of the meridians as a scientific basis for treatment.

To further determine whether Chinese tuina has an impact on p38MAPK in microglia, we evaluated the influence of Chinese tuina therapy on rehabilitation of sensory dysfunction post-sciatic nerve injury, specifically p38MAPK phosphorylation and the inhibitory effect of tuina on phosphorylation of p38MAPK, TNF-α, and IL-1β in the dorsal horn. The primary finding of the present study is that Chinese tuina significantly improved recovery of sensory dysfunction caused by peripheral nerve injury, as well as modified expression of p-p38MAPK, TNF-α, and IL-1β in a rat model of sciatic nerve injury.

Since the introduction of the solar heat pain threshold measurement (Fruhstorfer et al., 1976), it has become a mainstay in recovery assessments of sensory function post-sciatic nerve injury. This study shows that tuina intervention of "Three Methods and Three Points" can improve paw withdrawal latency and the gastrocnemius muscle in the hind limbs of rats with an injured sciatic nerve, as well as contribute to nociceptive recovery and promote recovery of sensory dysfunction and muscle atrophy after peripheral nerve injury. Moreover, the "Three Methods and Three Points" can also promote morphological recovery of sciatic nerve fibers, Schwann cells, and axons, maintain the relative integrity of the sciatic nerve, promote recovery of sensory dysfunction after peripheral nerve injury, and provide behavioral recovery to a certain extent.

The regulation of p38MAPK in the dorsal horn of the spinal cord by endogenous substances or related treatments (Ostenfeld et al., 2013; Wang et al., 2016), can inhibit the phosphorylation of p38MAPK (Moon et al., 2013; Taves et al., 2016), and thereby affect TNF-α and IL-1β in the dorsal horn of the spinal cord (Kato et al., 2013; Berta et al., 2016). Inhibition of p38MAPK phosphorylation is therefore a possible therapeutic strategy to treat sensory dysfunction after peripheral nerve injury.

To further explore whether tuina influences p38MAPK phosphorylation, TNF-α, and IL-1β in the dorsal horn of the spinal cord, we performed tuina intervention based on the "Three Methods and Three Points" in sciatic nerve injury rats. In this study, p-p38MAPK expression increased in the sciatic nerve injury groups, as evidenced by increased levels on day 0 post-treatment. These results showed that p38MAPK activation in the dorsal horn led to increased p38MAPK phosphorylation in the dorsal horn of the spinal cord after sciatic nerve injury, thereby promoting production of inflammatory factors and triggering increased TNF-α and IL-1β levels in the spinal cord. In addition to axonal disintegration and Schwann cell proliferation, p38MAPK was also involved in the formation and development of sensory dysfunction after peripheral nerve injury. Tuina treatment reduced expression of p-p38MAPK, TNF-α, and IL-1β, indicating decreased p38MAPK phosphorylation and production of inflammatory factors. Tuina treatment using the "Three Methods and Three Points" induced recovery of sciatic nerve fibers, Schwann cells, and axons, and reduced peripheral nerve injury, thereby promoting recovery of sensory function.

The use of an animal model in the present study presented some notable limitations. The tuina treatment process could cause stress and panic in the rats, which could influence treatment efficacy. Additionally, owing to the individual differences in size and sensitivity between animal subjects, both the force and duration of each tuina manipulation was accordingly changed. The third limitation in the present study concerned the number of animals used in the study; p-p38MAPK, TNF-α, and IL-1β expression levels were only analyzed on day 0 and 20 post-sciatic nerve injury. Consequently, levels of p-p38MAPK, TNF-α and IL-1β at intermediary times were not measured, and it is therefore impossible to determine the intermediate rate of change.

In summary, p38MAPK in the spinal cord is involved in signal transduction of sensory dysfunction following peripheral nerve injury, which greatly influences the production of TNF-α and IL-1β, which is central to hyperalgesia maintenance in a rat model of sciatic nerve injury. One of the mechanisms of tuina in treating peripheral nerve injury using the "Three Methods and Three Points" involves the regulation of p-p38MAPK expression in the dorsal horn, which subsequently inhibits TNF-α and IL-1β expression and promotes recovery of behavior and morphology after peripheral nerve injury. These results provide a scientific basis for the use of tuina in the clinical treatment of peripheral nerve injury.

Author contributions: *XG participated in design and performance of the main experiments. TYY was responsible for study design and guidance. SW modified language throughout the entire article. CM and YHT took charge of functional evaluation. SW and CY took charge of immunohistochemistry. JLL and TTL were responsible for perfusion and tissue processing. MQL provided the instruction for modeling. WDJ helped with data analysis. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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