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Original Article

Baicalein attenuates neuropathic pain and improves sciatic nerve function recovery in rats with partial sciatic nerve transection

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Abstract

Background: Modulating the inflammatory response to nerve injury may provide therapeutic opportunities by aborting the neurobiological alterations that support the development of persistent pain. Baicalein, a 12-lipoxygenase inhibitor, has anti-inflammation properties. It also demonstrates anti-inflammatory functions by inhibiting lipopolysaccharide-induced barrier disruption, expression of cell adhesion molecules, and adhesion and migration of leukocytes. The aim of the present study was to assess the possibility of early treatment of neuropathic pain via the systemic injection of baicalein in rats with left partial sciatic nerve transection (PST).

Methods: Wistar rats were divided into Sham, Vehicle, and Baicalein groups. The Vehicle and Baicalein rats underwent PST, whereas the Sham rats were not transected. Baicalein was administered 20 mg/kg/day intraperitoneally for 7 days after PST and after behaviour tests. After PST, rats developed mechanical and cold allodynia, and impaired sciatic nerve function.

Results: Baicalein attenuated mechanical and cold allodynia and improved sciatic nerve function after PST. Baicalein significantly inhibited the expression of tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-1β on days 14 and 28, and attenuated the activation of astrocytes in the L4–5 spinal cord less than day 28 after PST.

Conclusion: Our study revealed that early and multiple doses of systemic baicalein attenuated neuropathic pain and improved sciatic nerve function by inhibiting pro-inflammatory cytokine expression and attenuating the activation of astrocytes in the spinal cord.

Keywords: Astrocytes; Baicalein; Cytokines; Neuropathic pain; Sciatic nerve function

1. Introduction

Injury of a peripheral nerve may lead to neuropathic pain. Neuropathic pain management currently aims only at reducing symptoms, generally by suppressing neuronal activity. For many patients with neuropathic pain, these treatment modalities are ineffective or not well tolerated because of their side effects. Continued research into the underlying mechanisms and understanding of neuropathic pain are required to address this unmet medical need among patients with neuropathic pain.[1]

Neuropathic pain, which is characterized by hyperalgesia, allodynia, and spontaneous pain, is usually accompanied by peripheral nervous system (PNS) and central nervous system (CNS) damage or dysfunction. Tissue injury results in

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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inflammatory pain (hyperalgesia or allodynia), which is probably the symptom that most commonly leads patients to seek medical attention.[2] Sensitization of primary afferent nociceptors via the direct action of inflammatory mediators on their peripheral terminals is thought to be a mechanism that contributes to mechanical allodynia.[3] Several inflammatory mediators have been demonstrated to act directly on primary afferent nociceptors, to sensitize them and produce mechanical allodynia.[4] Nerve injury leads to the rapid release of pain-related mediators, such as the tumour necrosis factor α (TNF-α), interleukin (IL-1β), IL-6, and prostaglandins, resulting in inflammatory and immune responses, sensitization of the CNS, and facilitation of pain processing.[5]

Many researchers have found that baicalein, a chemical, has anti-inflammation properties.[6,7] Five extracts (n-hexane, chloroform, ethyl acetate, n-butanol, and water) of *Scutellaria rivularis* Benth were evaluated for their anti-inflammatory activity against carrageenan-induced paw oedema in rats and compared with indomethacin.[8] The results indicated that the chloroform extract proved to be the most effective in all of the extracts and baicalein was the major component of the chloroform extracts.[8]

The aim of this study was to assess the possibility of treating neuropathic pain and its underlying mechanisms via the systemic injection of baicalein after left partial sciatic nerve transection (PST) in rats.

2. Methods

This study protocol was reviewed and approved by the Animal Care and Use Committee of the National Defense Medical Center and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The code number of the ethic committee approval: IACUC-08-170, IACUC-10-127, and IACUC-11-178. Male Wistar rats (200–250 g) were housed individually with soft bedding on a 12 h night/day cycle with free access to food and water at all times. All efforts were made to minimize the number of animals used and their suffering.

2.1. Partial sciatic nerve transection neuropathic pain animal model

Under 2%–2.5% isoflurane anaesthesia, all rats underwent either PST or Sham operation.[9] In the PST rats, the left sciatic nerve was exposed at the midthigh level and a Prolene 7-0 (Ethicon, Somerville, NJ, USA) ligature was placed through the midpoint of the nerve, just cranially to the branch running to the musculus biceps femoris, resulting in transection of half of the nerve in the ventrocranial direction, up to the ligature. In the Sham-operated rats, the nerve was exposed and the wound was closed with sutures.

Mechanical allodynia and cold allodynia were tested in separate rats 3 days before surgery and on days 1, 3, 7, 10, 14, 21, and 28 after PST (Fig. 1). Rats displaying any sign of motor deficit 4 h after surgery and PST rats that did not develop neuropathic pain on postoperative day (POD) 1 were excluded.[10]

2.2. Drug administration

In the Baicalein group (n = 12), baicalein (20 mg/kg/day; Sigma, USA) was injected intraperitoneally (i.p.) after behaviour tests and after PST induction for 7 days (Fig. 1). Baicalein was dissolved in 10% dimethyl sulfoxide (DMSO) and further diluted in physiological buffer solution (final dilution, 0.01% DMSO). In the Vehicle group (n = 12), rats underwent PST induction and were treated with the same volume of vehicle (DMSO diluted in physiological buffer solution), while equal volumes of normal saline were given intraperitoneally to the Sham rats (n = 12). In each group, six rats were scarified on days 14 and 28 after PST induction.

The intraperitoneal dose of baicalein used in the our previous studies and others were 10–50 mg/kg in rats.[7,11] Moreover, Ribeiro et al.[12] revealed intraduodenal administration with baicalein (10–100 mg/kg) providing cytoprotective and anti-ulcerogenic effects in mice. Chen et al.[13] reported that post-injury treatment with baicalein (30 mg/kg, i.p.) improved functional and histological outcomes and reduced pro-inflammatory cytokines in rat traumatic brain injury. Moreover, in our previous study showed that baicalein (30 mg/kg, i.p.) attenuated neurological injury after SAH in rats.[7] Taken together, therefore, in this current study, the intraperitoneal dose of baicalein was 20 mg/kg in rats because the severity of the nerve injury was less than that of the traumatic head injury and SAH; the intraperitoneal dose of baicalein might be optimal or suboptimal for neuropathic pain management, and further investigation is needed.

2.3. Behavioural testing of tactile allodynia[14]

Mechanical allodynia was assessed using a dynamic plantar aesthesiometer (DPA, UGO BASILE, Italy), which did not produce tissue damage.[10] DPA is an automated version of the von Frey hair assessment. Animals were placed on an elevated wire-mesh-bottomed cage and responses to punctate mechanical stimulation were assessed using the DPA. The unit raised a straight metal filament (0.5 mm diameter) until it touched the plantar surface of the hindpaw and began to exert an upward force until the paw was withdrawn or the pre-set cut-off (50 g) was reached. The force required to elicit withdrawal responses was measured in grams.

2.4. Cold chemical allodynia behavioural testing[15]

An acetone spray was used for the determination of the reactivity to a cold chemical stimulus. The rat was placed in an acrylic cage on top of a wire-mesh grid, which allowed access to the paws, and acetone was applied to the plantar surface of the hind paw. To do this, 100 μl of acetone was sprayed onto the plantar surface of the rat's hind leg from below the grid using an Eppendorf multistepper pipette with a Combitip holding 2.5 ml. The time spent with the leg withdrawn from the floor during the 60 s that followed exposure to acetone was recorded. Both hind legs were tested in each animal, starting with the unoperated right leg, with an interval of 5–10 min

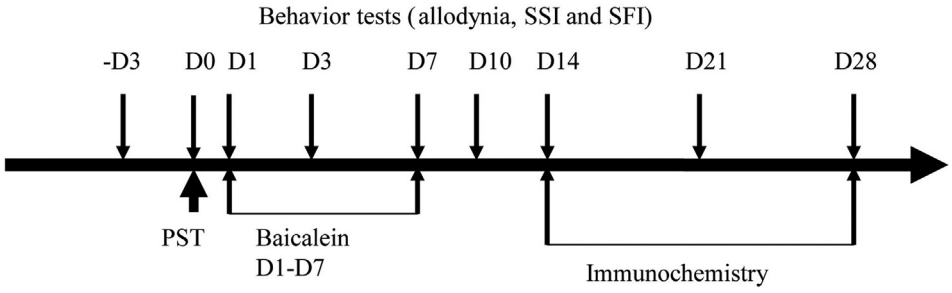

Fig. 1. The investigation period and items after PST. PST indicated partial sciatic nerve transection; SFI indicated sciatic functional index; SSI indicated sciatic static index; D indicated day; Baicalein was administrated 20 mg/kg/i.p.

between each test. A minimal value of 0.5 s was assigned to convey a fast or brisk reaction, while 0 was assigned if there was no reaction at all.

2.5. Foot posture and paw deformity (flexion contractures of toes)[16]

Before obtaining footprints, the rats were assessed serially using camera recordings of the plantar aspect of their hind feet during occasional rest periods in a glass-bottomed box measuring 25 × 16 × 9 cm. For recordings, we used a Nikon D3400 camera, which was placed under the transparent bottom of the box at a distance of 20 cm from the objective lens (set at focal length of 9 mm). The procedure was performed by viewing from below. The pale skin discoloured areas at the points of the hind feet that contacted with the transparent glass surface were clearly visible. We compared differences in the foot posture between the uninjured right and injured left hind limbs among the three groups.

2.6. Sciatic nerve functional tests: sciatic functional index (SFI) and static sciatic index (SSI)[17,18]

The animals were immobilized and their hind limb paws were painted with black China's ink up to the rear portion of the foot. They were then allowed to walk along a corridor and footprints were registered. Three days before and on days 1, 3, 7, 10, 14, 21, and 28 after surgery, the animals were evaluated to obtain three footprint parameters: the distance between the first and fifth toes (toe spread (TS)), the distance between the second and fourth toes (intermediary toe spread (ITS)), and the distance between the tip of the third toe and the most posterior part of the foot that was in contact with the ground (print length (PL)). The factors for each parameter (TSF, ITSF, and PLF) were calculated using the following formula: injured − uninjured/uninjured values (e.g., TSF = TS experimental − TS normal/TS normal), and the SFI was calculated using the formula developed by Inserra et al., $SFI = (-38.3 \times PLF) + (109.5 \times TSF) + (13.3 \times ITSF) - 8.8$.[17] The SSI is a time-saving and easy technique for the accurate functional assessment of peripheral nerve regeneration in rats, and is calculated using the static factors, not considering the print length factor (PL), according to the equation: $SSI = [(108.44 \times TSF) + (31.85 \times ITSF)] - 5.49$.[18] Rats with obvious motor deficit were excluded, however, there were no rats with motor deficit found in our present study.

2.7. Immunocytochemistry and image analysis[19]

At days 14 and 28 after PST, the rats (n = 6 in each group) were sacrificed by exsanguination under pentobarbital anaesthesia. The lumbar spinal cord L4–5 (lumbar enlargement at L1-2 vertebral body) were immediately removed, embedded in optimal cutting temperature compound (Sakura Finetek Inc, USA), and frozen at −20 °C. Sections (5 μm) were prepared, air dried on microscope slides for 30 min at room temperature, and fixed by immersion in ice-cold acetone/methanol (1:1) for 10 min. After washing three times in ice-cold phosphate-buffered saline, the sections were incubated sequentially with an FITC-labelled mouse monoclonal anti-GFAP antibody (Molecular Probes, Eugene, OR, USA; green fluorescence; 1:400 dilution) and unlabelled goat polyclonal antibodies against rat TNF-α, IL-1β, or IL-6 (all from R&D system, Minneapolis, USA). The sections were then reacted for 2 h at 25 °C with a Qdot 655 goat F (ab)'$_2$ anti-rat IgG antibody (red fluorescence) (Quantum Dot Corporation, Hayward, CA) and images were captured using an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan) and a Delta Vision disconsolation microscopic system operated by the SPOT software (Diagnostic Instruments Inc. USA).

2.8. Data and statistical analyses

Data are expressed as the mean ± standard error of the mean (S.E.M.). Analysis of variance for repeated measurements, followed by a *t*-test with Bonferroni's correction when appropriate, was used for the statistical analysis of the behavioural symptoms over time, and between the treatment groups and the control groups. Differences between treatment groups were evaluated against the left hind paw of the corresponding controls and baseline values using the Mann–Whitney *U* test (two tailed) with a correction for repeated measures. A significant difference was defined as a p value < 0.05. The statistics was performed by using SigmaStat 3.5 for Windows.

3. Results

3.1. Effect of PST on mechanical allodynia and cold allodynia

Two PST rats that did not develop neuropathic pain on POD 1 were excluded. No rats displayed motor deficits 4 h after surgery.

PST induced mechanical allodynia and cold allodynia on the ipsilateral hind limb from POD 1 to 28 ($p < 0.05$, Fig. 2a and b). The most severe mechanical allodynia values recorded were 14.4 ± 1.4 and 15.7 ± 1.9 g in the Vehicle and Baicalein rats on POD 1. However, the most severe cold allodynia values recorded were 53.5 ± 2.3 and 22.1 ± 6.7 s in the Vehicle and Baicalein rats on POD 14. In contrast, no significant change was observed in either mechanical allodynia or cold allodynia between the Sham and non-operated contralateral hindpaw.

3.2. Effect of baicalein on PST-induced mechanical allodynia

In the Baicalein group, treatment with baicalein once daily for 7 days after PST significantly improved the mechanical allodynia on POD 3, 7, 10, 14, 21, and 28 compared with the Vehicle group ($p < 0.05$, Fig. 2a), with a maximal improvement of 60% (13.3 ± 1.6 to 33.5 ± 4.6 g) observed on POD 28.

3.3. Effect of baicalein on PST-induced cold allodynia

Treatment with baicalein once daily for 7 days after PST caused a significant decrease in the duration of cold allodynia on POD 3, 7, 10, 14, 21, but not 28, compared with the Vehicle group ($p < 0.05$, Fig. 2b), with a maximal improvement of 59% (53.5 ± 2.3 to 22.1 ± 6.7 s) observed on POD 14.

3.4. Baicalein treatment improved sciatic nerve function after PST

The Sham group showed no paw deformity of bilateral hind limbs on POD 28 (Fig. 3a). In contrast, the Vehicle group showed obvious eversion of the foot and walking on heels and dystrophic changes with paw deformity in the injured left hind limb on POD 28 (Fig. 3b). The Baicalein group exhibited less eversion of the foot, no walking on heels, and less paw deformity in the injured left hind limb on POD 28 (Fig. 3c).

The SFI and SSI were calculated from the ventral view of the left footprints in the three groups. Here, we present only the ventral view of the bilateral footprints of the Sham group (Fig. 3d), the Vehicle group (Fig. 3e), and the Baicalein group (Fig. 3f) on POD28, respectively.

PST significantly reduced SFI and SSI from POD 1 to 28, with a maximal reduction of 89% in SFI observed on POD 3 and a maximal reduction of 93% in SSI observed on POD 1. Treatment with baicalein once daily for 7 days significantly improved the decrease in SFI and SSI from POD 7 to 21 and from POD 3 to 28, respectively, compared with the Vehicle group ($p < 0.05$, Fig. 3g and h), with a maximal change of 51% in SFI observed on POD 1 and a maximal change of 50% in SSI observed on POD 7.

3.5. Attenuation of the upregulation of TNF-α, IL-1β, and IL-6 and activation of astrocytes in the spinal cord of PST rats after baicalein treatment

On days 14 and 28 after PST, the overlay of the sections stained for cytokines (TNF-α, IL-1β, and IL-6) and astrocytes showed colocalization of TNF-α, IL-1β, and IL-6 with GFAP immunoreactivity (GFAP-positive astrocytes, in green) in the spinal cord of the Sham, Vehicle, and Baicalein rats (Figs. 4 and 5), indicating that these cytokines were expressed in astrocytes, but not in neurons.

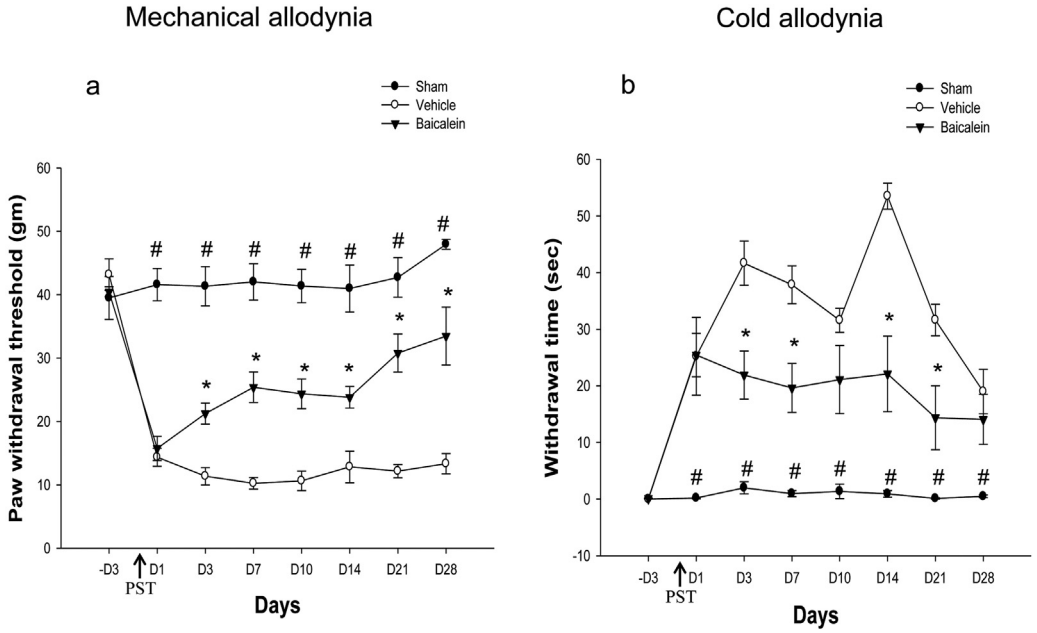

Fig. 2. Effect of baicalein on partial sciatic nerve transection (PST). PST-induced mechanical allodynia in response to the automated von Frey Dynamic Plantar Anesthesiometer (a: mechanical allodynia) and acetone spray (b: cold allodynia). The results represented the mean ± S.E.M. $^{\#}p < 0.05$ indicated among Sham (○), Baicalein (▲) and Vehicle (●) rats, $^{*}p < 0.05$ indicated Baicalein rats compared with Vehicle rats.

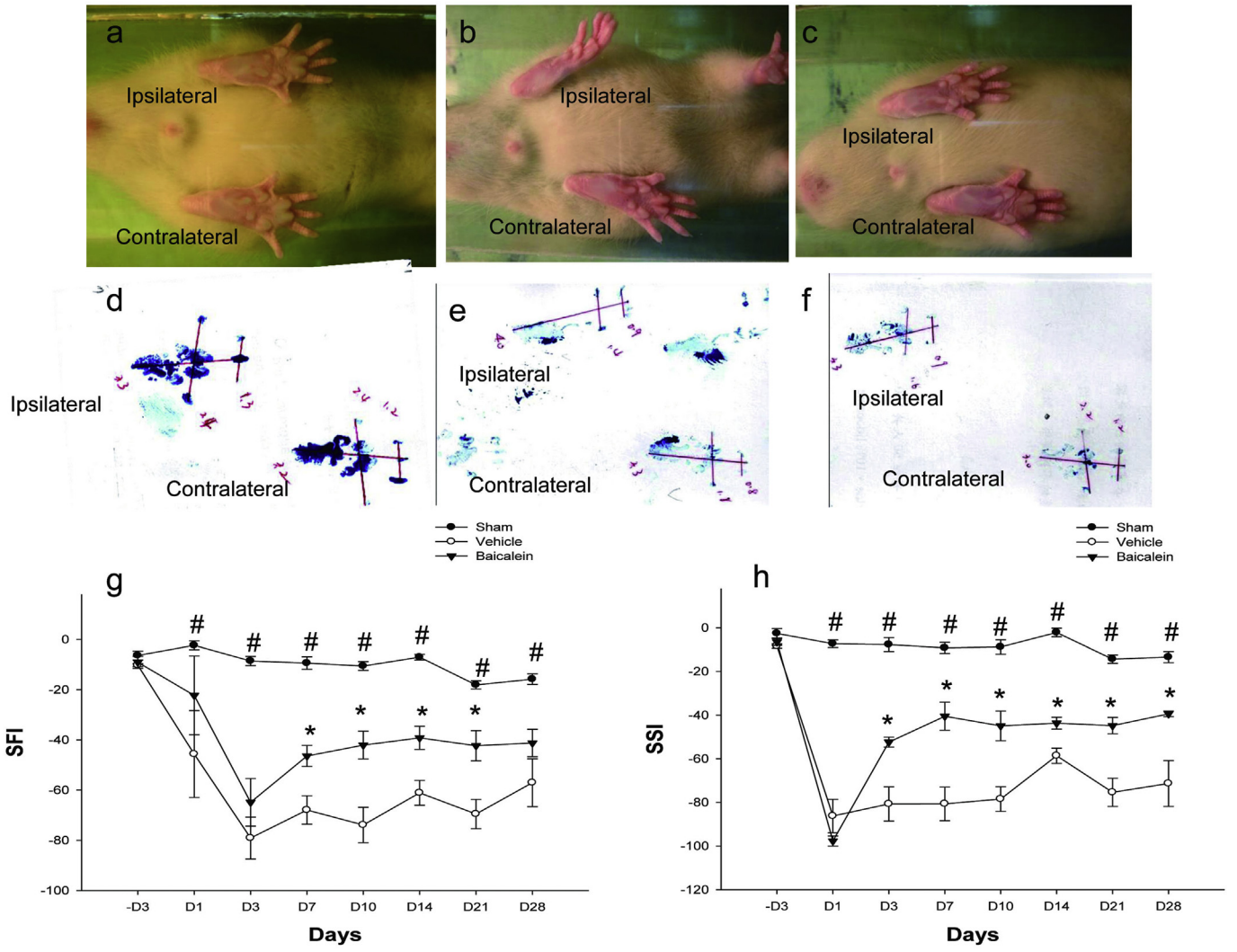

Fig. 3. Comparing foot posture and sciatic nerve function among three groups. Foot posture and footprint parameters of uninjured right and injured left foot among three groups on day of 28 after PST could be observed as: (a) no paw deformity of bilateral hindlimbs in the Sham rat; (b) obvious eversion of foot and walking on heels and paw deformity in the injured left hindlimb in the Vehicle rat; (c) less eversion of foot, without walking on heel, and less paw deformity in the injured left hindlimb in the Baicalein rat; Footprint parameters of uninjured contralateral and injured ipsilateral foot in the Sham rat (d) Vehicle rats (e), and footprint parameters of Baicalein rats (f). Baicalein improved nerve recovery on PST by SFI (g) and SSI (h). The results represent the mean ± S.E.M. $^{\#}p < 0.05$ indicated among Sham (○), Baicalein (▲) and Vehicle (●) rats; $^{*}p < 0.05$ indicated Baicalein rats compared with Vehicle rats. SFI indicated sciatic functional index; SSI indicated sciatic static index.

In the Sham rats, weak staining for astrocytes (GFAP-positive cells, in green) was distributed in the dorsal horn of the spinal cord, with cells that were stained more strongly exhibiting the ramified shape of non-activated astrocytes. In contrast, in cells with the less classic amoeboid shape of activated astrocytes, the expression of the TNF-α, IL-1β, and IL-6 proteins (in red) was lower than it was in the Vehicle rats on days 14 and 28 (Figs. 4a–c and 5a–c). In the Vehicle rats, strong GFAP staining was observed in the sections (Fig. 4d–f and 5d–f) and the expression of TNF-α, IL-1β, and IL-6 (in red) was higher (Figs. 4d–f and 5d–f) compared with that observed in the Sham rats on days 14 and 28 (Figs. 4a–c and 5a–c). The PST-induced increase in the expression of TNF-α, IL-1β, and IL-6 was attenuated by baicalein treatment on days 14 and 28 (Figs. 4g–i and 5g–i). Finally, the PST-induced activation of astrocytes was attenuated by baicalein treatment on day 14 (Fig. 4g–i), but not on day 28 (Fig. 5g–i).

4. Discussion

The PST rat model has been used for the study of neuropathic pain, inducing nerve injury without introducing foreign material, with reduction of thermal and tactile thresholds; it allows examining the drug effect on animal nociceptive behavior after endoneurial injury.[10,20] Because of the deficit of an epineurial inflammatory component, PST is a pure nerve injury model and is suitable for researching the role of endoneurial processes in the pathogenesis of neuropathic pain.[10,20] Our results showed that PST caused mechanical allodynia, cold allodynia and paw deformity with lower SFI

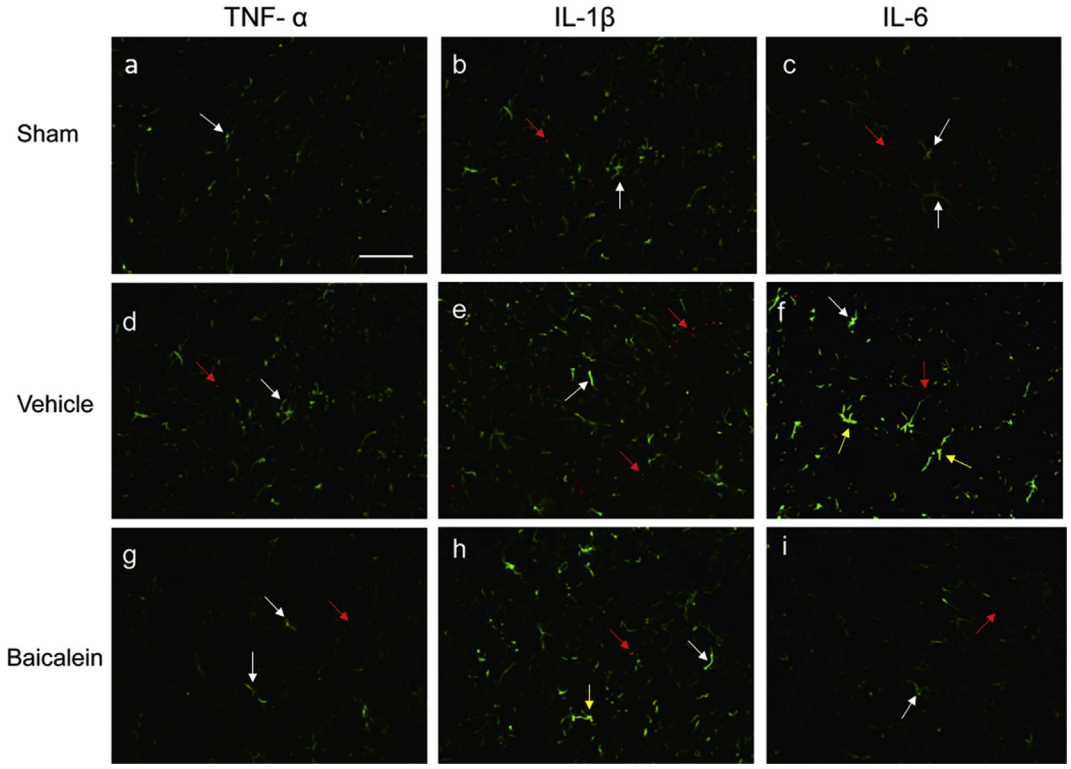

Fig. 4. Baicalein significantly inhibited the expression of TNF-α, IL-1β and IL-6, and attenuated the activation of astrocytes in the spinal cord dorsal horn on day 14 following partial sciatic nerve transection. Staining for astrocytes was shown in green and for TNF-α (left panels), IL-1β (centre panels), and IL-6 (right panels) in red. Top row, the Sham group; middle row, the Vehicle group; bottom row, the Baicalein group. The expression of GFAP, TNF-α, IL-1β, and IL-6 was significantly higher in the Vehicle rats (d–f) than in the Sham (a–c) and Baicalein (g–i) rats. The expression of GFAP, TNF-α, IL-1β, and IL-6 was significantly lower in the Sham rats than in the Baicalein rats 14 days after PST. Scale bar represents 50 μm (n = 6). We used white arrow marker to indicate where the representative astrocytes (green) and red arrow marker to indicate where the representative cytokine (red) were located. If they are mixed (yellow), we used yellow arrow marker to indicate where the representative mixed (yellow) were located.

and SSI values, which were similar to the neuropathic pain presentations.[9,10,20] Moreover, Lindenlaub et al.[10] reported that rats had neuropathic pain with paw deformity at 101 days after PST without observed motor deficit. In our study, we also found paw deformity with impaired sciatic nerve function and the lower SFI and SSI values on POD 28 in the PST rats and no observed motor deficit during the footprint test.

The major findings of the present study were (1) PST caused both mechanical and cold allodynia; (2) Early and multiple intraperitoneal baicalein administration ameliorated the PST-induced mechanical and cold allodynia; (3) PST impaired sciatic nerve function with paw deformity and lower SFI and SSI values; (4) Baicalein treatment improved the PST-induced sciatic nerve dysfunction; (5) Baicalein treatment significantly inhibited the upregulation of TNF-α, IL-1β, and IL-6 in the spinal cord dorsal horn of PST rats; and (6) Baicalein attenuated the activation of astrocytes on day 14, but not day 28, after PST. These results suggest that baicalein, at 20 mg/kg (i.p.), attenuated neuropathic pain and improved sciatic nerve function in PST rats via its anti-inflammatory property.

Baicalein has been demonstrated with anti-inflammation, and anti-oxidation.[21] In an animal model of carrageenan-evoked thermal hyperalgesia, the anti-inflammatory and analgesic effect of baicalein was associated with downregulation of pro-inflammatory mediators, including cytokines, nitric oxide, and prostaglandin E_2.[22]

Cherng et al.[23] reported that baicalin, similar to baicalein a component in Chinese herb Huang Qui,[24] can ameliorate neuropathic pain in spinal nerve ligation rats. Similarly, in our present study, baicalein also provided anti-allodynia, both mechanical and cold allodynia, effect in PST rats. Moreover, in the present study, we were the first demonstrated that baicalein treatment improved sciatic nerve function, as measured by SFI and SSI after PST. Stavniichuk et al.[21]also found that baicalein improved sciatic nerve function in diabetic mice. This improvement in sciatic nerve function by baicalein is explained by reduction of cell oxidative–nitrosative stress and inflammatory reactions to drugs.[21] Baicalein inhibits IL-1β and IL-6 expression by reducing nuclear factor-κB in the brain, which improved the perihematoma area recovery.[25] For peripheral nerve injury, Nadeau et al.[26] reported that this functional recovery of injured nerve was dependent on inhibiting pro-inflammatory cytokines IL-1β and TNF-α release.[27] Moreover, Kato et al.[28] concluded that TNF-α plays a crucial role in the initiation of degenerative cascades after peripheral nerve injury, and TNF-α antagonist enhanced axonal regeneration. In our present study, we demonstrated that baicalein suppressed pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 production in the spinal cord of PST rats on

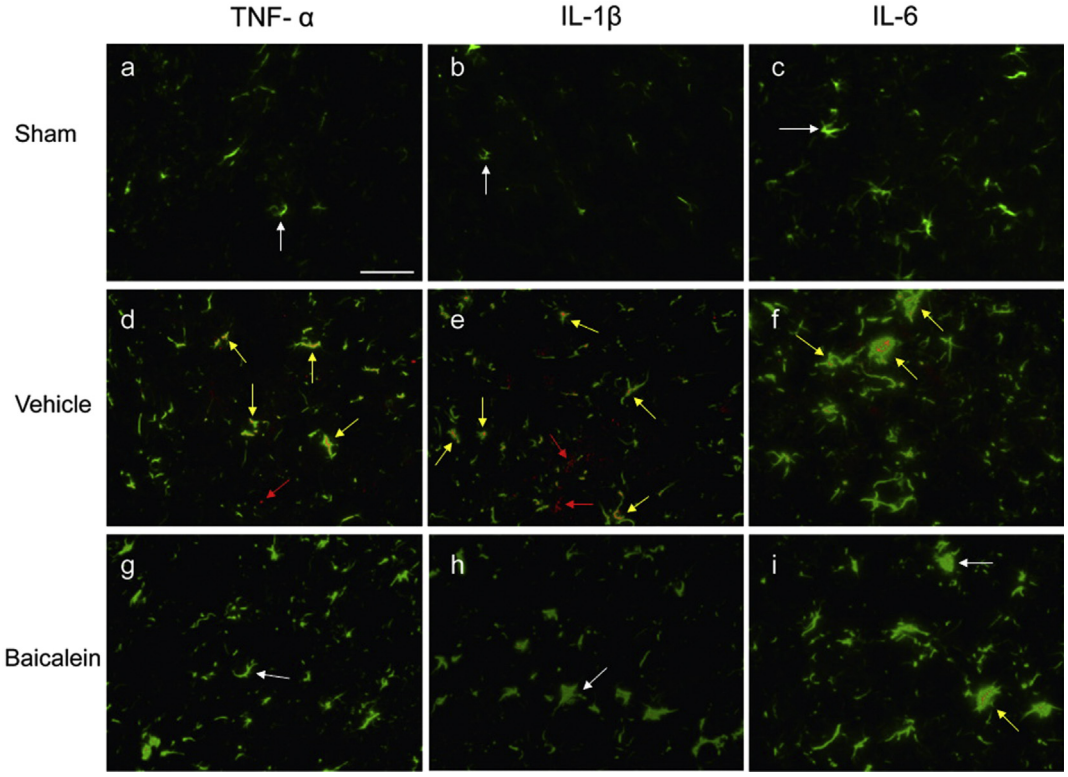

Fig. 5. Baicalein significantly inhibited the expression of TNF-α, IL-1βand IL-6, but unapparently attenuated the activation of astrocytes in the spinal cord dorsal horn on day 28 following partial sciatic nerve transection. Staining for astrocytes was shown in green and for TNF-α (left panels), IL-1β (centre panels), and IL-6 (right panels) in red. Top row, the Sham group; middle row, the Vehicle group; bottom row, the Baicalein group. The expression of GFAP,TNF-α, IL-1β, and IL-6 were significantly lower in the Sham (a–c) than in the Vehicle (d–f) and Baicalein (g–i) rats. The expression of TNF-α, IL-1β, and IL-6 was significantly higher in the Vehicle rats (d–f) than in the Baicalein (g–i) rats. The expression of GFAP was not significantly different between the Baicalein and the Vehicle rats 28 days after PST. Scale bar represents 50 μm (n = 6). We used white arrow marker to indicate where the representative astrocytes (green) and red arrow marker to indicate where the representative cytokine (red) were located. If they are mixed (yellow), we used yellow arrow marker to indicate where the representative mixed (yellow) were located.

POD 14 and 28. This might be explained by the neuroprotective effect of baicalein against the complex inflammatory pathway of central and peripheral sensitization. Furthermore, we also found, for the first time, that baicalein significantly attenuated of astrocytes activation in the injured rat spinal cord on day 14, but not day 28, after PST. By contrast, our previous study showed that baicalein preserved astrocytes and attenuated neurological injury in rats with subarachnoid haemorrhage (SAH) on day 7 after SAH.[7] It was consistent with that Becerra-Calixto et al. reported astrocytes playing an important role on neuroprotection after brain stroke.[29] But astrocytes were commonly involved in negative responses through their hyperreactivity in excitotoxic and/or mechanical injuries,[29] similar to our present study. Similarly, hyperbaric oxygen treatment produced an antinociceptive effect by inhibiting astrocytes activation in chronic constriction injury (CCI) rat model.[30] More support from a recent study, docosahexaenoic acid treatment reduced the number of reactive astrocytes in the spinal cord dorsal horn of CCI neuropathic pain rats,[31] moreover, reduction of the intensity and duration of neurogenic pain, and prevented cold allodynia and dystrophic changes in denervated limb.[31] Further, previous studies reported that astrocytic activation persisted for weeks to months after neural injury in neuropathic pain rats.[32,33] In addition, Bura and colleagues found that astrocyte proliferation was closely related to the maintenance of pain behaviour in a peripheral neuropathic pain model.[34] Taken together, we think that activated astrocytes do play an important role in pain central sensitization via production inflammatory cytokines.[35] Obviously, the pathophysiology of dynamic mechanical allodynia was different from those of cold allodynia which leads to specific sensory symptoms. In the current study, we found that baicalein attenuated astrocytes activation on POD14, but not 28, and did not significantly improve cold allodynia and SFI on POD 28, it might be resulting from the expiration of the drug effect. These results were consistent with those of Tamaddonfard et al.,[36] who reported that safranal and vitamin E improved SFI while suppressing cold and mechanical allodynia in rats with sciatic nerve crush injury only within POD 21, while Cobianchi et al.[37] showed that short-lasting treadmill running reduced mechanical allodynia and improved SSI for more than 28 days in CCI rats. Moreover, Tanimoto-Mori et al.[38] found that activation of C fibres induced cold allodynia in CCI rats, and Shir et al.[39] revealed the injured A-fibers resulting in mechanical allodynia in sciatic nerve ligation rats. Besides, Duan et al.[40] reported that

selectively A-fiber demyelination by single injection of cobra venom into the sciatic nerve induced mechanical allodynia and lasted for 3 weeks in rats. Therefore, we postulated that baicalein did not attenuate the activation of astrocytes and C fibres on POD 28, it may due to the expiration of the drug effect, thus resulted in lack of improvement on cold allodynia and SFI. Further detailed investigations are needed.

We found that SSI was improved since POD 3 and SFI was improved since POD 7. The difference might be due to methodological bias and the effect of baicalein. SFI is a quantitative method, it is dependent on the pressure exerted by the foot on the floor, therefore, automutilation and inversion or eversion deformations often limit the functional assessment.[41] SSI assess functional loss following injury during animal standing or periodic rest in sciatic injury rat. SSI was developed based on the premise that the recovery of muscle tone after nerve injury is a constituent part of integral nerve and muscle functional recovery and forces acting on the body, i.e., body weight and postural muscle tone during standing influenced footprint parameters. The main difference between SFI and SSI was that the distance between the tip of the third toe and the posterior margin of the sole discolored area. Besides, repeatability of the toe spread measurements was always better under static conditions.[16] In addition, the pain of dynamic gait was more intensive than that of static gait. Therefore, we found that baicalein improved SSI (static gait) earlier than SFI (dynamic gait) in the PST rats. And further detailed investigations are needed.

In conclusion, our results suggest that early and multiple doses of baicalein effectively attenuate neuropathic pain and improve sciatic nerve function recovery after PST in rats, which implies that baicalein is a potential therapy for peripheral neuropathic pain. The mechanisms underlying the action of baicalein may be involved in the complex inflammatory pathways of central and peripheral sensitization after PST.

Acknowledgments

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References

1. Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 2007;**10**:1361−8.
2. Kidd BL, Urban LA. Mechanisms of inflammatory pain. *Br J Anaesth* 2001;**87**:3−11.
3. Reichling DB, Levine JD. The primary afferent nociceptor as pattern generator. *Pain* 1999;(Suppl. 6):S103−9.
4. Taiwo YO, Levine JD. Serotonin is a directly-acting hyperalgesic agent in the rat. *Neuroscience* 1992;**48**:485−90.
5. Moalem G, Tracey DJ. Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev* 2006;**51**:240−64.
6. Chen H, Xu Y, Wang J, Zhao W, Ruan H. Baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat. *Int J Clin Exp Pathol* 2015;**8**:10139−47.
7. Kuo CP, Wen LL, Chen CM, Huh B, Cherng CH, Wong CS, et al. Attenuation of neurological injury with early baicalein treatment following subarachnoid hemorrhage in rats. *J Neurosurg* 2013;**119**:1028−37.
8. Lin CC, Shieh DE. The anti-inflammatory activity of Scutellaria rivularis extracts and its active components, baicalin, baicalein and wogonin. *Am J Chin Med* 1996;**24**:31−6.
9. Lindenlaub T, Sommer C. Partial sciatic nerve transection as a model of neuropathic pain: a qualitative and quantitative neuropathological study. *Pain* 2000;**89**:97−106.
10. Yang CP, Cherng CH, Wu CT, Huang HY, Tao PL, Wong CS. Intrathecal ultra-low dose naloxone enhances the antinociceptive effect of morphine by enhancing the reuptake of excitatory amino acids from the synaptic cleft in the spinal cord of partial sciatic nerve-transected rats. *Anesth Analg* 2011;**113**:1490−500.
11. Wang SY, Wang HH, Chi CW, Chen CF, Liao JF. Effects of baicalein on beta-amyloid peptide-(25-35)-induced amnesia in mice. *Eur J Pharmacol* 2004;**506**:55−61.
12. Ribeiro AR, do NascimentoValença JD, da Silva Santos J, Boeing T, da Silva LM, de Andrade SF, et al. The effects of baicalein on gastric mucosal ulcerations in mice: protective pathways and anti-secretory mechanisms. *Chem Biol Interact* 2016;**260**:33−4.
13. Chen SF, Hsu CW, Huang WH, Wang JY. Post-injury baicalein improves histological and functional outcomes and reduces inflammatory cytokines after experimental traumatic brain injury. *Br J Pharmacol* 2008;**155**:1279−96.
14. Dolan S, Nolan AM. Blockade of metabotropic glutamate receptor 5 activation inhibits mechanical hypersensitivity following abdominal surgery. *Eur J Pain* 2007;**11**:644−51.
15. Dowdall T, Robinson I, Meert TF. Comparison of five different rat models of peripheral nerve injury. *Pharmacol Biochem Behav* 2005;**80**:93−108.
16. Bervar M. Video analysis of standing−an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve. *J Neurosci Meth* 2000;**102**:109−16.
17. Varejão AS, Melo-Pinto P, Meek MF, Filipe VM, Bulas-Cruz J. Methods for the experimental functional assessment of rat sciatic nerve regeneration. *Neurol Res* 2004;**26**:186−94.
18. Luís AL, Amado S, Geuna S, Rodrigues JM, Simões MJ, Santos JD, et al. Long-term functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp. *J Neurosci Meth* 2007;**163**:92−104.
19. Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS. Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. *Pain* 2006;**124**:77−86.
20. Yang CP, Cherng CH, Wu CT, Huang HY, Tao PL, Lee SO, et al. Intrathecal ultra-low dose naloxone enhances the antihyperalgesic effects of morphine and attenuates tumor necrosis factor-α and tumor necrosis factor-α receptor 1 expression in the dorsal horn of rats with partial sciatic nerve transection. *Anesth Analg* 2013;**117**:1493−502.
21. Stavniichuk R, Drel VR, Shevalye H, Maksimchyk Y, Kuchmerovska TM, Nadler JL, et al. Baicalein alleviates diabetic peripheral neuropathy through inhibition of oxidative-nitrosative stress and p38 MAPK activation. *Exp Neurol* 2011;**230**:106−13.
22. Chou TC, Chang LP, Li CY, Wong CS, Yang SP. The antiinflammatory and analgesic effects of baicalin in carrageenan-evoked thermal hyperalgesia. *Anesth Analg* 2003;**97**:1724−9.
23. Cherng CH, Lee KC, Chien CC, Chou KY, Cheng YC, Hsin ST, et al. Baicalin ameliorates neuropathic pain by suppressing HDAC1 expression in the spinal cord of spinal nerve ligation rats. *J Formos Med Assoc* 2014;**113**:513−20.
24. Ku SK, Bae JS. Baicalin, baicalein and wogonin inhibits high glucose-induced vascular inflammation in vitro and in vivo. *BMB Rep* 2015;**48**:519−24.
25. Zhou QB, Jin YL, Jia Q, Zhang Y, Li LY, Liu P, et al. Baicalin attenuates brain edema in a rat model of intracerebral hemorrhage. *Inflammation* 2014;**37**:107−15.
26. Nadeau S, Filali M, Zhang J, Kerr BJ, Rivest S, Soulet D, et al. Functional recovery after peripheral nerve injury is dependent on the pro-

inflammatory cytokines IL-1β and TNF: implications for neuropathic pain. *J Neurosci* 2011;**31**:12533−42.
27. Cámara-Lemarroy CR, Guzmán-de la Garza FJ, Fernández-Garza NE. Molecular inflammatory mediators in peripheral nerve degeneration and regeneration. *Neuroimmunomodulation* 2010;**17**:314−24.
28. Kato K, Liu H, Kikuchi S, Myers RR, Shubayev VI. Immediate anti-tumor necrosis factor-alpha (etanercept) therapy enhances axonal regeneration after sciatic nerve crush. *J Neurosci Res* 2010;**88**:360−8.
29. Becerra-Calixto A, Cardona-Gómez GP. The role of astrocytes in neuroprotection after brain stroke: potential in cell therapy. *Front Mol Neurosci* 2017;**10**:88.
30. Zhao BS, Meng LX, Ding YY, Cao YY. Hyperbaric oxygen treatment produces an antinociceptive response phase and inhibits astrocyte activation and inflammatory response in a rat model of neuropathic pain. *J Mol Neurosci* 2014;**53**:251−61.
31. Manzhulo IV, Ogurtsova OS, Kipryushina YO, Latyshev NA, Kasyanov SP, Dyuizen IV, et al. Neuron-astrocyte interactions in spinal cord dorsal horn in neuropathic pain development and docosahexaenoic acid therapy. *J Neuroimmunol* 2016;**298**:90−7.
32. Tanga FY, Raghavendra V, DeLeo JA. Quantitiative real-time RT-PCR assessment of spinal microglia and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int* 2004;**45**:397−407.
33. Gwak YS, Hulsebosch CE. Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. *Neuroscience* 2009;**161**:895−903.
34. Bura SA, Nadal X, Ledent C, Maldonado R, Valverde OA. 2A adenosine receptor regulates glia proliferation and pain after peripheral nerve injury. *Pain* 2008;**140**:95−103.
35. Old EA, Clark AK, Malcangio M. The role of glia in the spinal cord in neuropathic and inflammatory pain. *Handb Exp Pharmacol* 2015;**227**:145−70.
36. Tamaddonfard E, Farshid AA, Maroufi S, Kazemi-Shojaei S, Erfanparast A, Asri-Rezaei S, et al. Effects of safranal, a constituent of saffron, and vitamin E on nerve functions and histopathology following crush injury of sciatic nerve in rats. *Phytomedicine* 2014;**21**:717−23.
37. Cobianchi S, Marinelli S, Florenzano F, Pavone F, Luvisetto S. Short- but not long-lasting treadmill running reduces allodynia and improves functional recovery after peripheral nerve injury. *Neuroscience* 2010;**168**:273−87.
38. Tanimoto-Mori S, Nakazato-Imasato E, Toide K, Kita Y. Pharmacologic investigation of the mechanism underlying cold allodynia using a new cold plate procedure in rats with chronic constriction injuries. *Behav Pharmacol* 2008;**19**:85−90.
39. Shir Y, Seltzer Z. A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgiform pain disorders in rats. *Neurosci Lett* 1990;**115**:62−7.
40. Duan WR, Xie YK. Modulation of C-nociceptive activities by inputs from myelinated fibers. *Adv Exp Med Biol* 2016;**904**:33−40.
41. Yu P, Matloub HS, Sanger JR, Narini P. Gait analysis in rats with peripheral nerve injury. *Muscle Nerve* 2001;**24**:231−9.