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Original Article

Effects of *Gentianae Macrophyllae Radix* on the functional recovery and expression of BDNF and c-Fos after sciatic crushed nerve injury in rats

Hyun-Chol Cho, Yun-Kyung Song, Hyung-Ho Lim

Department of Oriental Rehabilitation Medicine, College of Oriental Medicine, Kyungwon University

Background : Peripheral nerve injuries are a commonly encountered clinical problem and often result in a chronic pain and severe functional deficits.
Objective : The aim of this study was to evaluate the effects of *Gentianae Macrophyllae Radix (G. M. Radix)* on the pain control and the recovery of the locomotor function that results from the sciatic crushed nerve injury in rats.
Method : Using rats, we crushed their sciatic nerve, and then orally administered the aqueous extract of *G. M. Radix*. The effects of *G. M. Radix* on the recovery locomotor function were investigated by walking track analysis. The effects of *G. M. Radix* on pain control were investigated by brain-derived neurotrophic factor (BDNF) expression in the sciatic nerve, and c-Fos expression in the paraventricular nucleus (PVN) of the hypothalamus and in the ventrolateral periaqueductal gray (vlPAG).
Result : *G. M. RADIX* facilitates motor function from the locomotor deficit, and thereby increased BDNF expression and suppressed painful stimuli in the PVN and vlPAG after sciatic crushed nerve injury.
Conclusion : It is suggested that *G. M. Radix* might aid recovery locomotor function and control pain after sciatic crushed nerve injury. Further studies on identifying specific the component in G.M. Radix associated with enhanced neural activity in the peripheral nerve injury may be helpful to develop therapeutic strategies for the treatment of peripheral nerve injury.

Key Words : *Gentianae Macrophyllae* Radix, sciatic nerve, SFI, c-Fos, BDNF

Introduction

Peripheral nerves are frequently exposed to physical injury and this can result in a severe functional impairment and decreased quality of life, because it sometimes induces loss of sensory and motor functions.

Crush injury to the sciatic nerve has been used as the animal model of unilateral peripheral neuropathy. Many changes affecting the ascending facilitatory system and on the descending inhibitory system occur within the central nervous system, resulting in the development of persistent pain[1].

Characteristic gait changes occur after unilateral sciatic nerve injury in rats. Sciatic nerve lesions cause variable loss of both extensors and flexors of the foot. This deficit causes the foot to drop to the ground and thus changes the footprints. Gradual disappearance of this change reflects nerve regeneration and functional recovery[2]. In this way, footprints can be used to assess sciatic nerve function. The current and standard method for measuring

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• Correspondence to : Hyung-Ho Lim
Department of Oriental Rehabilitation Medicine, Kyung Won University, San 65, Bokjeong-dong, Sujeong-gu, Seongnam-si, Gyeonggi-do, Korea
Tel : +82-11-9899-1478, Fax : +82-32-772-9011, E-mail : omdlimhh@chol.com

functional recovery after sciatic nerve injury in rats is the sciatic function index (SFI) established by de Medinaceli *et al*.[3], and subsequently modified by Bain *et al*.[4] SFI formula is based on the characteristic walking patterns following sciatic nerve injury in rats, and the recovery rate can be determined by this gait analysis.

Neurotrophins are a family of proteins essential for the development, maintenance, and functioning of the vertebrate nervous system[5]. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that promotes the survival of specific neurons in the central and peripheral nervous system during development[6,7] and is known as an important neuromodulator of synaptic plasticity[8,9].

The products of the immediate early genes, such as c-Fos, are rapidly expressed in neurons in response to various stimuli, and c-Fos expression is recognized as a marker of increased neuronal activity[10].

In Oriental Medicine, *G. M. Radix* has been used for over 2000 years as an analgesic, anti-inflammatory, antipyretic, antirheumatic, diuretic, febrifuge, hypoglycaemic and for hypotensive rheumatic pains, fevers, allergic inflammations and paralysis[11,12]. It has been reported that *G. M. Radix* has inhibitory effects on rheumatoid arthritis[13,14] and on the production of pro-inflammatory cytokines[15].

The dominant active component in *G. M. Radix* is secoiridoids, mainly gentiopicroside. The biological and pharmacological effects of secoiridoids include anti-inflammatory, antifungal, antihistamine, and antihepatotoxic activities. Gentiopicroside can significantly suppress an increase of TNF in inflammatory tissue[11,13]. However, little is known about the effect of *G. M. Radix* against painful neuropathy induced by sciatic crushed nerve injury.

In the present study, we prepared the aqueous extract of the *G. M. Radix* and investigated its effects on the recovery rate of locomotor function, on the expression of BDNF in the sciatic nerve, and on the expression of c-Fos in the paraventricular nucleus (PVN) of the hypothalamus and ventrolateral periaqueductal gray (vlPAG) region following crushed sciatic nerve injury in rats.

Materials and methods

1. Preparation of aqueous extract of *Gentianae Macrophyllae* Radix

To obtain the aqueous extract, 100 g of *G. M. Radix* was added to distilled water, and extraction was performed by heating at 90°C for 2 h, concentrating with rotary evaporator (Eyela, Tokyo, Japan) and lyophilizing by a drying machine (Eyela) for 24 h. The resulting powder, weighting 19.5 g (yield of 19.5%) was diluted to the concentrations needed with autoclaved distilled water and filtered through a 0.45 μm syringe filter before use.

2. Experimental animals

Male Sprague-Dawley rats weighing 200 ± 10 g (7 weeks of age) were used. The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. The animals were housed at a controlled temperature (20 ± 2°C) and maintained under light-dark cycles of 12 h of light and 12 h of darkness (lights on from 07:00 to 19:00 h), with food and water made available *ad libitum*. The rats were randomly divided into five groups (n = 10 in each group): the sham operation group, the operation (crushed sciatic nerve injury) group, the operation and 50 mg/kg *G. M. Radix*-treated group, the operation and 100 mg/kg *G. M. Radix*-treated group, and the operation and 200 mg/kg *G. M. Radix*-treated group. The rats in the *G. M. Radix*-treated groups orally received *G. M. Radix* at the respective dose, and those in the sham operation group orally received an equivalent amount of drinking water, once a day from the 2nd day to the 14th day from the commencement of the experiment.

3. Surgical procedure

To induce crush injury on the sciatic nerve in

rats, a surgical procedure based on previously described method was performed[16]. In brief, the right sciatic nerve was exposed through splitting incision on the gluteal muscle under Zoletil 50® anesthesia (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). The sciatic nerve was carefully exposed and crushed for 30 sec using a surgical clip (Pressure: 125g; Fine Science Tools Inc., San Francisco, USA). The crush location was between the sciatic notch and the point of trifurcation. Subsequently, the surgical wound was sutured and allowed to recover. In the sham operation rats, the sciatic nerve was exposed but crushing pressure on the nerve was not applied.

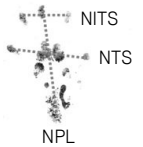
4. Tissue preparation

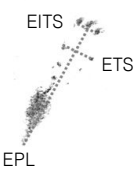
The animals were anesthetized using Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were dissected and postfixed in the same fixative overnight and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 μm thickness were made with a freezing microtome (Leica, Nussloch, Germany).

5. Walking track analysis

Functional recovery rate after sciatic nerve injury was analyzed using a walking track assessment, which can be quantified with SFI. Examination of the walking patterns was performed seven times at one-day intervals through the course of the experiment by a previously described method[17]. Footprints were recorded in a wooden walking alley (8.2 × 42 cm) with a darkened goal box at the end. The floor of the alley was covered with white paper. The anatomical landmarks on the hind feet of the rats were smeared with finger paint. The rats were allowed to walk down the track, leaving their footprints on the paper.

From the footprints, the following parameters were calculated: distance from the heel to the top of the third toe (print length; PL), distance between the first and the fifth toe (toe spread; TS), and distance from the second to the fourth toe (intermediary toe spread; IT). These parameters were taken both from the intact left (non-operated) foot (NPL, NTS, and NIT) and from the injured right (experimental) foot (EPL, ETS, and EIT). SFI values were obtained using following equation (Fig. 1).





$$SFI = -38.3\left(\frac{EPL-NPL}{NPL}\right) + 109.5\left(\frac{ETS-NTS}{NTS}\right) + 13.3\left(\frac{EIT-NIT}{NIT}\right)$$

Fig. 1. Walking track analysis.

After sciatic crushed nerve injury in rats, paired parameters of the print length (PL), toe spread (TS), and intermediary toe spread (IT) were calculated. (E) Experimental side, (N) normal side, (EPL) experimental print length, (NPL) normal print length, (ETS) experimental toe spread, (NTS) normal toe spread, (EIT) experimental intermediary toe spread, (NIT) normal intermediary toe spread, (SFI) sciatic functional index.

Interpolating identical values of PL, TS, and IT from the right and the left hind feet are close to zero in normal rats. A value of -100 indicates complete impairment of walking ability.

6. Western blot analysis

On the 14th day after commencement of the experiment, the animals were sacrificed and 5 mm of injured right sciatic nerve stumps were gathered and trimmed off. The samples were lysed in the lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% nonidet-P40 (NP40), 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 g/ml leupeptin. Protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). Protein of 50 g was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Rabbit BDNF antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) was used as primary antibody. Horseradish peroxidase-conjugated anti-rabbit antibody for BDNF (Santa Cruz Biotech) was used as secondary antibody. The detection of the band was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH, Freiburg, Germany).

7. c-Fos immunohistochemistry

The expression of c-Fos in the PVN and vlPAG in each group was measured immediately after determination of last SFI. For immunolabeling of c-Fos in the PVN and vlPAG of each brain, c-Fos immunohistochemistry was performed by a previously described method[18]. Free-floating tissue sections were incubated overnight with rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000, and the sections were then incubated for 1 h with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3-diaminobenzidine (DAB) and 0.01% H_2O_2 in 50 mM Tris-buffer (pH 7.6) for approximately 3 min. The sections were then washed three times with PBS and mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount®. As the negative control, the brain sections were likewise processed using normal goat serum in place of the primary antibody: no c-Fos-like immunoreactivity was observed.

8. Data analyses

The data are expressed as the mean ± standard error of the mean (S.E.M). For comparisons among the groups, one-way ANOVA and Duncan's post-hoc test were performed with $P < 0.05$ as an indication of statistical significance.

Results

1. Effect of *Gentianae Macrophyllae* Radix on functional recovery.

We measured SFI using a walking track analysis to assess recovery of motor after sciatic crushed nerve injury in rats. The mean SFI in each group was calculated on the 2nd, 4th, 6th, 8th, 10th, and 14th day after crushed sciatic nerve injury.

The SFI in the sham operation group was -10.18 ± 1.41 on the 2nd day, -10.43 ± 0.43 on the 4th day, -10.59 ± 1.15 on the 6th day, -10.49 ± 1.11 on the 8th day, -9.09 ± 0.54 on the 10th day, -8.69 ± 1.81 on the 12th day, and -10.37 ± 2.04 on the 14th day from the commencement of the experiment.

The SFI in the operation group was -97.40 ± 1.61 on the 2nd day, -95.63 ± 1.93 on the 4th day, -87.08 ± 3.45 on the 6th day, -85.07 ± 3.72 on the 8th day, -80.20 ± 2.15 on the 10th day, -74.72 ± 2.66 on the 12th day, and -61.47 ± 5.11 on the 14th day from the commencement of the experiment.

The SFI in the 50mg/kg *G. M. Radix*-treated group was -96.52 ± 2.58 on the 2nd day, -93.96 ±

2.85 on the 4th day, -87.99 ± 2.65 on the 6th day, -82.38 ± 2.61 on the 8th day, -78.51 ± 1.61 on the 10th day, -63.16 ± 3.21 on the 12th day, and -53.90 ± 3.17 on the 14th day from the commencement of the experiment.

The SFI in the 100mg/kg *G. M. Radix*-treated group was -96.87 ± 2.26 on the 2nd day, -93.36 ± 3.15 on the 4th day, -86.00 ± 3.39 on the 6th day, -79.50 ± 3.35 on the 8th day, -73.41 ± 3.04 on the 10th day, -59.66 ± 4.91 on the 12th day, and -43.14 ± 5.45 on the 14th day from the commencement of the experiment.

The SFI in the 200 mg/kg *G. M. Radix*-treated group was -97.76± 1.59 on the 2nd day, -92.23 ± 2.60 on the 4th day, -81.99 ± 3.31 on the 6th day, -76.78 ± 3.40 on the 8th day, -70.38 ± 2.66 on the 10th day, -51.33 ± 4.85 on the 12th day, and -40.93 ± 4.30 on the 14th day from the commencement of the experiment (Fig. 2).

In the present results, the SFI of the sham operation group continued near zero level during the experimental period. At the beginning, the SFI of all operation groups dropped near to -100. In the operation and *G. M. Radix*-treated groups, the SFI value continued at a low level until the 8th day after injury and then slowly increased. In the *G. M. Radix*-treated groups, SFI value was enhanced from the 8th day and rapidly increased throughout the experiment. On the 14th day from the commencement of the experiment, treatment with 200 mg/kg *G. M. Radix* showed statistically significant recovery effect.

2. Effect of *Gentianae Macrophyllae* Radix on BDNF expression

Western blot analysis of protein levels of BDNF (16 kDa) was performed and the level of BDNF protein expressions in the sham group was set as 1.00. The level of BDNF protein expressions was 1.83 ± 0.13 in the operation group, 2.34 ± 0.14 in the 50 mg/kg *G. M. Radix*-treated group, 2.64 ± 0.20 in the 100 mg/kg *G. M. Radix*-treated group, and 2.92 ± 0.17 in the 200 mg/kg *G. M. Radix* -treated group (Fig. 3).

In the present results, the level of BDNF protein expression was increased by crushed sciatic nerve injury and treatment with *G. M. Radix* significantly enhanced the level of BDNF protein expression; *G. M. Radix* at 200 mg/kg most potently increased the level of BDNF protein expressions. These results indicate that *G. M. Radix* might promote functional locomotor recovery following sciatic crushed nerve injury through enhancing BDNF protein expression.

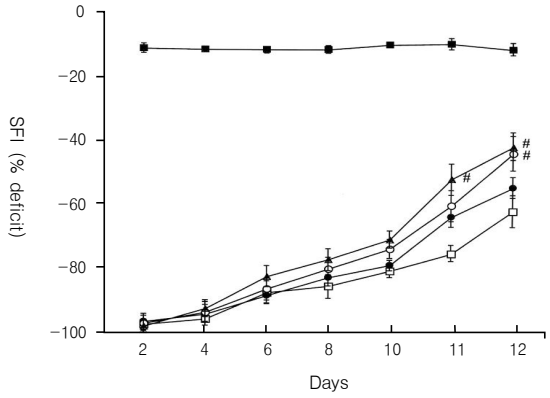


Fig. 2. Effect of *Gentianae Macrophyllae* Radix (*G. M. Radix*) on the sciatic functional index (SFI).
The values are represented as the mean ± S.E.M. # represents $P < 0.05$ compared to the operation group.
(■) Sham operation group, (□) operation group, (●)50 mg/kg *G. M. Radix*-treated group, (○)100 mg/kg *G. M. Radix*-treated group, (▲)200 mg/kg G. M. Radix-treated group.

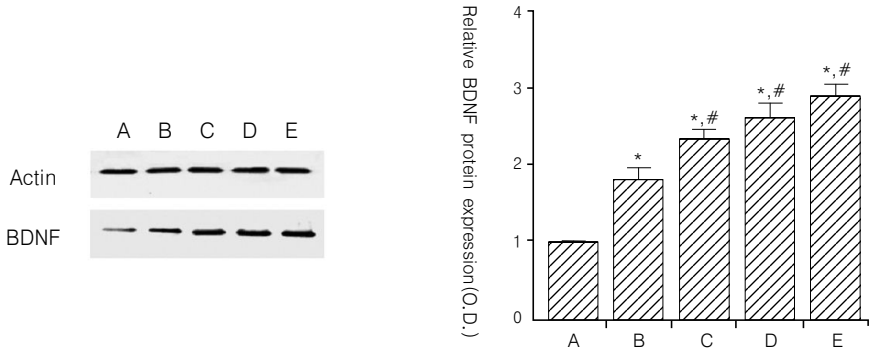


Fig. 3. Effect of *Gentianae Macrophyllae Radix (G. M. Radix)* on Western blot analysis of protein level of BDNF.

The values are represented as the mean ± S.E.M. * represents $P < 0.05$ compared to the sham-operation group. # represents $P < 0.05$ compared to the operation group.
(A) Sham operation group. (B) operation group, (C) 50 mg/kg *G. M. Radix*-treated group, (D) 100mg/kg *G. M. Radix*-treated group, (E) 200 mg/kg *G. M. Radix*-treated group.

3. Effect of *Gentianae Macrophyllae* Radix on c-Fos expression in the PVN

The number of Fos-positive cells in the PVN of the hypothalamus was 72.28 ± 5.38/mm^2 in the sham operation group, 214.28 ± 12.76/mm^2 in the operation group, 155.14 ± 12.49/mm^2 in the 50 mg/kg *G. M. Radix*-treated group, 114.57 ± 8.94/mm^2 in the 100 mg/kg *G. M. Radix*-treated group, and 88.01 ± 10.96/mm^2 in the 200 mg/kg *G. M. Radix*-treated group (Fig. 4).

In the present results, c-Fos expressions in the PVN were increased by crushed sciatic nerve injury and treatment with *G. M. Radix* significantly decreased c-Fos expression dose dependently.

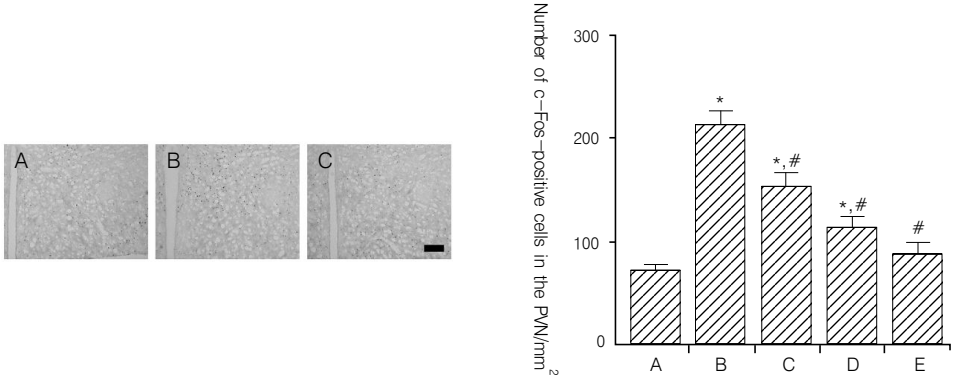


Fig. 4. Effect of *Gentianae Macrophyllae Radix (G. M. Radix)* on the c-Fos expression in paraventricular nucleus (PVN) of the hypothalamus.

Upper: Photomicrographs of the c-Fos-positive cells. The scale bar represents 100 μm.
Lower: Mean number of c-Fos-positive cells in each group.
The values are represented as the mean ± S.E.M. * represents $P < 0.05$ compared to the sham-operation group.
represents $P < 0.05$ compared to the operation group. (A) Sham operation group, (B) operation group, (C) 50 mg/kg *G. M. Radix*-treated group, (D) 100 mg/kg *G. M. Radix*-treated group, (E) 200 mg/kg *G. M. Radix*-treated group.

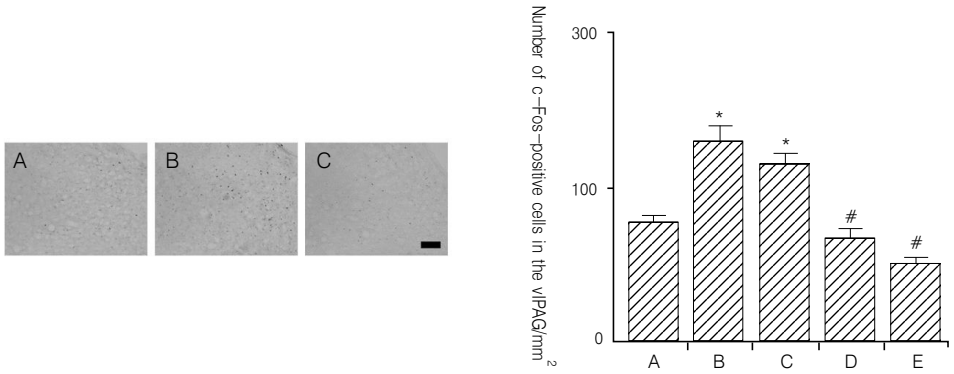


Fig. 5. Effect of *Gentianae Macrophyllae Radix (G. M. Radix)* on the c-Fos expression in ventrolateral periaqueductal gray (vlPAG).

Upper: Photomicrographs of the c-Fos-positive cells. The scale bar represents 50 μm.
Lower: Mean number of c-Fos-positive cells in each group.
The values are represented as the mean ± S.E.M. * represents $P < 0.05$ compared to the sham-operation group. # represents $P < 0.05$ compared to the operation group.
(A) Sham operation group, (B) operation group, (C) 50 mg/kg *G. M. Radix*-treated group, (D) 100 mg/kg *G. M. Radix*-treated group, (E) 200 mg/kg *G. M. Radix*-treated group.

4. Effect of *Gentianae Macrophyllae* Radix on c-Fos expression in the vlPAG

The number of Fos-positive cells in the vlPAG was 77.81 ± 3.48/mm^2 in the sham operation group, 130.75 ± 8.99/mm^2 in the operation group, 115.66 ± 7.48/mm^2 in the 50 mg/kg *G. M. Radix*-treated group, 67.86 ± 5.99/mm^2 in the 100 mg/kg *G. M. Radix*-treated group, and 52.82 ± 2.60/mm^2 in the 200 mg/kg *G. M. Radix*-treated group (Fig. 5).

In the present results, c-Fos expressions in the vlPAG were increased by crushed sciatic nerve injury and treatment with *G. M. Radix* significantly decreased c-Fos expression dose dependently.

Discussion

Peripheral nerves systems are often damaged by crush, compression, ischemia, and various diseases. In the crushed sciatic nerve injury, the injured limb displays hyperalgesia, pain-related gait, and swelling[19]. In peripheral nerve injury, the distal stump of injured axons undergoes Wallerian degeneration with breakdown of myelin sheath, recruitment of inflammatory cells from the circulation, and over-production of growth factors[20,21]. Regeneration of new axons begins earlier, within 2 days after injury, but it proceeds slowly[22]. In many studies, the effects of various therapeutic approaches have been developed to stimulate the regeneration of the nerve. Administration of neurotrophins[23] and extracellular matrix molecules[24] and application of electrical stimulation[25] have been tried, but effective medication for the treatment of neuropathic pain has not been ensured, and we are asked to make a greater and more sophisticated effort to develop new analgesics.

Herbs are a major component of traditional medicine worldwide. The *Gentianae Macrophyllae Radix (Gentianaceae)* (*G. M. Radix*) commonly known as "*Qin Jiao*" is distributed mainly in China and Siberia. *G. M. Radix* contains some bitter compounds and is slightly cold in nature. It can expel wind and dampness pathogens, relax muscle and blood vessels and clear deficient heat to treat arthralgia caused by wind dampness pathogens, contraction of the joints of the whole body and paralysis of the limbs[11,26]. *G. M. Radix* is widely used in Oriental medicine for the treatment of diverse diseases, such as fever,

diabetes, apoplexy, paralysis, osteoarthritis, and rheumatoid arthritis[12,27].

Despite the widespread application of *G. M. Radix* with other medicinal herbs in the treatment of diverse diseases, there is little information about its effect against painful neuropathy induced by crushed sciatic nerve injury. This study studied SFI level (representing functional recovery), BDNF expression and c-Fos expression after crushed sciatic nerve injury in rats and treated with aqueous extracts of *G. M. Radix*.

The SFI derived from walking track analysis in rats provides a reliable and easily quantifiable method for assessing motor function after sciatic nerve injury[28]. This gait analysis is based on the fact that rats normally walk on their digits and metatarsal footpads. Print length is therefore short in normal animals. Sciatic nerve injury causes functional loss of both extensor muscles and flexor muscles of the foot, causing drop of foot. In crushed sciatic nerve injury model, Vogelaar *et al*.[29] reported that although sensory and motor reinnervation of the paw were fully established at 3 weeks following nerve injury, persistent pain still existed and the animals could not support their weight on the injured paw. In the acute stage of crushed sciatic nerve injury, in this study flexion contracture of the toes and a curvation of the feet made it impossible to calculate SFI in some rats. The rats subjected to crush injury sometimes walk by the dorsum of the affected foot or load their weight on the medial part of the affected foot. These observations might be due to compensatory immobilization due to painful neurological loss.

In the present study, right crushed sciatic nerve injury in rats resulted in the characteristic pattern of the footprints, representing reduction in the SFI value. The SFI values of the rats in the *G. M. Radix*-treated groups significantly increased from the 8th day of the experiment, whereas the SFI value of the rats in the operation-only group remained low level until 14th day of the experiment. The present results indicate that treatment with G.M. Radix accelerated functional recovery from the locomotor deficit after crushed sciatic nerve injury.

Damage to peripheral nerves induces pain, sometimes resulting in peripheral neuropathic pain. It is generally accepted that injured nerve recruits immune cells such as mast cells and macrophages and then immune cells release allergic mediators which sensitize nociceptors and induce inflammation[30]. Under physiological conditions, BDNF synthesis is highest in the central nervous system, however BDNF is highly produced in the nonneuronal cells in the damaged nerve after peripheral nerve injury[31]. Pezet *et al*.[8] reported that BDNF might play an important role in the induction of neuropathic pain after peripheral nerve injury. In contrast, a recent study revealed that neurotrophic factors including BDNF supplied endogenously or exogenously at the site of an injured lesion reduced motor neuronal cell death and enhanced peripheral nerve regeneration[32].

In the present study, BDNF protein expression was enhanced following crushed sciatic nerve injury and that *G. M. Radix* treatment further enhanced BDNF protein expression in the sciatic nerve. The present results indicate that *G. M. Radix* accelerated peripheral nerve regeneration as well as preventing neuronal cell death after crushed sciatic nerve injury.

Expression of c-Fos is commonly used to represent activation of neurons in the brain by external inputs and plasticity following sciatic nerve ligation[33]. Previous studies have revealed that expression of c-Fos was significantly increased by painful stimuli in the frontal cortex, PVN of the hypothalamus, and PAG brain regions[34]. Neuropathic pain significantly increased expression of the immediate early gene, c-Fos, within hypothalamic PVN[35]. The periaqueductal gray matter (PAG) is a midbrain that is an important area in pain perception, antinociception, and defense reactions[36].

In the present study, c-Fos expression in the PVN of the hypothalamus and vlPAG region was increased following crushed sciatic nerve injury, indicating increased activity in the neurons of the PVN of the hypothalamus and vlPAG region. Treat-

ment with *G. M. Radix* significantly suppressed c-Fos expression in the PVN of the hypothalamus and vlPAG region. The present results represent that *G. M. Radix* suppressed the painful stimuli presenting on the PVN of the hypothalamus and on the vlPAG region following sciatic nerve injury.

Conclusion

In this study, it was shown that *G. M. Radix* increased SFI following crushed sciatic nerve injury in rats. The SFI recovery mechanism of *G. M. Radix* is presumed to be increase of BDNF expression for nerve regeneration and reinnervation. The results, where c-Fos expressions in the PVN and vlPAG were decreased by the treatment of *G. M. Radix*, showing that *G. M. Radix* might exert analgesic effect on crushed sciatic nerve injury. Further studies on identifying specific components in *G. M. Radix* associated with enhanced neural activity in the peripheral nerve injury may be helpful to develop therapeutic strategies for the treatment of peripheral nerve injury.

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