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Original Article

Effects of Herbal Bath on Functional Recovery and c-Fos Expression in the Ventrolateral Periaqueductal Gray Region of the Brain after Sciatic Crushed-Nerve Injury in Rats

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Peripheral nerve injuries are a commonly encountered clinical problem and often result in chronic pain and severe functional deficits. At the Dept. of Oriental Rehab. Medicine, we have used for pain control a herbal bath containing the following herbs: *Harpagophytum radix*, *Atractylodes japonica* and *Corydalis tuber*. In the present study, we investigated the effects of this herbal bath on the recovery rate of the locomotor function and the expression of c-Fos in the ventrolateral periaqueductal gray (vlPAG) region of the brain following sciatic crushed nerve injury in rats. In the present study, characteristic gait change with decreasing of the sciatic function index (SFI) was observed and c-Fos expression in the vlPAG was suppressed following sciatic crushed nerve injury in rats. Immersion into herbal bath enhanced SFI value and restored c-Fos expression in the vlPAG to the control value. These results suggest the herbal bath might activate neurons in the vlPAG, and could facilitate functional recovery from peripheral nerve injury.

Key Words : Herbal bath, *Harpagophytum radix*, *Atractylodes japonica*, *Corydalis tuber*, sciatic crushed nerve injury, sciatic function index (SFI), c-Fos expression, ventrolateral periaqueductal gray (vlPAG)

Introduction

Crush injury to the sciatic nerve serves as the animal model of unilateral peripheral neuropathy. The affected limb displays characteristics of painful neuropathy such as hyperalgesia, pain-related gait, and swelling[1]. These features are considered as the abnormal responses to peripheral stimuli, reflecting the changes in central nervous system (CNS) nociceptive neural transmission.

The mammalian nervous system contains networks that modulate nociceptive transmission. Of these, the descending pain control system consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla (RVM) including the nucleus raphe magnus (NRM), and the spinal dorsal horn. Neurons in the PAG and NRM project directly to the spinal cord dorsal horn. Through these descending projections, the excitability of spinal dorsal horn neurons is inhibited[2]. Decreased activity in the descending pain control system, termed 'disinhibition', has been considered to generate persistent pain after nerve injury[3]. It has been reported that activation of PAG, particular ventrolateral PAG (vlPAG), by electrical stimulation or by injection of opioids exerts analgesic action through activation of descending pain control systems.

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C-Fos protein, the product of the immediate early gene, is rapidly expressed in neurons in response to various stimuli, and c-Fos expression is recognized as a marker of increased neuronal activity[4]. In many studies, up-regulation of c-Fos expression in the vlPAG, NRM, and dorsal raphe nucleus (DR) has been suggested as the activation of descending pain control systems[5,6].

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 dropping of foot to the ground and thus induces changes of the footprints. In contrast, gradual disappearance of these changes reflects nerve regeneration and functional recovery[7]. In this way, footprints have been used to assess the sciatic nerve function. The current and standard method for measuring functional recovery after sciatic nerve injury in rats is the sciatic function index (SFI) established by De Medinaceli et al[8]. and subsequently modified by Bain et al[9]. 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.

One effect of herbal bath has been reported, that coptidis rhizoma enhances skin permeation and it's activity[10]. *Harpagophytum radix* (Pedaliaceae), popularly known as "devil's claws", is a medicinal plant originating from Southern Africa. The plant has been traditionally used by San Bushmen for a digestive tonic, headache, fever and allergy relief, and as an ointment to alleviate pain during childbirth. Recent clinical trials have established that *Harpagophytum radix* has anti-inflammatory and anti-arthritic properties for patients with degenerative joint disease[11].

Atractylodes rhizoma (Compositae) is a perennial herb distributed through East Asia. The dried rhizomes of the plants are generally used as main ingredients in various herbal formulations for the treatment of gastrointestinal diseases[12]. Aqueous extract from *Atractylodes rhizoma* has been reported to possess anti-inflammatory and analgesic activities through inhibition on lipopolysaccharide- induced cyclooxygenase-2 and inducible nitric oxide synthase expressions[13].

Corydalis tuber (Papaveraceae) is the root of Corydalis yanhuso W.T. Wang. Aqueous extract from *Corydalis tuber* has been used as an analgesic and for the treatment of inflammatory and allergic diseases[14]. The analgesic action of *Corydalis tuber* is closely associated with descending pain control systems[15].

We have used a herbal bath of *Harpagophytum radix* extract, *Atractylodes rhizoma* extract, and *Corydalis tuber* extract for pain control. In the present study, the effects of herbal bath immersion on recovery rate of locomotor function and the expression of c-Fos in the various PAG regions following sciatic crushed nerve injury in rats were investigated using walking track analysis and immunohistochemistry for c-Fos.

Materials and Methods

1. Experimental animals

Male Sprague-Dawley rats weighing 200 ± 10 g (6 weeks of age) were used. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute 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, each consisting of 12 h of light and 12 h of

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

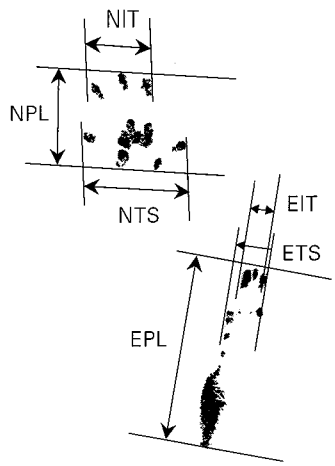

Fig. 1. Walking track analysis
After sciatic crushed nerve injury in rats, paired parameters of the print length (PL), toe spread (TS), and intermediary toe spread (IT) were taken, and these were incorporated into Bain's formula (17). E, experimental side; N, normal side; EPL, experimental print length; 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

darkness (lights on from 07:00 to 19:00 h), with food and water made available ad libitum. The rats were randomly divided into four groups (n = 8 in each group): the sham operation group, the operation (sciatic crushed nerve injury) -only group, the operation and water bath group, and the operation and herbal bath group.

2. Surgical procedure

To induce crush injury on the sciatic nerve in rats, a surgical procedure based on a previously described method was performed[16]. In brief, the right sciatic nerve was exposed through splitting incision on the gluteal muscle under pentobarbital anesthesia (50 mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA). The sciatic nerve was carefully exposed and crushed for 30 sec using a surgical clip between the sciatic notch and the point of trifurcation. Subsequently, the surgical wound was sutured and recovered. In the sham operation rats, the sciatic nerve was exposed but crushing pressure on the nerve was not applied.

3. Herbal bath immersion

72 h after the operation, the rats in the operation and herbal bath group were placed in a plastic cage 50 cm in height and 30 cm in diameter filled with herbal bath maintained at 32°C. Herbal bath immersion was applied to the level of the rat's xiphoid process for 30 min once a day for 12 consecutive days. The herbal bath was composed of aqueous extracts of *Harpagophytum radix* (3.33 g/L), *Atractylodes rhizoma* (3.33 g/L), and *Corydalis tuber* (3.33 g/L). The rats in the operation and water bath group were placed in the water bath without herbal mixture at the same temperature and in the same manner as the rats in the operation and herbal bath group.

4. 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 five times at one-day intervals through the course of the experiment as per a previously described method[9]. 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 the following equation (Fig. 1):

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.

5. c-Fos immunohistochemistry

For immunolabeling of c-Fos in the vlPAG of each brain, c-Fos immunohistochemistry was performed as per a previously described method[4]. 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, Burlingame, CA, USA) 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% H2O2 in 50 mM Tris-buffer (pH 7.6) for approximately 3 min. The sections were then washed three times with PBS and mounted onto gelatine-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.

Schematic illustration of vlPAG, chosen for the quantification of the number of Fos-positive cells is shown in Fig. 2.

6. Data analyses

The data are expressed as the mean ± SEM. 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. Immersion in the herbal bath enhanced SFI following sciatic crushed nerve injury

The mean SFI in each group was calculated on the 3rd, 5th, 7th, 9th, and 11th day after sciatic crushed nerve injury.

The SFI in the sham operation group was -15.17 ± 2.96 % on the 3rd day, -9.73 ± 5.10 % on the 5th day, -12.34 ± 3.86 % on the 7th day, -18.75 ± 1.39 % on the 9th day, and -16.60 ± 2.58 % on the 11th day at the commencement of the experiment.

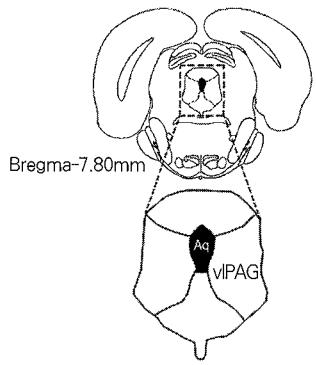

Fig. 2. Schematic illustrations of the ventrolateral periaqueductal gray (vlPAG) region where the number of Fos-positive cells was directly counted (drawn from the atlas of Paxinos and Watson, 1986). Aq : aqueduct; vlPAG: ventrolateral periaqueductal gray.

The SFI in the operation-only group was -98.21 ± 1.57 % on the 3rd day, -97.27 ± 4.97 % on the 5th day, -99.46 ± 7.64 % on the 7th day, -89.61 ± 3.59 % on the 9th day, and -81.06 ± 5.45 % on the 11th day at the commencement of the experiment.

The SFI in the operation and water bath group was -96.76 ± 2.78 % on the 3rd day, -101.93 ± 5.04 % on the 5th day, -97.44 ± 6.21 % on the 7th day, -89.73 ± 5.72 % on the 9th day, and -88.24 ± 2.55 % on the 11th day at the commencement of the experiment.

The SFI in the operation and herbal bath group was -97.14 ± 3.81 % on the 3rd day, -95.23 ± 3.01 % on the 5th day, -79.71 ± 3.71 % on the 7th day, -63.59 ± 12.97 % on the 9th day, and -61.36 ± 14.47 % on the 11th day at the commencement of the experiment (Fig. 3).

In the present results, the SFI of the sham operation group continued near zero level during the experiment. At the beginning, the SFI in all operation groups dropped near to -100 %. In the operation group and in the operation and water bath group, the SFI value continued at a low

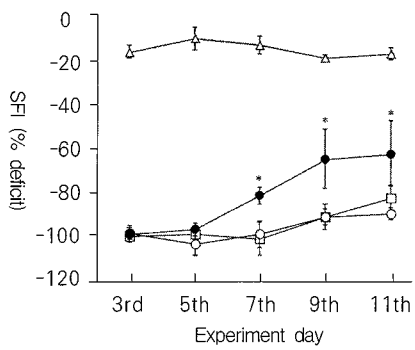

Fig. 3. Effect of herbal bath on the sciatic functional index (SFI)
The values are represented as the mean ± S.E.M. *represents P < 0.05 compared to the operation group. △: the sham-operation group; ○: the operation-only group; □: the operation and water bath group; ●: the operation and herb bath group.

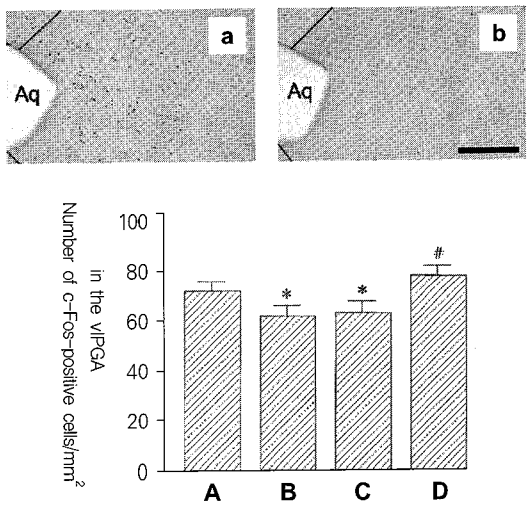

Fig. 4. Effect of herbal bath on c-Fos expression in ventrolateral periaqueductal gray (vlPAG)
Upper: Photographs of the c-Fos-positive cells expression. Aq: aqueduct; vlPAG: ventrolateral periaqueductal gray. The scale bar represents 100 μm.
Lower: Mean number of c-Fos-positive cells in each group. 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-only group
A : The sham-operation group
B : The operation-only group
C : The operation and water bath group
D : The operation and herbal bath group

level until 7th day after injury and then slowly increased. In the operation and herbal bath group, SFI value was enhanced from the 7th day and rapidly increased throughout the remainder of the experiment (Fig. 3). These results indicate that immersion into the herbal bath promotes functional locomotor recovery following sciatic crushed nerve injury.

2. Immersion in the herbal bath enhanced c-Fos expression in the vlPAG following sciatic crushed nerve injury

The expression of c-Fos in the vlPAG in each group was measured immediately after determination of last SFI.

The number of Fos-positive cells in the vlPAG was 71.41 ± 3.74/mm2 in the sham operation group, 61.85 ± 3.97/mm2 in the operation-only group, 63.00 ± 4.36/mm2 in the operation and water bath group, and 77.80 ± 3.77/mm2 in the operation and herbal bath group (Fig. 4).

In the present results, c-Fos expression in the vlPAG was reduced by sciatic crushed nerve injury but immersion in the herbal bath significantly enhanced c-Fos expression.

Discussion

Crush injury on the sciatic nerve serves as an animal model of unilateral peripheral neuropathy. Many changes affecting the ascending facilitative system and the descending inhibitory system

occur within the CNS as a result of neuropathy, resulting in the development of persistent pain. Treatment goals generally target alleviating pain and improving physical function[18].

The mechanisms underlying the generation of pain after peripheral nerve injury are complex, involving various peripheral and central components of nervous systems, and are not clear. Recent studies have proposed that the inhibition of descending pain control systems caused by decreased activation of neurons is one of the mechanisms of pain production following nerve injury[2,3]. Basbaum and Fields[19] reported that electrical stimulation on the several brain stem areas elicits anti-nociceptive processes through activation of descending pain control systems and Coimbra et al[20]. also demonstrated that electrical or chemical stimulation into vlPAG inhibits responses to noxious stimuli.

Expression of c-Fos is commonly used to represent activation of neurons in the brain by external inputs. Up-regulation of c-Fos in vlPAG, NRM, and DR induced by electroacupuncture and drugs such as morphine, antidepressant, and NMDA antagonist is associated with analgesic effect[5,6].

In the present study, c-Fos expression in the vlPAG was suppressed following sciatic crushed nerve injury, indicating decreased activity in the neurons of vlPAG. Immersion in the herbal bath significantly enhanced c-Fos expression in the vlPAG. The present results show that herbal bath facilitates neuronal activity in the vlPAG following sciatic nerve injury.

The analgesic effect of several kinds of herbal extract on neuropathic pain has been well reported. Tatsumi et al.[21] demonstrated that extracts of Moutan cortex and Coicis semen have an analgesic effect on neuropathic pain in mice. Analgesic effect of certain herbs has been suggested to be involved in the descending pain control system. Isono et al.[22] and Omiya et al[23]. showed that antinociceptive action of *Aconiti tuber* is implicated in the descending pain control system. Shin et al[24]. demonstrated that *Chelidonii herba* increases neuronal excitability in PAG, which results in activation of descending pain control systems and may contribute as a potential mechanism of the analgesic actions of *Chelidonii herba*. Cheong et al[15]. also reported that application of *Corydalis tuber* onto PAG neurons modulates glycine-activated ion current in the PAG neurons, which exerts analgesic action.

The SFI derived from walking track analysis in rats provides a reliable and easily quantifiable method for the assessment of motor function after sciatic nerve injury[25]. 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 sciatic nerve crushed injury model, Vogelaar et al[26]. reported that although sensory and motor reinnervation of the paw are fully established after nerve injury, persistent pain still exists and the animals can not support their weight on the injured paw. In the acute stage of sciatic crushed nerve injury, I observed that the flexion contracture of the toes and curvation of the feet make it impossible to calculate SFI in some rats. The rats subjected to crush injury sometimes walk on the dorsum of the affected foot or load their weight on the medial part of the affected foot. These observations may be due to compensatory immobilization to painful dysesthesia as well as neurological loss.

In the present study, right sciatic crushed nerve injury in rats resulted in a characteristic pattern of footprints, presenting reduction in the SFI value. The SFI value of the rats in the operation and herb bath group significantly increased from the 7th day of the experiment, whereas the SFI values of the rats in the operation-only and operation and water bath groups remained low until the 11th day of the experiment. The present results indicate the herbal bath accelerates functional recovery from the locomotor deficit after sciatic crushed nerve injury. The present study implies that decreased activation of descending pain control systems induced by sciatic nerve injury may consequently contribute to muscle atrophy and motor dysfunction. The herbal bath may facilitate motor performance through stimulation of the descending pain control system by activating neurons in the vlPAG.

In this study, we have shown the herbal bath activates neurons in the vlPAG, and thereby facilitates motor function. Based on the present results, the herbal bath could be used as a therapeutic intervention for pain control and functional recovery from peripheral nerve injury.

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