Effects of amygdalin on the functional recovery and c-Fos expression in the ventrolateral periaqueductal gray region after sciatic crushed nerve injury in rats

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SUMMARY

Peripheral nerve injuries are a commonly encountered clinical problem and often result in a chronic pain and severe functional deficits. The expression of c-Fos is sometimes used as a marker of increased neuronal activity. We have prepared the aqueous extract of amygdalin from Armeniacae semen for pain control. In the present study, we investigated the effects of amygdalin on the recovery rate of the locomotor function and on the expression of c-Fos in the ventrolateral periaqueductal gray (vlPAG) region following sciatic crushed nerve injury in rats. Walking track analysis for the evaluation of functional recovery and immunohistochemistry for the c-Fos expression were used in this study. In the present results, characteristic gait change with dropping of the sciatic function index (SFI) was observed and c-Fos expression in the vlPAG was suppressed following sciatic crushed nerve injury in rats. Amygdalin enhanced SFI value and restored c-Fos expression in the vlPAG to the control value. The present our study indicated that amygdalin activates neurons in the vlPAG, and it facilitates functional recovery following peripheral nerve injury.

Key words: Amygdalin; Sciatic crushed nerve injury; Sciatic function index; c-Fos expression; Ventrolateral periaqueductal gray

INTRODUCTION

Peripheral nerves are often damaged by crush, compression, stretching, contusion, ischemia, and various diseases. In the sciatic crushed nerve injury, the affected limb displays characteristics of painful neuropathy such as hyperalgesia, pain-related gait, and swelling (Bennett and Xie, 1988). These features are considered as the abnormal responses to peripheral stimuli, reflecting the changes in central nervous system (CNS) nociceptive neural transmission.

Although the pathologic mechanisms contributing to the painful sequelae of peripheral nerve injuries have not been fully elucidated, decreased activity in the descending pain control systems has been suggested as the etiologic factor of the persistent pain after peripheral nerve injuries.

The mammalian nervous system contains networks...
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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 (Vanegas and Schaible, 2004). It has been reported that activation of PAG, particular ventrolateral PAG (vPAG), by electrical stimulation or by injection of opioids exerts analgesic action through activation of descending pain control system.

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 (Lee et al., 2003). In many studies, upregulation of c-Fos expression in the vPAG, NRM, and dorsal raphe nucleus (DR) has been suggested as the activation of descending pain control system (de Medeiros et al., 2003; Hattori et al., 2004).

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 (Bervar, 2000). In this way, footprints can be used to assess 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. (1982), and subsequently modified by Bain et al. (1989). 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.

Amygdalin is one of many nitrilosides. Amygdalin is ingredient of *Prunus persica* Batsch (*Persicae semen*, Rosaceae), *Prunus armeniaca* L. var. ansu Max (*Armeniacae semen*, Rosaceae) and *Prunus amygalus* Batsch var. amara (*Amygdali semen amara*, Rosaceae), and these are abundant in the seeds of bitter almond and apricots. It was reported that aqueous extract of *Armeniacae semen* suppressed lipopolysaccharide-induced expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells (Chang et al., 2005). Amygdalin was also used to treat cancers, referring as vitamin B_{17} (Fukuda et al., 2003).

The mechanisms underlying the generation of pain after peripheral nerve injury are complex and are not clarified clearly. To date, 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 for new analgesics.

Little is known about the effect of amygdalin against painful neuropathy induced by sciatic crushed nerve injury. In the present study, we have prepared the aqueous extract of the amygdalin from *Armeniacae semen*, and we investigated the effects of amygdalin on the recovery rate of locomotor function and on the expression of c-Fos in the PAG region following sciatic crushed nerve injury in rats using walking track analysis and immunohistochemistry for c-Fos.

**MATERIALS AND METHODS**

**Experimental animals**

Male Sprague-Dawley rats weighing 200 ± 10 g (6 w of age) were used. The experimental procedures were performed in accordance with the animal care guidelines of 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, each consisting 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 six groups (n = 5 in each group): the sham operation group, the operation...
(sciatic crushed nerve injury) group, the operation and 5 mg/kg amygdalin-treated group, the operation and 10 mg/kg amygdalin-treated group, the operation and 50 mg/kg amygdalin-treated group, and the operation and 100 mg/kg amygdalin-treated group. The rats in the amygdalin-treated groups received amygdalin intraperitoneally at the respective doses, and those in the control group received an equivalent amount of saline intraperitoneally, once a day from 3rd day to 13th day at the commencement of the experiment.

Surgical procedure
To induce crush injury on the sciatic nerve in rats, a surgical procedure based on previously described method was performed (De Koning et al., 1986). 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.

Extraction of amygdalin from Armeniacae semen
Both 500 g of Armeniacae semen hatched from the shell and 10 L of 4% citric acid solution were refluxed for 2 h. After filtering when it was still hot, the filtrate was passed through the column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. 4.2 g of amygdalin (with the yield rate of 0.84%) was abained by recrystallizing the extract with ethanol. The amygdalin was used after it has been determined to be over 99.0% of purity using by high-pressure liquid chromatography (HPLC; Shiseido, Tokyo, Japan).

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 as a previously described method (Bain et al., 1989). 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 foot (EPL, ETS, and EIT). The Walking track analysis was performed by measuring the following parameters:

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

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.
Effects of amygdalin on the functional recovery and c-Fos expression (experimental) foot (EPL, ETS, and EIT). SFI values were obtained using 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.

c-Fos immunohistochemistry
For immunolabeling of c-Fos in the vlPAG of each brain, c-Fos immunohistochemistry was performed as a previously described method (Lee et al., 2003). 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$_2$O$_2$ in 50 mM Tris-buffer (pH 7.6) for approximately 3 min. The sections were then washed three times with PBS and mounted onto gelatine-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount$^\text{®}$. 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.

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
Amygdalin enhanced SFI following sciatic crushed nerve injury
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 3rd, 5th, 7th, 9th, 11th, and 13th day after sciatic crushed nerve injury.

The SFI in the sham operation group was -4.51 ± 3.85 on the 3rd day, -14.88 ± 6.98 on the 5th day, -1.90 ± 3.46 on the 7th day, -2.09 ± 5.80 on the 9th day, -1.63 ± 6.12 on the 11th day, and -1.85 ± 4.43 on the 13th day at the commencement of the experiment.

The SFI in the operation group was -99.93 ± 0.06 on the 3rd day, -102.16 ± 1.68 on the 5th day, -109.07 ± 3.07 on the 7th day, -102.08 ± 3.82 on the 9th day, -83.90 ± 4.59 on the 11th day and -88.02 ± 6.28 on the 13th day at the commencement of the experiment.

The SFI in the operation and 5 mg/kg amygdalin-treated group was -95.09 ± 3.71 on the 3rd day, -98.90 ± 4.12 on the 5th day, -109.49 ± 3.07 on the 7th day, -102.08 ± 3.82 on the 9th day, -83.92 ± 6.24

![Fig. 2. Schematic illustrations of the vlPAG region where the number of Fos-positive cells was counted.](image-url)
on the 11th day, and -64.05 ± 6.09 on the 13th day at the commencement of the experiment.

The SFI in the operation and 10 mg/kg amygdalin-treated group was -91.15 ± 2.98 on the 3rd day, -105.49 ± 2.58 on the 5th day, -98.65 ± 7.13 on the 7th day, -98.71 ± 4.17 on the 9th day, and -74.06 ± 8.44 on the 11th day, and -54.02 ± 9.31 on the 13th day at the commencement of the experiment.

The SFI in the operation and 50 mg/kg amygdalin-treated group was -95.49 ± 3.14 on the 3rd day, -101.12 ± 4.17 on the 5th day, -106.44 ± 3.38 on the 7th day, -101.15 ± 1.44 on the 9th day, -81.38 ± 8.38 on the 11th day, and -63.30 ± 9.03 on the 13th day at the commencement of the experiment.

The SFI in the operation and 100 mg/kg amygdalin-treated group was -90.58 ± 4.43 on the 3rd day, -102.30 ± 3.13 on the 5th day, -109.35 ± 4.27 on the 7th day, -93.26 ± 3.67 on the 9th day, -79.51 ± 6.60 on the 11th day, and -75.54 ± 6.54 on the 13th 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 experimental period. At the beginning, the SFI in all operation groups dropped near to -100. In the operation group and the operation and amygdalin-treated group, the SFI value was continued at the low level until 7th day after injury and then slowly increased. In the operation and amygdalin-treated group, SFI value was enhanced from the 7th day and rapidly increased throughout the experiment. On the 13th day at the commencement of the experiment, 10 mg/kg amygdalin showed statistically significant recovery effect. These results indicated that amygdalin-treated group promotes functional locomotor recovery following sciatic crushed nerve injury.

**Fig. 3.** Effect of amygdalin on the SFI. The values are represented as the mean ± S.E.M. * represents P < 0.05 compared to the sham operation group. (□) Sham operation group, (○) operation group, (▲) operation and 5 mg/kg amygdalin-treated group, (●) operation and 10 mg/kg amygdalin-treated group, (▲) operation and 50 mg/kg amygdalin-treated group, (●) operation and 100 mg/kg amygdalin-treated group.

**Fig. 4.** Effect of amygdalin on the c-Fos expression in vIPAG. Upper: Photographs 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) operation and 5 mg/kg amygdalin-treated group, (D) operation and 10 mg/kg amygdalin-treated group, (E) operation and 50 mg/kg amygdalin-treated group, (F) operation and 100 mg/kg amygdalin-treated group.
Amygdalin enhanced c-Fos expression in the vIPAG following sciatic crushed nerve injury

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

The number of Fos-positive cells in the vIPAG was 174.25 ± 11.04/mm² in the sham operation group, 124.55 ± 12.74/mm² in the operation group, 149.37 ± 5.67/mm² in the operation and 5 mg/kg amygdaulin treated group, 193.87 ± 7.59/mm² in the operation and 10 mg/kg amygdaulin treated group, 167.88 ± 5.38/mm² in the operation and 50 mg/kg amygdaulin treated group, 164.88 ± 6.03/mm² in the operation and 100 mg/kg amygdaulin treated group (Fig. 4).

In the present results, c-Fos expression in the vIPAG was reduced by sciatic crushed nerve injury and amygdaulin significantly enhanced c-Fos expression. Amygdalin at 10 mg/kg showed most potent enhancing effect on c-Fos expression. These results indicated that amygdaulin-treated group promotes functional locomotor recovery following sciatic crushed nerve injury.

DISCUSSION

Increasing population of elderly people means a rising prevalence of age-related painful conditions, but much of currently available clinical treatments are partially effective and may be accompanied by use-limiting side effects. Successful pain management is required both for the quality of life of patients suffering from intractable persistent pain and for social economic cost.

Crush injury on the sciatic nerve serves as an animal model of unilateral peripheral neuropathy. Many changes affecting on the ascending facilitatory system and on the descending inhibitory system occur within the CNS, resulting in the development of a persistent pain. Treatment goals generally target alleviating of pain and improving of physical functions (Irving et al., 2004).

Analgesic effects of several kinds of herbal extracts on the painful neuropathy have been suggested. Tatsumi et al. (2004) demonstrated that extracts of Moutan cortex and Coicis semen have an analgesic effect on the neuropathic pain in mice. Analgesic effect of certain herbs has been suggested to be involved in the descending pain control system. Isono et al. (1994) and Omiya et al. (1999) showed that antinociceptive action of Aconiti tuber is implicated in the descending pain control system. Shin et al. (2003) demonstrated that Chelidonii herba increases neuronal excitability in PAG, which results in activation of descending pain control system and may contribute as a potential mechanism of the analgesic actions of Chelidonii herba. Cheong et al. (2004) 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 assessing of motor function after sciatic nerve injury (Varejo et al., 2003). 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 crushed nerve injury model, Vogelaar et al. (2004) reported that although sensory and motor reinnervation of the paw were fully established at 3 weeks after following nerve injury, persistent pain still existed and the animals could not support their weight on the injured paw. In the acute stage of sciatic crushed nerve injury, in this study flexion contracture of the toes and a curvation of the feet caused impossible to calculate SFI in some rats. The rats subjected to crush injury sometimes walk by their dorsum of the affected foot or load their weight on the medial part of affected foot. These observations might 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 the characteristic pattern of the footprints, representing reduction in the SFI value. The SFI value of the rats in the operation and amygdalin-treated group was significantly increased from 7th day of the experiment, whereas the SFI value of the rats in the operation group remained low level until 13th day of the experiment. The present results indicated that amygdalin accelerated functional recovery from the locomotor deficit after sciatic crushed nerve injury. The present study implies that decreased activation of descending pain control system induced by sciatic nerve injury may consequently contribute muscle atrophy and motor dysfunction. Recent studies have proposed that the inhibition of descending pain control system caused by decreased activation of neurons is one of the mechanisms of pain production following nerve injury (Birklein, 2002; Vanegas and Schaible, 2004). Basbaum and Fields (1984) reported that electrical stimulation on the several brain stem areas elicited anti-nociceptive effect through activation of descending pain control system, and Coimbra et al. (1992) also demonstrated that electrical or chemical stimulation into vIPAG inhibited responses to noxious stimuli.

Expression of c-Fos is commonly used to represent activation of neurons in the brain by external inputs. Upregulation of c-Fos in vIPAG, NRM, and DR induced by electroacupuncture and drugs such as morphine, antidepressant, and NMDA antagonists is associated with analgesic effect (de Medeiros et al., 2003; Hattori et al., 2004). The levels of c-fos in the frontal cortex, thalamus, and PAG, which are key structures for the coordination of pain perception and antinociception induced by opioid, were significantly increased in the rat following sciatic nerve ligation (Minoru, 2003).

In the present study, c-Fos expression in the vIPAG was suppressed following sciatic crushed nerve injury, indicating decreased activity in the neurons of vIPAG, and amygdalin treatment significantly enhanced c-Fos expression in the vIPAG. The present results showed that amygdalin facilitates neuronal activity in the vIPAG following sciatic nerve injury. Amygdalin, a major ingredient of Armeniacae semen, has been studies for many years for its anti-cancer activity, and it was also reported that amygdalin is effective for the relief of pain (Ellison et al. 1978; Fukuda et al., 2003).

The present our results indicate that amygdalin activates neurons in the vIPAG, and thereby facilitates motor function from the locomotor deficit after sciatic crushed nerve injury through stimulation of the descending pain control system. Based on the present results, amygdalin can be used as a new therapeutic intervention for pain control and functional recovery following peripheral nerve injury.

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