행인에서 추출한 Amygdalin이 좌골신경 단열로 신경이 손상된 쥐의 기능 회복 및 중심회백질의 c-Fos 발현에 미치는 영향

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Effect of Amygdalin on the Functional Recovery and c-Fos Expression in the Ventrolateral Periaqueductal Gray Region after Crushed Sciatic Nerve Injury in Rats

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배경:
만초 신경 손상은 임상에서 자주 발생하는 질환으로 통증 조절계에 영향을 주어 만성 통증을 유발하고, 연관 부위에 운동 기능 장애를 남기는 질환이다.

목적:
본 연구의 목적은 좌골 신경 손상으로 유발된 만성 통증과 운동기능 저하에 있어 Amygdalin의 통증 감소 및 기능 회복 효과에 대하여 알아보고자 한다.

방법:
좌골 신경에 손상을 입힌 쥐에 행인에서 추출된 Amygdalin을 복강내에 투여하여, 보행 분석과 면역조직화학을 활용한 vPAG내 c-Fos 발현을 측정하여 통증 감소 및 기능 회복 정도를 판별하였다.

결과:
복강 내에 행인에서 추출된 Amygdalin을 투여한 쥐는 보행분석과 면역조직화학을 활용한 vPAG내 c-Fos 발현 정도에서 통증 감소 및 기능 회복이 보이며 유의성 있는 변화를 보였다.

결론:
Amygdalin은 만초 신경 손상에 의한 질환에 있어 통증 조절 및 운동 기능 회복에 뚜렷한 효과가 있는 것으로 생각된다.

Key words : Amygdalin, Sciatic function index (SFI), c-Fos Expression, Ventrolateral periaqueductal gray (vPAG)
I. Introduction

Crushing, compression, stretching, contusion, ischemia, and various diseases often damage peripheral nerves. In a crushed sciatic nerve injury, the affected limb displays characteristics of painful neuropathy such as hyperalgesia, pain-related gait, and swelling\(^1\). These features are considered abnormal responses to peripheral stimuli, reflecting the changes of nociceptive neural transmission in CNS.

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\(^2\). In many studies, upregulation of c-Fos expression in the ventrolateral PAG (vlPAG), the nucleus raphe magnus (NRM), and dorsal raphe nucleus (DR) has been suggested as the activation of the descending pain control system\(^3,4\).

Characteristic gait changes occur after unilateral sciatic nerve injury in rats. Sciatic nerve lesions cause loss of muscle power in both extensors and flexors of the foot. This deficit causes foot drop and footprint changes. Gradual disappearance of these changes reflects nerve regeneration and functional recovery\(^5\). 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\(^6\), and subsequently modified by Bain et al\(^7\). The 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 an ingredient of Prunus persica Batsch (Persicae semen, Rosaceae), Prunus armeniaca L. var. ansu Max (Armeniae semen, Rosaceae) and Prunus amygalus Batsch var. amara (Amygdali semen amara, Rosaceae). These are abundant in the seeds of bitter almonds and apricots. It was reported that an aqueous extract of Armeniacae semen suppressed lipopolysaccharide-induced expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells\(^8\). Amygdalin was also found to inhibit genes related to the cell cycle in snu-c4 human colon cancer cells\(^9\).

Still, the mechanisms underlying the generation of pain after peripheral nerve injury are not clear. Additionally, effective medication for the treatment of neuropathic pain has not yet been adequately determined, and the effect of amygdalin on painful neuropathy has not yet been uncovered.

Little is known about the effect of amygdalin on painful neuropathy induced by crushed sciatic nerve injury. The aim of this study was to evaluate the effects of amygdalin on the recovery rate of locomotor function and the expression of c-Fos in the PAG region following crushed sciatic nerve injury in rats.

II. Materials and Methods

1. Experimental animals

Male Sprague-Dawley rats weighing 200±10 g
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(6 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, 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 the 3rd day to the 13th day from the start of the experiment.

2. Surgical procedure

To induce crush injury on the sciatic nerve in rats, a surgical procedure was performed based on the previously described method. In brief, the right sciatic nerve was exposed through a split incision on the gluteal muscle under pentobarbital anesthesia (50mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA). The sciatic nerve was carefully exposed and crushed for 30 sec using a microvascular clip (18055-01, 60 gr/mm², Fine Science Tools, USA) between the sciatic notch and the point of trifurcation. Subsequently, the surgical wound was sutured and the rats were allowed to recover. In the sham operation, the sciatic nerve was exposed but crushing pressure on the nerve was not applied.

3. Extraction of amygdalin from Armeniacae semen

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

4. Walking track analysis

The Functional Recovery Rate after sciatic nerve injury was analyzed using a walking track, which can be quantified with SFI. Examination of the walking patterns was performed seven times at one-day intervals throughout the course of the experiment as previously described. Footprints were recorded in a wooden walking alley (8.2×42cm) 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).

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

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.

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.

5. c-Fos immunohistochemistry

For immunolabeling of c-Fos in the vIPAG of each brain, c-Fos immunohistochemistry was performed as in a previously described method\(^2\). 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. 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 50mM Tris-buffer (pH 7.6) for approximately 3 min. The sections were then washed three times with PBS and mounted on gelatin-coated slides.
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III. Results

1. Amygdalin enhanced SFI following sciatic crushed nerve injury

We measured sciatic functional index (SFI) using a walking track analysis to assess motor recovery 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 from the start 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 from the start of the experiment.

The SFI in the operation and 5mg/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 on the 11th day, and -64.05±6.09 on the 13th day from the start of the experiment.

The SFI in the operation and 10mg/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...
Fig. 3. Effect of *amygdalin* on the sciatic functional index (SFI). The values are represented as the mean±SEM. * 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

the 13th day from the start of the experiment.

The SFI in the operation and 50mg/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 from the start of the experiment.

The SFI in the operation and 100mg/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 from the start of the experiment (Fig. 3).

In the results to date, we see that the SFI in the sham operation group continued at a level near zero level during the experimental period. In the beginning, the SFI in all operation groups dropped to nearly -100. In the operation-group group, the SFI value was continued at a low level until the 7th day following injury, and subsequently 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 from the commencement of the experiment, 10 mg/kg *amygdalin* showed a statistically significant recovery effect. These results indicate that the *amygdalin* promotes functional locomotor recovery following sciatic crushed nerve injury.

2. *Amygdalin* 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 the last SFI.

The number of c-Fos-positive cells in the vlPAG 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 5mg/kg *amygdalin* treated group, 193.87±7.59/mm² in the operation and 10 mg/kg *amygdalin* treated group, 167.88±5.38/mm² in the operation and 50mg/kg *amygdalin* treated group, and 164.88±6.03/mm² in the operation and 100 mg/kg *amygdalin* treated group (Fig. 4). The present results show that c-Fos expression...
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Fig. 4. Effect of amygdalin on c-Fos expression in ventrolateral periaqueductal gray (vlPAG).
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 ± SEM.
(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

in the vlPAG was reduced by sciatic crushed nerve injury, and amygdalin significantly enhanced c-Fos expression. Amygdalin at 10 mg/kg showed the most potent enhancing effect on c-Fos expression. These results indicate that amygdalin promotes neuronal activity following sciatic crushed nerve injury.

IV. Discussion

The increasing population of elderly people means a rising prevalence of age-related painful conditions. However, many available clinical treatments are only 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 to lessen any social economic cost.

Crush injuries on the sciatic nerve can serve as an animal model for unilateral peripheral neuropathy. Many changes affecting both the ascending facilitatory system and the descending inhibitory system occur within the CNS, resulting in the development of a persistent pain.

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 etiological factor in the persistent pain after peripheral nerve injuries.

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\(^{11}\). It has been reported that activation of PAG, particularly ventrolateral PAG (vPAG), by electrical stimulation or injection of opioids exerts an analgesic action through activation of the descending pain control system.

Treatment goals generally target the alleviation of pain and the improvement of physical functions\(^{12}\).

The Analgesic effects of several kinds of herbal extracts on painful neuropathy have been suggested. For example, Tatsumi et al\(^ {13}\) demonstrated that extracts of Moutan cortex and Coicis semen have an analgesic effect on neuropathic pain in mice. The analgesic effect of certain herbs has also been suggested to involve the descending pain control system. Isono et al\(^ {14}\) and Omiya et al\(^ {15}\) showed that the antinociceptive action of Aconiti tuber is implicated in the descending pain control system. Shin et al\(^ {16}\) demonstrated that Chelidonii herba increases neuronal excitability in PAG, which results in activation of the descending pain control system and may serve as a potential mechanism of the analgesic actions of Chelidonii herba. Cheong et al\(^ {17}\) also reported that application of Corydalis tuber onto PAG neurons modulates a glycine-activated ion current in the PAG neurons, which exerts an analgesic action. Amygdalin, a major ingredient of Armeniacae semen, has been studied for many years for its anti-cancer effect. It has also been reported that amygdalin is effective for pain relief\(^ {18}\).

The SFI derived from the walking track analysis in rats provides a reliable and easily quantifiable method for the assessment of motor function after sciatic nerve injury\(^ {19}\). 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, resulting in foot drop.

In the sciatic crushed-nerve injury model, Vogelaar et al\(^ {20}\) reported that although sensory and motor re-innervation of the paw were fully established by 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 sciatic crushed 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. Rats subjected to crush injury sometimes walk on the 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 or neurological loss.

In the present study, right sciatic crushed-nerve injury in rats resulted in the characteristic pattern of the footprints, representing a reduction in the SFI value. The SFI value of the rats in the operation and amygdalin-treated group was significantly increased from the 7th
day of the experiment, whereas the SFI value of the rats in the operation group remained at a low level until 13th day of the experiment. The present results indicate that amygdalin accelerated functional recovery from the locomotor deficit following sciatic crushed-nerve injury. The present study implies that decreased activation of the descending pain control system induced by sciatic nerve injury may consequently contribute to muscle atrophy and motor dysfunction. Recent studies have proposed that the inhibition of the descending pain control system caused by decreased activation of neurons is one of the mechanisms of pain production following nerve injury\(^{21,22}\). Basbaum and Fields\(^{22}\) reported that electrical stimulation of several brain stem areas elicited anti-nociceptive effects through activation of the descending pain control system. Coimbra et al\(^{23}\) also demonstrated that electrical or chemical stimulation in vlPAG inhibited responses to noxious stimuli.

The expression of c-Fos is induced by a variety of stimuli and is commonly used as a marker for increased neuronal activity and is sometimes used to represent activation of neurons in the brain by external inputs\(^2\). Upregulation of c-Fos in vlPAG, NRM, and DR induced by electro-acupuncture and drugs such as morphine, antidepressants, and NMDA antagonists is associated with analgesic effects\(^3,4\). The levels of c-fos in the frontal cortex, thalamus, and PAG, which are key structures for the coordination of pain perception and anti-nociception induced by opioids, were significantly increased in the rat following sciatic nerve ligation\(^{26}\).

In this current study, c-Fos expression in the vlPAG was suppressed following sciatic crushed-nerve injury - indicating decreased neuronal activity - and the amygdalin treatment significantly enhanced c-Fos expression in the vlPAG. It shows that amygdalin facilitates neuronal activity in the vlPAG following sciatic nerve injury.

It is thought that amygdalin activates neurons in the vlPAG, and thereby facilitates motor function of the locomotor deficit after sciatic crushed-nerve injury through stimulation of the descending pain control system. This work suggests that amygdalin may be used as a new therapeutic intervention for pain control and functional recovery following peripheral nerve injury.

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