Facilitated Axonal Regeneration of Injured Sciatic Nerves by Yukmijihwang-tang Treatment

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Yukmijihwang-tang (YM) is used in Oriental medicine for treatments of diverse systemic symptoms including neurological disorders. The present study was performed to examine potential effects of YM on growth-promoting activity of injured sciatic nerve axons. YM treatment in the injured sciatic nerve induced enhanced distal elongation of injured axons when measured 3 and 7 days after injury. Retrograde tracing of sciatic nerve axons showed YM-mediated increases in the number of Dil-labeled dorsal root ganglion (DRG) sensory neurons and spinal cord motor neurons at 3 days after injury. Hoechst nuclear staining showed that non-neuronal cell population was largely elevated by YM treatment in distal nerve area undergoing axonal regeneration. Furthermore, phospho-Erk1/2 protein levels were upregulated by YM treatment in the injured nerve area. These data suggest that YM may play a role in facilitated axonal regeneration in injured peripheral nerves. Further investigations of individual herbal components would be useful to explore effective molecular components and develop therapeutic strategies.

Key words : Yukmijihwang-tang (YM), axonal regeneration, sciatic nerve, Erk1/2

Introduction

In the peripheral nervous system, axons after nerve injury can regenerate and reinnervate its original target organs to a certain level39. This is one of important characteristics that is distinct from the central nervous system where injured axons cannot regenerate after injury. Numerous molecular factors known to be related to peripheral nerve regeneration have been identified39.

For instance, increased levels of growth factors such as nerve growth factor (NGF), brain-derived growth factor (BDNF), and ciliary neurotropic growth factor (CNTF) in the injured nerve area function to facilitate axonal regrowth processes. These factors are produced by the injured neurons and by non-neuronal cells such as Schwann cells in the injured area. Schwann cells, a major non-neuronal cell type in the peripheral nervous system, induces their proliferation and phagocytosis activity to remove cell debris and guide for regrowing axons toward their original targets.

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However, intrinsic capability of axonal regeneration in the peripheral nerve is not perfect in that some of regrowing axons innervate incorrect targets. Several studies have been devoted to improve functional recovery; For instance, electrical stimulation of the nerves was reported to facilitate correct targeting for motor and sensory axons in the distal area from the injury area39. Overexpression of growth promoting molecules such as GAP-43 and physical training of the animals were also shown to enhance axonal regeneration39.

Thus, axonal regeneration of injured peripheral nerves is a consequence of combinatorial activities of intrinsic properties of neurons and surrounding molecular factors.

Yukmijihwang-tang (YM) is composed of Sukjihwang (Rehmanniæ Radix Preparat), Sanyâk (Dioscoreæ Rhizoma), Sansuyou (Corni Fructus), Bongnyeong (Poria), Taeksâ (Alismatis Rhizoma), Mokdanpi (Moutan Cortex) and used as an important herbal drug in Oriental medicine39. Its potential efficacy for treatment of neurological disorders is based on theoretical background in Oriental medicine.

The brain’s marrow, which indicates brain, spinal cord, and bone marrow according to modern medical expression, is functionally supplemented by the vital essence. YM is one of the representative prescriptions for ‘nourishing the Eum and reinforcing the kidney’ which is further effective for...
supplementing vital essence. Thus, YM may be involved in maintaining neuronal function, including the repair of injured nerve. In the present study, effects of YM treatment on injured sciatic nerves were investigated.

Our data showed that the treatment of YM into the sciatic nerve improved neuronal cell responses in a way that the molecular factor Erk1/2 was upregulated in neurons and axonal elongation was facilitated compared to the control.

Materials and Methods

1 Materials

1) Experimental animals

Sprague-Dawley rats (8 weeks old) were used in this experiment. They were placed in an animal room with regulated temperature (22°C), 60% of humidity, and 12-h light and 12-h dark cycle. They were allowed to eat commercial rat chow (Samyang Co., Korea) and drink water ad libitum.

2) Prescription

YM was obtained from Daejeon University Oriental medicine Hospital (Daejeon, Korea). Dried YM, which consists of six herbal components, 15 g of Rehmanniae Radix Preparat (熟地黄), 7.5 g of Dioscoreae Rhizoma (山藥), 7.5 g of Corni Fructus (山茱萸), 5.625 g of Poria (茯苓), 5.625 g of Moutan Cortex (丹皮), and 5.625 g of Alismatis Rhizoma (澤蘭), was resuspended in 2 liters of water, heat and extracted with 2 liters of water for 3 h, and filtered three times. The filtered fluid was distilled using the rotary vacuum evaporator, and concentrated solutions were frozen at -70°C for 4 h, and freeze-dried for 24 h. The yield for YM was 6 g for 50 g of the initial raw material. The product was kept at 4°C, and dissolved in water. The stock solution was stored at -20°C and used for experiment by diluting with physiological saline solution before use. For drug administration into the injured sciatic nerves, 5 μl YM (10 mg/ml) or an equivalent volume of saline (0.5% NaCl) was injected into the injury site.

2. Methods

1) Sciatic nerve surgery

Seven-week-old Sprague-Dawley rats (male, body weight 200-250 g) were housed individually in cages in a temperature-controlled room. Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg).

Sciatic nerve was exposed and crushed with a pair of forceps held tightly for 30 sec twice at 1-min intervals. Then, 5 μl YM (10 mg/ml) in saline solution or the equal volume of saline were microinjected into the crush sites. Animals were recovered from anesthesia and sacrificed 3 or 7 days later. Animals were deeply anesthetized again with a mixture of ketamine and xylazine, and sciatic nerves were separately dissected, immediately frozen, and kept at -70°C until use.

2) Immunohistochemistry

For immunohistochemistry experiment, dissected tissues were frozen immediately at -75°C and embedded into the OCT medium. The sciatic nerve or dorsal root ganglion (DRG) sections (20 μm) were cut using a cryostat and mounted on positively charged slides. Sections were used for immunofluorescence staining, or Hoechst staining. Individual experimental procedures are described below. For immunofluorescence staining, sections on a slide were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 40 min, permeabilized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 hr at room temperature. Sections were incubated with the primary antibody, and then incubated with fluorescein-goat anti-mouse (Molecular probes) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes, USA) in 2.5% horse serum and 2.5% bovine serum albumin for 1 hr at room temperature and cover-slipped with gelatin mount medium. Primary antibody reaction was performed with single or double antibodies depending on the experimental purposes, and followed with corresponding specific secondary antibody reaction. The antibodies used in the present study were anti-NF-200 antibody, anti-tubulin antibody (Tuj), anti-phospho Erk1/2 antibody, and anti-tubulin antibody (TUJ1). For some experimental purpose, Hoechst staining reaction for nuclear visualization was performed after the first washing step after secondary antibody reaction. Tissue sections were treated with 25 μg/ml of Hoechst 33258 dye (Sigma, USA) in 0.1% triton X-100 in phosphate-buffered saline solution (PBS/T) for 10 min. Cell nuclei were observed blue under the fluorescence microscope. Sections were observed with a Nikon fluorescence microscope (E-600, Nikon, Japan) and the images were captured by using Nikon camera (DXM 1200F, Nikon, Japan). The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5).

3) Western blot analysis

Nerve tissues were washed with ice-cold PBS, and sonicated under 50-200 μl of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.4, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM NaVO₄, 1% triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotime, 3 μM benzamidine, 0.5 mM DTT, 1 mM PMSF)

Protein (10 μg) was used resolved in 12% SDS polyacrylamide gel and transferred to Immobilon
polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). Blots were blocked with 5% nonfat dry milk in PBST (17 mM KH2PO4, 50 mM Na2HPO4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween-20) for 1 hr at room temperature and then incubated overnight at 4°C in 0.1% triton X-100 in PBS plus 5% nonfat dry milk containing antibodies. Protein bands were detected using the Amersham ECL kit (Amersham Pharmacia Biotech, USA), with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Transduction Laboratories, USA). Relative intensities of the protein bands were analyzed by autoradiography. The antibodies used in the present study were anti-Erk1/2 antibody, anti-phospho Erk1/2 antibody, and anti-actin antibody.

4) Retrograde tracing

The sciatic nerve of anesthetized rats with ketamine and xylazine was exposed and Dil (5 µl of 3% in dimethylsulfoxide) was applied to 0.5 cm distal to the injury site with microsyringe. The incision was sutured, and the animals were returned to the cages after they had recovered from the narcosis. Dil labeled motor neurons were visualized and counted under fluorescence microscope by an observer unaware of the experimental treatment.

Then, the mean numbers of total labeled cells in individual animals were compared among groups by Student’s t-test or one-way ANOVA.

Results

To examine the effects of YM treatment on axonal regeneration, immunofluorescence staining with anti-NF-200 antibody was performed for the sciatic nerve sections prepared 3 or 7 days after injury.

As shown in Fig. 1, NF-200 immunostaining of the intact sciatic nerve showed clearly individual axons in the longitudinal nerve sections. In sections prepared 3 days after injury, NF-200 signal intensity was gradually decreased at the distal portion of the nerves in both saline- and YM-treated groups. Yet in the saline-treated injured nerve, NF-200 signals were much weaker at 3 mm distal portion compared to SMS-treated nerve at the same position. In sections prepared 7 days after crush injury, NF-200 staining intensity was gradually decreased and very low at 14 mm distal to the injury site in saline-treated group(Fig. 1C). In YM-treated group, NF-200 staining intensity was maintained to the area distal to 7 mm from the injury site, and the staining intensity at the region 14 mm distal to the injury site was decreased but was higher than saline control.

To examine quantitatively the effects of YM treatment on axonal regeneration, retrograde tracing of Dil-labeled DRG sensory neurons and spinal cord motor neurons were analyzed. Dil was injected into 3 mm distal to the injury site and regenerated axons for DRG sensory neurons were analyzed 3 or 7 days after injury. In the intact nerve, many Dil-labeled DRG sensory neurons were visualized under the fluorescence microscope (Fig. 2A, B). In a group prepared 3 days after injury, the number of Dil-labeled neurons in the DRG were compared between saline- and YM-treated groups.

![Fig. 1. Immunofluorescence staining of the sciatic nerves with anti-NF-200 antibody.](image1)

![Fig. 2. Retrograde tracing of DRG sensory neurons after sciatic nerve injury.](image2)
The number of Dil-labeled cells in YM-treated group was significantly higher in YM-treated group than saline-treated group (Fig. 2B). Similar comparison was made for DRG tissue prepared 7 days after injury. In this case, the cell number was similar between two groups. It was also noted that the pattern of Dil-labeled neurons in YM-treated group was more intense and larger than saline control, suggesting qualitative improvement in regeneration pattern by YM treatment.

The motor neurons for sciatic nerve axons were identified in lower thoracic spinal cord by retrograde tracing of sciatic nerve motor axons. When the sciatic nerve injury was given, Dil was microinjected into the injury site and retrogradely labeled-motor neurons were visualized in the ventral horn of the spinal cord. In an intact animal group, motor neuron for sciatic axons were visualized in the longitudinal sections of the spinal cord. In the saline-injected injury group, much less cells were seen in the spinal cord at 3 days after injury. YM treatment then significantly increased Dil-labeled cells (Fig. 3A, and B). Labeled cells were much increased 7 days after injury. YM treatment further elevated labeled cells though the difference between saline and YM treated groups was not statistically different.

![Image](image-url)

**Fig. 3.** Retrograde tracing of lower motor neurons in the spinal cord ventral horn after sciatic nerve injury. (A) Spinal cord sections from uninjured intact animal (Intact) was used to count Dil-labeled neuronal cell bodies (positive control). Dil-labeled neurons were visualized under the fluorescence microscope (red). (B) All of Dil labeled cells was counted in individual fields and the mean cell number was compared among different treatments (mean±SEM, *p* < 0.05, N=5).

To determine the responses of non-neuronal cells in the injured sciatic nerve and the effects of YM treatment on non-neuronal cells, the sciatic nerve was stained with Hoechst 33258 dye to identify non-neuronal cells. In an intact sciatic nerve, a moderate level of Hoechst stained cells were observed (Fig. 4A). Nerve injury increased the number of Hoechst-stained nuclei in the nerve sections. The mean cell number from 3 mm proximal to 3 mm distal areas was higher than that from the intact group. YM treatment further increased the cell number in the same set of sections at different areas. Quantitative analysis showed significant elevation in YM-treated group in the injury site and 3 mm distal portion from the injury site. In the sciatic nerve sections prepared 7 days after injury, the mean cell number from the injury site to 10 mm distal area in saline-treated group was higher than that from the intact group (Fig. 4B). YM treatment further increased the cell number in the same set of sections at different areas. Quantitative analysis showed significant elevation in YM-treated group at 7 and 10 mm distal portion from the injury site.

![Image](image-url)

**Fig. 4.** Identification of non-neuronal cells in the sciatic nerve and comparison of the cell number among different treatments. The sciatic nerve was given crush injury and saline or YM was treated. Three days or 7 days later, the sections of the nerve tissues were prepared and used for Hoechst nuclear staining to visualize non-neuronal cells in the nerve. Nerve sections from uninjured intact animal (Intact) was used as a negative control. In saline- and YM-treated groups, the images at different positions from the injury site were collected and the number of Hoechst-stained cells was compared among different treatments (Student’s t-test).

Effects of YM treatments on levels of active form of Erk1/2 protein (phospho-Erk1/2) levels were analyzed in injured sciatic nerve and in the DRG tissues. In general, Erk1/2 activation in response to external stimulation occurs rapidly and maintains similar levels for several days. Thus, we initially investigated phospho-Erk1/2 level in sciatic nerve at 3 days after injury, and possibly accompanying changes in DRG sensory neurons at 3 days and 7 days after injury.

Western blot analysis showed low basal levels of phospho-Erk1/2 protein in the intact sciatic nerve. When the protein levels were examined at 3 days after injury, in saline-treated neve, phospho-Erk1/2 protein levels were increased, displaying particularly large increases in upper protein band (44 kDa isoform) (Fig. 5A). YM-treatment further elevated protein band intensity. Measurement of inactive total Erk1/2 protein showed similar levels in samples with different treatment.

Double immunofluorescence staining of phospho-Erk1/2 protein and neuron-selective protein NF-200 showed almost complete overlapping of the protein signals, indicating
predominant presence of phospho-Erk1/2 protein in the sciatic nerve axons (Fig. 5B).

Fig. 5. Examination of Erk1/2 protein synthesis in the sciatic nerve. The sciatic nerve was prepared 3 days after injury. When the injury was given, saline or YM was injected into the nerve. (A) Protein lysate (10 μg) prepared from the nerve tissue (1 cm length around the crush injury) was used for analysis, and Erk1/2 and phospho-Erk1/2 protein bands (43 kDa) was detected as doublets (42 kDa, 44 kDa). Western blot analysis for actin protein was performed as an internal loading control. (B) Merged images of phospho-Erk1/2 protein signals with NF-200 protein. A representative double immunofluorescence image from the sections prepared from YM-treated group was shown to localize Erk1/2 protein signals in the sciatic nerve tissue.

Above data showed the presence of phospho-Erk1/2 protein in the sciatic nerve at injury area. To determine whether phospho-Erk1/2 protein production in the injured nerve area had a prolonged effect on its presence in the neuronal cell body, DRG sensory neurons were investigated at 3 and 7 days after sciatic nerve injury. Phospho-Erk1/2 protein signals were clearly observed in the DRG at lumbar 4-6 except the scattered distribution on the edge at low levels (Fig. 6B).

In YM-treated group, phospho-Erk1/2 protein was greatly increased throughout the DRG, and these signals were found to be overlapped with neuron-selective marker protein tubulin. In an intact DRG tissue, intense red staining was seen on the edge, but they were not merged with TuJ1 protein signals suggesting non-specific signals. In the DRG at lumbar 4-6 prepared 7 days after sciatic nerve injury, comparison of phospho-Erk1/2 protein levels between saline-treated and YM-treated groups showed marked difference; strong phospho-Erk1/2 protein signals were seen in many of tubulin-positive sensory neurons in YM-treated group (Fig. 6C).

In contrast, no clear phospho-Erk1/2 protein signals were seen in the DRG sections of saline control. Merged images further showed that phospho-Erk1/2 protein signals in YM-treated group were overlapped with tubulin-positive sensory neurons.

**Discussion**

Ever since described first in ‘Soayakzengiikgyeol (小兒燉癮散), YM has been noted for nourishing the Eum and reinforcing the kidney function, and further used for the treatments of diverse disorders such as lumbago, difficulties in urination, nocturnal emission caused by exhaustion of strength due to congenital defect, loss of kidney function, and Eum deficiency of liver and kidney as well as amnesia and dizziness.**

Recent studies further implicated that YM activities can be applied for circulatory, digestive, nervous function, and can be used for anticoagulation, improved circulation, neuroprotection, regulation of blood pressure, analgesic and antiinflammatory function, antisepsis, anticancer, antiaging and immune activity. A potential function of YM for nervous system has been noted from the fact that YM can supplement the Eum and kidney function. In Oriental medicine, the concept of the kidney function is broader than the western medicine and recognized as to store the vital essence which produces medulla which, in turn, nourishes the brain and nervous system. Yet, no studies based on experimental verification have been performed yet.

In this study, YM has been examined for its involvement in growth-promoting activities of injured axons in sciatic nerve. In the sciatic nerves, the regeneration responses were examined 3 or 7 days after injury using histological, biochemical, and cell biological methods. Administration of YM into the injury site improved axonal regeneration responses compared to saline-treated group. Determination of phospho-Erk1/2 proteins levels in the injured tissues showed
upregulation by YM treatment.

The first major finding of the present study was that YM treatment in the injured sciatic nerve stimulated the production of phospho-Erk1/2 protein levels. Erk1/2 is activated by phosphorylation by upstream kinase MEK1/2 which is activated by further upstream kinases. Erk1/2 activates several proteins including Rsk phosphorylation to activate cAMP-responsiveness element binding protein (CREB), and results in increases in cell differentiation and survival. In relation to axonal regeneration, involvement of Erk1/2 activity has been suggested in several reports in which the levels of phospho-Erk1/2 protein were increased in the injured peripheral nerves. However, its specific effect on axonal regeneration is not known. Here in this investigation, phospho-Erk1/2 protein level was elevated in the injured sciatic nerve and further increases were observed in YM-treated nerve. Immunohistochemical analysis revealed that induced phospho-Erk1/2 signals were exclusively located in axons, but not in non-neuronal cells in the sciatic nerves.

Previous studies using cultured cells have reported that phospho-Erk1/2 protein, when activated at axonal terminal, can communicate to the cell body via its retrograde transport into the cell body for the induction of axonal responses. This possibility was examined by immunofluorescence staining of phospho-Erk1/2 signals in DRG neurons for sciatic nerve sensory axons at lumbar 4-6. As demonstrated in Fig. 6, increased phospho-Erk1/2 protein signals in YM-treated group above saline-treated group was clearly seen in DRG sensory neurons. Moreover, merged images in the sciatic nerve and DRG by YM treatment showed that induction of Erk1/2 activity occurred at the sensory neurons where their cell bodies are placed in the DRG, implicating that retrograde signaling of phospho-Erk1/2 protein could activate its substrate protein in the cell body area. For instance, cAMP-responsive element binding protein (CREB) is activated by Erk1/2 phosphorylation and can act as a transcription factor for target gene expression.

Further studies may be useful to identify Erk1/2-mediated activated gene in relation to axonal regeneration in YM-treated animals, and can delineate YM-specific molecular targeting for enhanced axonal regeneration. Although overall upregulation in neuronal and non-neuronal cells in the injured sciatic nerves by YM treatment cannot be interpreted for facilitated axonal regeneration responses, they may work in the concerted manner.

In neuronal cells, the signals from Erk1/2 would be communicated with cell body responses for intrinsic, enhanced axonal responses. Large increases in Hoechst-stained nuclei were positively correlated with overlapping images with S100β signals for Schwann cell identification. Schwann cells are known to play a role in removing cell debris and provide tropic factors into the axonal and therefore support axonal elongation processes. YM-mediated Schwann cell activation might contribute to facilitated axonal regeneration as demonstrated by increased NF-200 axonal staining, enhanced retrograde signals and neurite outgrowth in cultured cells as is discussed below further. The effect of YM on axonal regeneration was directly examined by retrograde tracing techniques. Retrograde diffusion of fluorescein dye DIL injected into the nerve area distal to the injury site and its visualization in the cell body can reveal the neurons that regenerate at least in the region of DIL injection area.

There are both sensory and motor axons in the nerve, and by the experimental treatment of fluorescence dye DIL to the injury area, it was possible to analyze sensory located in DRG at lumbar 4-6 and motor neurons in the ventral horn of the lower thoracic level of the spinal cord. Quantitative assessment of DIL-labeled cell bodies at DRG for sensory neurons and at ventral area of the spinal cord for motor neurons showed that at 3 days after injury, YM treatment clearly increased the number of regenerating nerves in both sensory and motor neurons. At 7 days after injury, comparison in the number of DIL-labeled cell bodies in both DRG and spinal cord showed no significant differences between saline- and YM-treated groups although YM-treated group was numerically higher than saline-treated group. One possible explanation for these changes in groups differences at 3 and 7 day-treated experimental sets might be caused by saturation of regenerating axons at the site of DIL injection at 7 days, but not 3 days, after injury.

It should also be mentioned that YM treatment at 3 days after nerve injury not only increased the number of DIL labeled cells in the DRG and spinal cord but also improved qualitatively labeling pattern of neurons. Labeling intensity and the size of DIL labeling were clearer and larger in YM-treated group than saline-treated group. This may be related with the consequence of qualitatively improved repairing of individual axons because clearer DIL signaling might be associated with more efficient diffusion through the axon.

Combined together, YM appears to enhance facilitated regeneration of both sensory and motor axons in the distal portion of the injured nerve. Since retrograde dye was injected 3 mm distal to the injury site, a long-range regeneration to the peripheral target cannot be presumed by the present data. Further studies on longer time responses at longer range.
axonal responses may help to clarify this issue.

References