

Pharma Sci- The Effects of the Novel Herbal Drug Gamijakyakgamchobuja-Tang (KCHO-1) on Neuropathic Pain in Mice- Sungchul Kim- Center of Wonkwang University

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Introduction

According to the International Association of Pain, neuropathic pain is one of the major types of chronic pain. It can be caused by central [nervous system](#) injury, peripheral nerve injury, tumors, or metabolic disorders. Patients in neuropathic pain may experience allodynia, hyperalgesia, dysesthesia, paresthesia, shooting and burning pain, electric shock-like pain, tingling, or numbness. Recent therapies for neuropathic pain include ion channel blockers such as anti-convulsant, anti-arrhythmic drugs, and tricyclic anti-depressants, because the pathways from the peripheral nerve to the spinal cord are damaged.

The expression of γ -aminobutyric acid (GABA) has been shown to be reduced in animal models of pain compared with healthy animals. GABA, an inhibitory neurotransmitter, is synthesized by the glutamic acid decarboxylases (GADs) GAD65 and GAD67. GABAergic inhibitory interneurons are located in the dorsal horn of the spinal cord and play an important role in the control of pain. Furthermore, loss of function of GABAergic inhibitory interneurons resulted in increasing pain.

After nerve injury, reactive oxygen species (ROS) play an important role in aberrant pain transmission. ROS levels are increased in the dorsal horn after [spinal cord injury](#), and the increased ROS levels downregulate GABAergic inhibitory transmission.

As a result, the balance between excitatory and inhibitory transmission is disrupted, favoring enhanced excitatory transmission and leading to neuropathic pain. In many studies, the relationship

between ROS levels and pathological pain has been clearly demonstrated. Increased ROS levels induce pathological pain, and ROS scavengers have anti-hyperalgesic effects in animal models of neuropathic pain, such as the spinal nerve ligation (SNL) and chronic constriction injury (CCI) models.

Gamijakyakgamchobuja-tang (KCHO-1) is a novel [herbal drug](#) based on Jakyakgamchobuja-tang and containing extracts from Curcuma Longa Rhizome, Gastrodiae Rhizoma, Salviae Miltiorrhizae Radix, Chaenomelis Fructus, Atractylodis Rhizoma, Polygalae Radix, and others. Jakyakgamchobuja-tang, which is composed of Paeoniae Radix Alba and Glycyrrhizae Radix e Rhizoma, is used in traditional medicine to treat abdominal pain, muscle spasms in the leg, bronchial asthma, and biliary colic.

Jakyakgamchobuja-tang has also been shown to be effective in anti-osteoarthritis. Extracts from Curcuma Longa Rhizome, Gastrodiae Rhizoma, Salviae Miltiorrhizae Radix, Chaenomelis Fructus, Atractylodis Rhizoma, and Polygalae Radix, which have [anti-inflammatory](#) and anti-oxidative properties, were added to Jakyakgamchobuja-tang to create KCHO-1.

In the present study, we tested whether the novel [herbal drug](#) KCHO-1 can alleviate neuropathic pain caused by SNL. In animals with neuropathic pain, KCHO-1 decreased pain symptoms and increased GABAergic neuronal recovery in spinal cord lesions. Moreover, KCHO-1 significantly reduced ROS-mediated GABAergic neuronal cell death compared with

saline treatment. These results suggest that KCHO-1 may provide a therapeutic benefit for the treatment of neuropathic pain by reducing ROS and enhancing the regeneration of GABAergic neurons.

Methods

Induction of Mouse Neuropathic Pain and Drug Administration

Adult C57/BL6 mice weighing 25–30 g (approximately 8 weeks of age) were housed in a controlled environment and provided with standard animal chow and water. The animals were subjected to SNL at L5 using a modification of the protocol described by Kim et al. Briefly, the animals were anesthetized using Avertin. Their skin, connective [tissue](#), and muscle were incised to expose the L6 lamina and iliac crest.

Then, the L6 transverse process and fascia were removed using microscissors and the L5 spinal nerve was ligated using thread. After surgery, the [skin](#) was sutured, and the animals were kept warm and allowed to recover from the anesthesia before being returned to their cages. Beginning 7 days after surgery, the mice were given KCHO-1 (500 mg/kg) orally once a day for 7 days. About 3 h after KCHO-1 was administered, behavior tests were conducted.

Behavior Tests

Von Frey test

For the test of mechanical allodynia, the paw withdrawal threshold (PWT) to mechanical stimulation of each hindpaw plantar surface was measured using von Frey filaments. Before the test, each mouse was placed in a plastic box with a mesh wire floor, and mechanical stimulation was applied to each hindpaw from beneath the mouse.

The mouse plantar surface was pierced using filament. The interval between stimuli was

approximately 30 to 60 sec. The incidence of foot withdrawal was expressed as a percentage of the five applications of each stimulus as a function of force. PWT was defined as the force corresponding to a 50% withdrawal rate, as determined by linear interpolation.

To reduce the effects of pre-existing differences among individuals in mechanical responsiveness, the withdrawal thresholds were also normalized by subtracting the value on the treated (ipsilateral) side from the corresponding value on the contralateral side.

Hargreaves test

After [surgery](#), the animals develop thermal hyperalgesia. Thermal pain was therefore measured by the Hargreaves test. Briefly, the mice were placed in a plastic cage on the glass table of the Hargreaves apparatus. The temperature of the glass was maintained at 27°C. Each hindpaw was stimulated using a heat source (IITC Model 390 Analgesia Meter, Series 8; Life Science Products, Frederick, CA) from beneath the plantar surface.

The heat was applied for a maximum of 20 sec, and the amount of time until the mouse withdrew its hindpaw was recorded. Thermal stimuli were applied 3–5 times for each hindpaw at approximately 5 min intervals. The hindpaw withdrawal latency time was approximately 15–20 sec in the control mice.

RNA Isolation and RT-PCR

Total RNA from spinal cords was extracted using the Pure Link RNA mini kit (Ambion, Carlsbad, CA). After isolation of total RNA, cDNA was synthesized using AccuPower RT PreMix (Bioneer, Daejeon, Korea). According to the manufacturer's protocol, 1 µg of total RNA and oligo dT [primer](#) were mixed, incubated at 70°C for 5 min, and then placed on ice.

Next, the mixture was transferred to an AccuPower RT PreMix tube and diluted to the appropriate reaction volume with RNase-free distilled water. Synthesis of cDNA was performed at 42°C for 60 min. The RTase was then inactivated at 94°C for 5 min, and reaction mixtures were stored at 4°C.

PCR was carried out using AccuPower PCR PreMix (Bioneer) as follows: Pre-denaturation at 94°C for 5 min; followed by 35 cycles of 30 sec at 94°C for denaturation, 30 sec at the annealing temperature of each primer set for annealing, and 30 sec at 72°C for extension; and final extension at 72°C for 5 min, followed by storage at 4°C.

Western Blotting

Spinal cord tissues were minced with microscissors and transferred to Pro-Prep solution (#17081, iNtRON Biotechnology, Seongnam, Korea). The minced tissue in Pro-Prep solution was homogenized (Polytron PT-2100, Kinematica AG, Littau-Lucerne, Switzerland), then incubated for at least 30 min on ice. The tissue lysate was centrifuged at 4°C/13,000 rpm for 10 min, and the supernatant was collected and stored at -20°C. The protein concentration was determined using the Bradford method. Protein lysates (20 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were transferred to nitrocellulose membranes (Whatman, GE Healthcare Life Science, Buckinghamshire, UK). The membranes were blocked with 3% bovine serum albumin in TBS-T washing buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1%, 0.1% Tween-20) for at least 1 h and then incubated with primary antibodies diluted in blocking solution at 4°C overnight. The primary antibodies used were as follows: anti-GAD65, anti-GAD67, anti-vesicular GABA transporter (VGAT), and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), and

anti-inducible nitric oxide synthase (iNOS), anti-endothelial NOS (eNOS), anti-neuronal NOS (nNOS), anti-cyclooxygenase (COX2), and anti- α -tubulin (Abcam, Cambridge, UK). The next day, membranes were washed with TBS-T three times for 10 min per wash. Then, the membranes were incubated with appropriate secondary antibodies for 2 h and washed three times with TBS-T. The membranes were developed using enhanced chemiluminescence (ECL) solution (GE Healthcare). The normalization was performed using anti-GAPDH.

Immunohistochemistry

Mouse tissues were fixed with 4% paraformaldehyde by perfusion. Next, spinal cords were collected in 4% paraformaldehyde and incubated overnight at 4°C. The tissues were transferred to 30% sucrose and incubated for at least 1 day at 4°C, then embedded using Frozen Section Compound (FSC 22, Leica, Buffalo Grove, IL) and stored at -80°C.

The tissues were sectioned to 10 µm thick using a microtome (HM525, ThermoFisher Scientific), and attached onto saline coated glass slides, then dried overnight. For immunostaining, the slides were washed with PBS three times for 10 min per wash and incubated in blocking solution (5% normal goat serum, 0.5% Triton-X100 in PBS) for at least 1 h.

Then, the slides were incubated with primary antibody diluted in blocking solution overnight at 4°C. The primary antibodies used were as follows: anti-GABA, anti-GAD65, anti-GAD67, and anti-VGAT. The next day, the slides were washed in PBS three times for 10 min per wash, incubated with the appropriate secondary antibodies diluted in blocking solution for 1 h, and washed again three times with PBS.

The secondary antibodies used were as follows: Alexa488-conjugated anti-mouse IgG, Alexa488-conjugated anti-rabbit IgG, Alexa594-conjugated anti-mouse IgG, and Alexa594-conjugated anti-rabbit IgG (Molecular Probes/ThermoFisher Scientific).

For nuclear staining, the slides were incubated with Hoechst stain (Molecular Probes/ThermoFisher Scientific) diluted in blocking solution for 5 min and washed with PBS three times for 5 min. The slides were then mounted, and cover slips were applied. Images were taken using a confocal microscope (Eclipse TE200, Nikon, Tokyo, Japan).

Dihydroethidium (DHE) Staining

For dihydroethidium (DHE) staining, slides were washed with PBS for 10 min and incubated with 2 μ mol DHE solution (Molecular Probes/ThermoFisher Scientific) for 30 min at 37°C. Slides were incubated for 30 min at 4°C to stop the reaction and washed with PBS three times. Next, the slides were stained with Hoechst to label nuclei, then mounted, and cover slips were applied. Images were taken on a confocal microscope (Eclipse TE200).

Statistical Analysis

Data are shown as the mean \pm SEM from three or more independent experiments. Behavioral tests were analyzed by twoway analysis of variance (ANOVA), and biochemical experiments were analyzed by t-tests using the statistical software program Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). For all experiments, $P < 0.05$ was considered significant.

Discussion

Here, we report that a novel herbal medicine, KCHO-1, has the potential to treat neuropathic pain in the SNL mouse model. This new drug,

Gamijakyakgamchobuja-tang (KCHO-1), contains extracts of Curcuma Longa Rhizome, Gastrodiae Rhizoma, Salviae Miltiorrhizae Radix, Chaenomelis Fructus, Atractylodis Rhizoma, Polygalae Radix, Paeoniae Radix Alba, and Glycyrrhizae Radix et Rhizoma. These herbs are included in the Korean pharmacopoeia. Curcuma Longa Rhizome in particular is used to increase circulation and has anti-oxidant and anti-inflammatory properties. Recently, this herb has been studied for the relief of pain and inflammatory bowel disease. Gastrodiae Rhizoma, which belongs to the Orchidaceae family, is actively being studied for the regulation of the GABA neurotransmitter. Salviae Miltiorrhizae Radix has also been demonstrated as an anti-oxidant. Thus, this study was performed to evaluate the ability of KCHO-1 to inhibit oxidation and relieve neuropathic pain.

Neuropathic pain is a form of chronic pain that occurs when the primary lesion or dysfunction occurs in the central or peripheral nervous system. Most causes of neuropathic pain are intractable diseases that alter the mental health of patients, inhibit their ability to function socially, and diminish their quality of life. Neuropathic pain is considered one of the most serious clinical diagnoses, because it is not controllable with analgesics and is resistant to many drugs. Thus, patients with neuropathic pain suffer for extended periods of time.

Gene expression in the dorsal root ganglion is directly altered by neuronal injury. Various animal models of neuropathic pain involve binding or cutting the entire nerve or binding around the nerve loosely. These models include the chronic pressure damage model, the partial sciatic nerve ligation model, the sciatic nerve freezing model, and the tibial and sural nerve ligation model. Among these, we used the SNL model because this method has the advantage of having a

relatively consistent level of nerve damage and symptom expression.

After SNL, the animals develop mechanical allodynia and thermal hypersensitivity. These symptoms of neuropathic pain can be quantified using the von Frey and Hargreaves tests, respectively. KCHO-1 treatment reduced both mechanical allodynia and thermal hypersensitivity compared with SNL animals treated with normal saline. These results indicate that KCHO-1 significantly reduces neuropathic pain.

GABA (the major inhibitory neurotransmitter) is generated by decarboxylation of glutamic acid by the GADs GAD65 and GAD67. One mechanism contributing to neuropathic pain is a decrease in the number of GABAergic neurons following neuronal injury. In this study, KCHO-1 led to the regeneration of GABAergic neurons, possibly contributing to the decreased neuropathic pain. By RT-PCR, western blotting, and immunohistochemistry, the expression of GAD65, GAD67, and VGAT was increased in mice treated with KCHO-1 compared with saline-treated SNL animals. In addition, the expression of GABA receptors (GABRA1, GABRB2, GABRG2, GABBR1, and GABBR2) also increased after KCHO-1 treatment. However, the expression of the glutamate receptor NMDAR2B was decreased in KCHO-1 mice. These results indicate that the reduction in GABAergic neurons induced by SNL was reversed by oral treatment with KCHO-1. Thus, KCHO-1 reduces pain by promoting the regeneration of GABAergic neurons.

The expression of NOS, which is involved in the generation of ROS, increases in injured tissue. The resulting nitric oxide (NO) plays a vital role in hypersensitivity to chronic pain and also increases the expression of COX2. As a result, ROS increase. To examine the mechanism through which KCHO-1 alleviated pain, ROS and the expression of proteins related to ROS

generation were measured in injured spinal cord tissue. KCHO-1-treated SNL mice had less ROS and lower expression of proteins involved in ROS generation than SNL animals treated with saline. These data suggest that KCHO-1 might alleviate pain by inducing the ROS scavenging machinery.

This study demonstrates that a novel herbal drug, KCHO-1, reduced ROS generation and enhanced the recovery of GABAergic neurons in the spinal cords of animals with neuropathic pain, resulting in pain relief. In conclusion, KCHO-1 may have important therapeutic activity in neuropathic pain.

Conclusion

Our study showed that KCHO-1 treatment efficiently induced functional recovery in neuropathic pain because KCHO-1 efficiently induced GABAergic neuronal regeneration and reduced occurrence of ROS. As a result, KCHO-1 may revealed a new potential role for neuropathic pain modulation.

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