

The Effect of Jeopgol-Tang 2.0 on Recovery of Bone Fracture in Sprague-Dawley Rats

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Natural Korean medicine has already been a great success, being used to treat various types of orthopedic diseases, especially fracture and osteoporosis. In this study, we find a new mixture of herbal medicine (JGT 2.0) of five well-known Korean herbal medicine for bone healing, including *Angelica gigas*, *Dendrobium moniliforme*, *Dipsaci Radix*, *Antler*, *Astragalus membranaceus*, *Conidium officinale*, *Achyranthes japonica*, *Messa medicata fermentata* on bone fracture healing. To evaluate the efficacy of JGT 2.0, the ulna of Sprague-Dawley rats was intentionally fractured and administered JGT 2.0, followed by radiographic imaging and histological examination using H&E staining to confirm the progress of recovery over time at the fracture site. X-ray imaging showed no significant difference between groups up to 1 week after JGT 2.0 administration. However, after 4 weeks of JGT 2.0 administration, bone regeneration decreased by -15% in the control group, but increased by 23.6% in the JGT 2.0 group. In addition, the length of calluses increased by 252% in the JGT 2.0 group compared to the control group. Taken together, the results suggest that JGT may offer a novel therapeutic approach to accelerate the growth of bone calluses and promote healing of bone fractures.

keywords : Bone fracture, JGT 2.0, X-ray, Rat

Introduction

A bone fracture is a partial or completely break bone. Most of bone fracture are caused by car accidents or sport injuries, but fractures may also occur due to diseases such as osteoporosis or tumors, or accumulated fatigue due to continuous vigorous exercise^{1,2}. Fractures heal by three stages: an inflammatory phase, a restoration phase, and a remodeling phase³. When a fracture occurs, not only bone tissue but also surrounding soft tissues are damaged, and a hematoma was formed in the fractured area due to rupture of surrounding blood vessels⁴.

Bone tissue newly formed after a fracture is formed by the differentiation of osteogenic cells derived from the surface of the epioseum or endosteum or by the differentiation of fibroblasts in the connective tissue around the bone into osteogenic cells by appropriate stimulation^{5,6}. During the fracture healing process, various factors related to bone formation affect the healing rate, but the related changes in indicators of bone metabolism are not well

known. In the bone formation process or calcification mechanism, there are intramembranous ossification and endochondral ossification, and inorganic salts are deposited together with the action of osteoblasts and osteoclasts to change into hard bones⁷⁻⁹. When a fracture occurs, active proliferation of osteogenic cells present in the periosteum at the shortest distance from each fracture surface occurs, the periosteum becomes thick, and at this time, capillaries appear around it, and the proliferation of osteogenic cells becomes more vigorous^{1,10,11} differentiates into osteoblasts and secretes bone matrix on the cut bone surface to form osteogenic tissue^{12,13}.

Our previous study reported that the JGT improved low mineral density in a middle-aged man¹⁴. They took two packs of master (120mL/pack) every morning and evening. After taking JGT, calluses and bone cross-linking were confirmed in the follow-up image, and patients described their experience of pain reduction in post-recovery interviews. However, in this study, we found a new mixture of herbal medicine (JGT 2.0) of five well-known herbal

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medicine for bone healing, including *Angelica gigas*, *Dendrobium moniliforme*, *Dipsaci Radix*, *Antler*, *Astragalus membranaceus*, *Conidium officinale*, *Achyranthes japonica*, *Messa medicata fermentata*. To investigate the effects of JGT 2.0, the ulna of Sprague-Dawley rats was intentionally fractured, followed by administration of JGT or JGT 2.0, followed by radiographic and histological examination to confirm the progress of recovery over time at the fracture site.

Materials and Methods

1. Prescription of Jeopgol-tang (JGT) and JGT 2.0 (Table 1.)

Dried herb samples (Human Herb, Daegu, Korea) were immersed in 10 volumes of dH₂O and boiled at 80°C for 2 hours, and then the water extract was collected. The above process was repeated once, and the extracts were combined, concentrated with a rotary evaporator, and then vacuum-dried to yield 27.78 % (w/w) of the extract.

Table 1. Prescription of Jeopgol-tang (JGT) and JGT 2.0

Jeopgol-tang (JGT)	(g)
<i>Cnidium officinale</i>	20
<i>Angelica gigas</i>	20
<i>Astragalus membranaceus</i>	8
<i>Panax ginseng</i>	8
<i>Lycium barbarum</i> Linné	8
<i>Codonopsis pilosula</i>	8
<i>Cuscuta chinensis</i>	4
<i>Dipsacus asperoides</i> C. Y. Cheng et T. M. Ai	4
<i>Dendrobium nobile</i> Lindley	4
<i>Pasoralia cotylifolia</i>	4
<i>Cervus elaphus</i> Linné	4
Jeopgol-tang 2.0 (JGT 2.0)	(g)
<i>Angelica gigas</i>	20
<i>Dendrobium moniliforme</i>	16
<i>Dipsaci Radix</i>	12
<i>Antler</i>	10
<i>Astragalus membranaceus</i>	8
<i>Cnidium officinale</i>	8
<i>Achyranthes japonica</i>	4
<i>Messa medicata fermentata</i>	4

2. Animals

All experiments on animals were conducted with the approval of the Ethics Committee of Daegu Haany University (DHU2021-020) and in accordance with the US National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH Publication no. 80-23, revised 1996).

42 Sprague-Dawley male rats (Hyochang Sci., Daegu, Korea) that weighed 230-250 g (6 weeks old) each were used for the experiments. Male mice were group housed (three per cage) with an inverted light/dark cycle (light on from

07 : 00-19 : 00 hr). The room temperature was $22 \pm 2^\circ\text{C}$ and the humidity was $50 \pm 15 \%$. The rats had free access to food and water. All rats were handled daily for at least one week prior to the experiment. Rats were randomly divided in two groups: 1) Control: ulna bone fracture operation and no treatment and 2) JGT: ulna bone fracture operation + treatment of JGT (10 mg/kg) and 3) JGT 2.0: ulna bone fracture operation + treatment of JGT 2.0 (10 mg/kg). Experimental schedule was presented in Fig. 1.

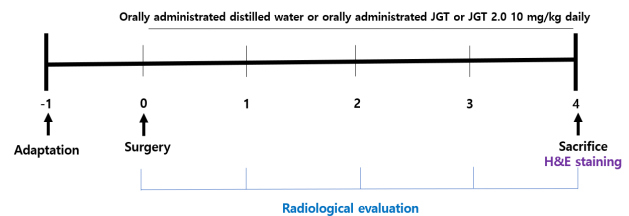


Fig. 1. Experimental schedule

3. Bone defect model

The rats were anesthetized with Ketamine HCl (Keaset 10 %; Fort Dodge, IO50501 USA, 85 mg/kg, i.p.) and Xylazine (XYL-M 2 %; VMD n.v., Berendonk 74-B-2370, Arendonk, Belgium, 3 mg/kg, i.p.). After cleanly shaving the right forelimb, a skin incision of about 3 cm was made and the bone was cut about 2-3 mm using osteotomy. After cutting the bone, the skin was sutured again.

4. Bone damage range

Bone damage range shown on x-ray film was measured once a week using dial calipers (dial calipers, OZAKI MGF.Co., Ltd, Japan). Bone gap immediately after surgery is set to "0".

5. Measurement of callus formation

After oral administration of JGT or JGT 2.0 for 4 weeks, 2 mm of the anterior and posterior parts were extracted centering on the fracture line of the ulna and H&E staining was performed. All calluses were fixed in 4 % buffered formaldehyde for 48 h and then decalcified in 7 % nitric acid solution for 1 week. After that, demineralized tissues were washed for 5 min, dehydrated in alcohol and then, embedded in paraffin wax. All sample cut into 4 μm thick sections. These sections were stained with hematoxylin and eosin. The stained area was observed with an Olympus microscope (BX53, Miami, USA) to measure the length of the regenerated callus from the fracture line. As the callus is formed and bone is regenerated, the value increases.

6. Statistical analysis

The data were analyzed using the one-way ANOVA test followed by the LSD post-hoc test using the SPSS 15.0 program ($P < 0.05$). All the data were marked mean \pm S.E., and the significance level between groups.

Results

1. The bone length growth

As shown in Fig. 2(A) and (B), x-ray imaging was performed weekly to measure the ulnar gap. In observing the x-ray images of bone setting progress for 4 weeks, bone length growth is rated $-0.67 \pm 0.07 \mu\text{m}$ in control group, $-0.67 \pm 0.12 \mu\text{m}$ in JGT group and $-0.44 \pm 0.10 \mu\text{m}$ in JGT group starting from the first week. Until 1 week after administration during the experiment, there was no significant difference among groups. After 2 weeks, bone length growth significantly progressed in JGT 2.0 group compared to the control group ($F(6, 75) = 1.843, P < 0.05$). After administration of JGT or JGT 2.0 for 4 weeks, bone length growth showed $-0.32 \pm 0.29 \mu\text{m}$ in control group $0.39 \pm 0.33 \mu\text{m}$ in JGT group and $0.77 \pm 0.18 \mu\text{m}$ in JGT 2.0 group. The recovery rate observed through x-ray images shows that the JGT 2.0 group recovers three times faster than the control group ($P < 0.05$).

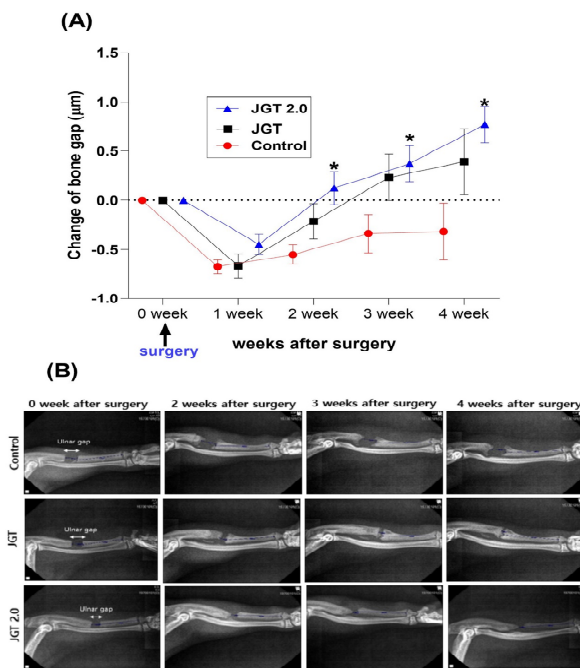


Fig. 2. Comparison of bone length growth (A) and the representative radiographs image of rats'ulna bone fracture (B). Data represent the mean \pm S.E.M. * $P < 0.05$ compared with Control.

2. The length of the callus at the fracture line

The histological result was shown in Fig. 3(A) and (B). In the histological photographs, at week 4 post-fracture, the control group exhibited the domination of callus hyperplasia along with the appearance of the woven bone, whereas a larger woven bone was developed in the JGT 2.0 group. Also, the length of the callus in JGT 2.0 group was increased by 252 % compared to that of control ($P < 0.001$). This result proved that JGT 2.0 has improved greatly bone healing and bone regeneration.

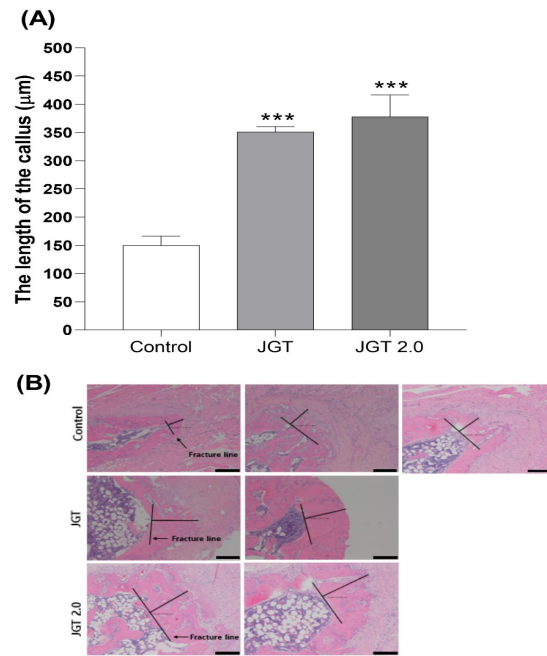


Fig. 3. The representative histological photograph of peri-fracture tissues (A) and the length of the callus at the fracture line (B) Histological evaluation of fracture. H&E staining of fracture callus sections. Magnification, $\times 40$; scale bar = 200 μm . Data represent the mean \pm SEM. *** $P < 0.001$ compared with Control.

Discussion

After 2 weeks, bone recovery was significantly progressed in the JGT 2.0 group compared to the control group. After 4 weeks of JGT 2.0, bone length growth increased significantly after 3 times compared to the control group. On histological photography, 4 weeks after fracture, the control group showed dominance of callus hyperplasia with the appearance of the weaving bone, whereas in the JGT 2.0 group, a larger weaving bone developed. These results suggest that administration of JGT 2.0 may help speed up bone healing and bone regeneration.

A fracture is a break in a bone, similar to a crack or break. Bones can be completely fractured or partially

fractured in several ways. Also, fractures take weeks to months to heal, depending on the severity of the injury and how well you follow treatment. Healing fractures requires surgery, slings, braces, or other devices, as well as proper nutrition and overall health. However, natural oriental medicine has already achieved great success in treating many types of orthopedic conditions, including osteoporosis, bone fracture, and rheumatism¹⁵. In addition to fundamental treatment of fractures, a healing process that can reduce recovery time is necessary for patients to quickly recover from their daily lives¹⁶.

Our previous study proved that the JGT treatment improved the bone mineral density (BMD) in 44-year-old male who had BMD of in lumbar spine scan¹⁴ and also JGT prescription was useful for treatment of delayed bone union¹⁷. However, JGT 2.0 was excluded because *Albizia julibrissin* Durazz because it is hepatotoxic¹⁸. Also, Deer antler was added based on research results showing that deer antler is more effective in treating osteoporosis than deer antler¹⁹. In this study, five well-known herbal medicines for bone healing, including *Angelica gigas*, *Dendrobium moniliforme*, *Dipsaci Radix*, Antler, *Astragalus membranaceus*, *Conidium officinale*, *Achyranthes japonica*, *Messa medicata fermentata*, *Angelica gigas*, were newly discovered as a mixture (JGT 2.0). Antler has the effects of nourishing the blood, relieving pain by activating blood circulation, and increasing intestinal energy absorption, and is used for symptoms, such as syndrome of heart-liver blood deficiency, menopause, afterpain, contusion injury, pain caused by a large abscess, and constipation^{20,21}. *Dendrobium moniliforme* has the effects of promoting the secretion of gastric juice by strengthening the stomach, and removing fever by cultivating yin energy, and is used to treat symptoms such as thirst caused by fever^{22,23}. *Dipsaci Radix* has an effect of strengthening the liver and kidney and connecting broken bones or muscles and is used to treat symptoms such as the lack of proper blood of the liver and kidney and back and leg pain^{24,25}. Antler is used in the present invention is obtained by cutting and drying the ossified horns of *Cervus nippon* Temminck, *Cervus elaphus* Linne or *Cervus canadensis* Erxleben belonging to the family Cervidae. The antler has the effects of nourishing the vitality of the kidneys, boosting yang energy, promoting bone growth by promoting the synthesis of protein and nucleic acids, promoting hematopoiesis and is known to improve immune function and to be effective against liver disease and osteoporosis²⁶. *Astragalus membranaceus* is known to have a remarkable effect on the treatment of knee diseases

(arthritis, rheumatoid arthritis, and inflammation causes by bruises). It is also frequently used in the case in which the waist and leg feel heavy^{27,28}. *Conidium officinale* is used in boosting yang qi, protecting and firming the skin, removing toxins such as inflammation, giving new flesh, facilitating moisture drainage, and removing edema, and is used to treat symptoms such as spleen qi deficiency and symptoms of weakness in both nasal and lungs^{19,29}. *Achyranthes japonica* is used to relieve cerebral blood vessel spasms while lowering blood pressure when there are symptoms such as headache, dizziness, and eye irritation in hypertension³⁰⁻³². *Messa medicata fermentata* mainly acts on the spleen and stomach to strengthen the function of the digestive system, has an action to comfort the stomach with the action to help digesting, and exhibits effects against an upset stomach, a stuff chest, vomiting and diarrhea and abdominal pain due to extravasated blood after childbirth³³. Antler is used for treatment of osteoporosis³⁴. Also, polysaccharide of *Astragalus* promote angiogenesis via activation of TLR4 signaling pathway³⁵. Another study also proved the isoflavone of *Astragalus membranaceus* has prevented osteoporosis development³⁶. Park et al reported that the *Achyranthes japonica* can help bone density in ovariectomized induced osteoporotic mouse model³⁷. *Achyranthes japonica* inhibit osteoblast and osteoclast cells in ovariectomized mice³⁸. Consistent with previous study, after 4 weeks of JGT 2.0 administration, bone length growth significantly progressed in JGT 2.0 group compared to the control group. After 4 weeks of JGT 2.0 administration, bone regeneration showed a decrease of -15% in the control group, but the bone regeneration rate increased by 23.6% in the JGT 2.0 group. Also, histochemical result showed that at week 4 post-fracture, the control group exhibited the domination of callus hyperplasia along with the appearance of the woven bone, whereas a larger woven bone was developed in the JGT 2.0 group. The length of the callus in JGT 2.0 group was increased by 252 % compared to that of control. The recovery process of a fracture proceeds in the following order: inflammatory phase, repair phase, and remodeling phase. During the inflammatory phase, the bone cells that have fallen from the fracture site are absorbed, so the fracture surface becomes wider when seen on an x-ray. Next, during the recovery period, the hematoma changes into soft callus and the fracture interval decreases. JGT 2.0 group recovered the inflammatory phase quickly, and produced soft callus faster compared to the JGT group. Taken together, these results assume that JGT 2.0 may be effective in reducing bone damage during the

early inflammatory phase of fracture and promotes bone healing after restoratinocaused by fractures via activation of cartilage callus formation.

To our knowledge, this will be the first pre-clinical trial to evaluate the efficacy of JGT 2.0 in an animal model of bone fracture. The results of this study are expected to contribute to the development of new herbal medicine mixture that can be used for bone regeneration treatment. The limitation of this study is that the small number of samples, and bone density measurement and morphological observation were not performed for elucidation of the effect of JGT 2.0 on bone fracture recovery mechanism. However, that X-ray examination showed that the bone regeneration rate of JGT 2.0 treated group was markedly faster than that of the control group that is considered to have a positive effect on fracture treatment.

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