

Original Article

Effects of Yuhyangjeongtong-san on Fracture Healing in Rats

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Objectives: The purpose of this study is to determine the effect of *Yuhyangjeongtong-san* on the recovery of tibial fractures in rats.

Methods: In this study, osteocalcin and Calcitonin, CTX-2, TGF- β and BMP-2, which are used as indicators of bone formation, were analyzed after hematologic fractures using experimental rats. In addition, the fracture union process was confirmed using X-rays.

Results: Osteocalcin, Calcitonin and BMP-2 showed a significant increase compared with the control at 4 weeks. CTX-2 and TGF- β showed a significant increase compared with the control at 3 weeks. On X-ray, YJS treated group, as the experiment progressed, the boundary line became blurred, the bone outline was clearly visible, and the fracture recover was progressing.

Conclusion: The findings suggest that YJS can play a significant role in the repair of fractures. Therefore YJS is likely to be used to treat fractures.

Key Words : *Yuhyangjeongtong-san*, Fracture, Rat, Tibia, Korean medicine.

Introduction

A fracture is a condition in which the continuity of a bone is completely or incompletely lost or a linear deformation occurs. Fractures are mostly accompanied by damage to the skin, subcutaneous tissue, fascia, blood vessels, and nerves¹. Elderly people are more likely to experience fractures with a small impact. In addition, the risk of fracture increases as the proportion of sporting activities performed in their spare time increases².

Fractures differ according to their location and extent, but most require conservative or surgical treatment, such as a splint fixation or gypsum fixation³. This treatment process leads to limited activity and a long treatment duration, which causes a great loss not only for each individual but also for society. In general, various methods, such as weight bearing, fixation, and drug administration, are used for promoting the Western healing of fractures⁴. Therefore, new therapies are needed to complement or replace existing ones.

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Fractures heal through inflammatory, restorative, and remodeling phases. Chinese herbal medicine has the advantage of selecting an effective drug through the dialectic according to the healing stage of these fractures¹⁾. Therefore, personalized treatment according to the present condition of the patient is possible. In addition, it is expected that the treatment period may be shortened by administering an herbal medicine that promotes bone union in patients who have undergone conventional orthopedic treatment. In particular, in elderly patients, it is possible to combine drugs in the consideration of systemic conditions. To take advantage of such herbal treatments, an experimental study on herbal medicines that acts at each stage of healing must be performed.

In the previous study, there was experiment for the results of Yukmijihwang-tang and Cervi Pantotrichum Cornu⁵⁾ administration for fracture treatment. There were three trials in which Neutral Eohyeol(Yuxue) Herbal Acupuncture and Dangkisoo-san⁶⁾ was administered to treat fractures. For monodrugs, Pyritum⁷⁾ was used. As a combination drug, Jeopgolsan⁸⁾ and Bokwonhwalhyul-tang⁹⁾ has been reported to have a significant effect. There is a need for systematic research on more diverse Korean herbal prescriptions.

Yuhyangjeongtong-san(YJS) is a medicine used for injuries. It is said that this is effective for trauma, such as bruises¹⁰⁾. Previous studies have shown that YJS is effective for arthritis¹¹⁾. Clinically, it has been reported to be effective in reducing pain from injuries caused by traffic accidents¹²⁾. Therefore, YJS is expected to be effective for fracture union. However, there has been no study on YJS and fracture recovery.

The authors thought that the initial treatment of fractures was anti-inflammatory. In this study,

osteocalcin and Calcitonin, CTX-2, TGF- β and BMP-2, which are used as indicators of bone formation, were analyzed after hematologic fractures using experimental rats. In addition, the fracture union process was confirmed using X-rays. Based on the experimental results, we obtained knowledge about the effects of YJS fracture repairs.

Materials and Methods

1. Materials

1) Cells

RAW 264.7 cells were purchased from Korea Cell Line Bank (Seoul, Korea).

2) Medicinal herbs

The composition of the medicine was referred to Donguibogam¹⁰⁾. The medicinal herbs that the frankincense (hereinafter referred to as YJS) used in this experiment were purchased through OmniHub (Daegu, Korea) (Table 1).

Table 1. Prescription of Yuhyangjeongtong-san per Pack in Donguibogam

pharmacological name	Dose(g)
Angelicae Dahuricae Radix	4g
Angelicae Gigantis Radix	4g
Rhmanniae Radix	4g
Moutan Cortex	4g
Paeoniae Radix Rubra	4g
Cnidii Rhizoma	4g
Olibanum	4g
Myrrha	4g
Atractylodis Macrocephalae Rhizoma	4g
Glycyrrhizae Radix	4g
Total	40g

3) Animals

5-week-old male SD(Sprague-Dawley) Rat (120~130 g) was supplied from Samtaco (Gyeonggi-do,

Korea). Humidity was maintained at $55 \pm 15\%$ at 2°C ., adapted for 2 weeks in a 12-12 hour (light-dark cycle) environment. Rats were divided into three groups of 12 animals each. Normal group was fed saline without causing fracture. The control group induced fractures and fed saline. The YJS group fed 2 ml of YJS concentrate at $200 \mu\text{g} / \text{ml}$ after the fracture.

2. Methods

1) Specimen

YJS 5packs (200 g) was added 4,000 ml of DW and extracted for 4 hours filtrate was obtained and concentrated under reduced pressure using a rotary vacuum evaporator. As a concentrated solution, 19.7 g of lyophilized powder was obtained using a freeze dryer (yield 9.85%), and the obtained powder was used after diluting to a required while being stored in a cryogenic freezer (-80°C).

2) Culture of RAW 264.7

The frozen 264.7 cells were transferred to a 50ml tube, and 9 ml of PBS was added. The cells were suspended centrifuged at 1,200 rpm for 5 minutes, and the supernatant was removed. 1 ml of 1% penicillin and 10% fetal bovine serum (FBS) were added to the tube containing cells, and 1 ml of DMEM was suspended. A total of 9 ml of medium was placed on a 100-mm dish, and the cells were suspended and incubated with a cell incubator (37°C , $5\% \text{CO}_2$). The number of passages was 10 times or less. Twenty-four hours were adapted before processing the samples.

3) Cytotoxicity

RAW 264.7 cells were dispensed at 1×10^5 cells/well in 96-well plates and incubated for

24hours. Before the start of the experiment was replaced with a new culture, YJS was treated at concentrations of 10, 50, 100, 200, and $400 \mu\text{g}/\text{ml}$ and incubated for 24 hours. After incubation, $10 \mu\text{l}$ of water-soluble tetrazolium salt solution was added, followed by reaction for 30 minutes using a cell incubator (37°C , $5\% \text{CO}_2$). The change in absorbance at 450nm was measured to express the survival rate of the cells as a percentage with respect to the control group.

4) Tibial fracture induction

The animals were anesthetized by intraperitoneal injection $1 \text{mg}/\text{kg}$ zoletil. After anesthesia, the left hind legs of the experimental animals were placed on a flat plate and a blunt blade composed of was placed in the middle of the tibia. A tube with a diameter of 3.5 cm and a length of 70 cm was erected on a blunt blade, and then a metal weight of 18 g and 3 cm in diameter was dropped. After confirming the fracture, the fracture site was fixed with fixed tape for an orthopedic sprint (Kumjeong Chemical, Korea).

5) Group assignment and sample processing

The experiment was divided into three groups, and 12 animals were assigned to each group. Once daily, at 2 pm, 2 ml was administered orally. We followed allowable dose per 100 g per SD rat according to the KFDA Animal Testing Guidelines. A free diet was provided during the experiment. Oral administration was performed for a total of 4 weeks at time point 0 when a tibia fracture was induced.

6) Serum separation

In the experiments, 0, 1, 2, 3, and 4 weeks after anesthesia with ethyl ether, blood was

collected by cardiac puncture and centrifuged at 3,000 rpm for 15 minutes to determine the factors related to fracture healing.

7) In vivo

(1) Osteocalcin measurement

Osteocalcin was measured using Osteocalcin ELISA kit (R & D, U.S.A.). A total of 100 μ l of biotinylated osteocalcin was dispensed into each well and plate mixed for 30 minutes. After washing the coated plate with the washing buffer solution, primary antibody, primary incubation buffer, standard, control, and serum added and plate mixed for 1 hour. After washing with the washing buffer solution again, 100 μ l of secondary antibody was added to each well for 15 minutes, plate mixing was performed, 100 μ l of stop solution was added, and the absorbance was measured at 450 nm using an ELISA reader.

(2) Calcitonin and CTX II measurement

Calcitonin and CTXII were measured using a calcitonin and CTXII ELISA kit (MyBioSource, U.S.A.). A total of 50 μ l of the standard, control, and serum was dispensed into each well, 100 μ l of horse radish peroxidase-conjugate was added and mixed, and the resultant was placed in an incubator at 37 °C for 1 hour. After the reaction was washed with a washing buffer solution, 100 μ l of the chromogen solution was reacted for 15 minutes in an incubator at 37 °C again. Finally, 50 μ l of stop solution was added and the absorbance was measured at 450 nm using an ELISA reader.

(3) TGF- β and BMP-2 measurement

TGF- β and BMP-2 were measured using the TGF- β and BMP-2 ELISA kits (R & D, U.S.A.).

First, 1 N HCl solution was added to 40 μ l of serum for 10 minutes in a 37 °C incubator for TGF- β measurement, and then 10 μ l of 1.2 N NaOH/0.5 M HEPES was mixed to prepare a sample. Afterward, 50 μ l of assay diluent was added to each well for β measurement, 100 μ l of BMP-2 was measured, and 50 μ l of the standard, control, and serum was added for 2 hours. The reaction was carried out in an incubator at 37 °C, and the BMP-2 measuring plate was mixed. After washing with a washing buffer solution, 100 μ l of TGF- β conjugate and 200 μ l of BMP-2 conjugate were added and reacted for another 2 hours. After the reaction, the resultant was washed with washing buffer solution again, 100 μ l and 200 μ l of substrate solution were reacted for 30 minutes, and 100 μ l and 50 μ l of stop solution were measured absorbance was measured at 450 nm using an ELISA reader.

8) Radiography

X-rays were taken at weeks 0, 2, and 4 using an Inalizer (Medikors, Korea).

3. Statistical processing

The results were expressed as mean \pm standard deviation (Mean \pm S.D.) using the SPSS 18.0 statistical program, and the significance was verified at the *: $p < 0.05$, and **: $p < 0.01$, using ANOVA and the post-hoc Duncan test.

Result

1. Cytotoxicity

YJS was administered to the RAW 264.7 cells at different concentrations, and the cell viability was measured. As a result, cell viability rates of $100.00 \pm 0.73\%$, $99.23 \pm 1.72\%$, $98.51 \pm 1.27\%$,

97.47 ± 2.53%, 93.29 ± 2.56%, and 88.17 ± 2.65% were observed at the concentrations of the control at 10, 50, 100, 200, and 400 µg/ml, respectively. At concentrations above 400 µg/ml, there was a significant decrease in cells (Fig. 1).

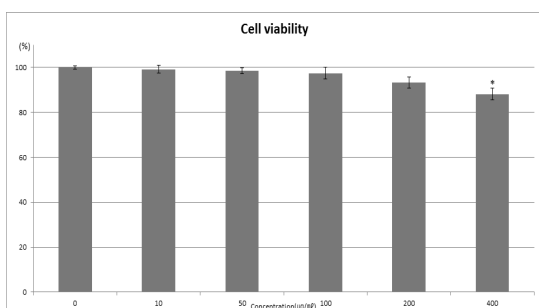


Fig. 1. Cell viability of YJS extract in RAW 264.7 cells. The results were represent the mean ± S.D.(Significance of result,*: $p < 0.05$ compared to control)

2. Osteocalcin production

Effects on osteocalcin production were observed. As a result, the osteocalcin production rates of 0.70 ± 0.04 pg/ml, 0.71 ± 0.02 pg/ml, 0.72 ± 0.01 pg/ml, 0.71 ± 0.06 pg/ml, and 0.73 ± 0.02 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on normal. production rates of 0.69 ± 0.03 pg/ml, 10.36 ± 0.17 pg/ml, 9.78 ± 0.46 pg/ml, 9.06 ± 0.37 pg/ml, and 8.32 ± 0.28 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on control. production rates of 0.73 ± 0.03 pg/ml, 10.21 ± 0.26 pg/ml, 11.54 ± 0.53 pg/ml, 10.53 ± 0.47 pg/ml, and 9.82 ± 0.34 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on YJS at concentration 200 µg/ml. There was a significant increase in cells at concentratio at week 4 (Fig. 2).

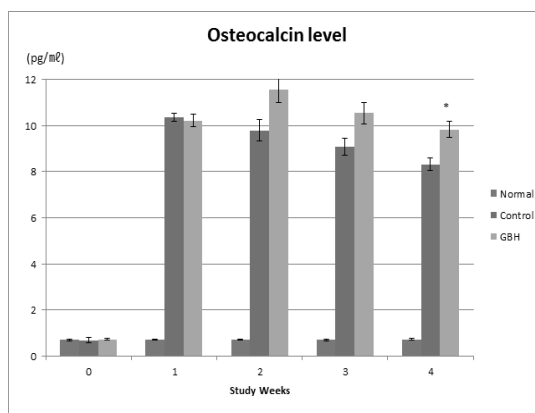


Fig. 2. Effect of YJS on the level of Osteocalcin in serum of tibia fractured rat. The result were represent the mean ± S.D.(Significance of result,*: $p < 0.05$ compared to control)

3. Calcitonin Production

Effects on calcitonin production were observed. As a result, the calcitonin production rates of 2.31 ± 0.15 pg/ml, 2.36 ± 0.24 pg/ml, 2.43 ± 0.17 pg/ml, 2.24 ± 0.14 pg/ml, and 2.25 ± 0.13 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on normal. production rates of 2.45 ± 0.17 pg/ml, 2.43 ± 0.29 pg/ml, 2.58 ± 0.23 pg/ml, 2.93 ± 0.18 pg/ml, and 2.61 ± 0.21 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on control. production rates of 2.42 ± 0.15 pg/ml, 2.41 ± 0.27 pg/ml, 2.32 ± 0.18 pg/ml, 3.72 ± 0.15 pg/ml, and 4.61 ± 0.28 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on YJS at concentration 200 µg/ml. There was a significant increase in cells at concentratio at week 4 (Fig. 3).

4. CTX-2 production

Effects on CTX-2 production were observed. As a result, the CTX-2 production rates of 917.31 ± 70.02 pg/ml, 954.44 ± 40.58 pg/ml, 932.37 ± 20.05 pg/ml, 923.32 ± 30.53 pg/ml, and $924.37 \pm$

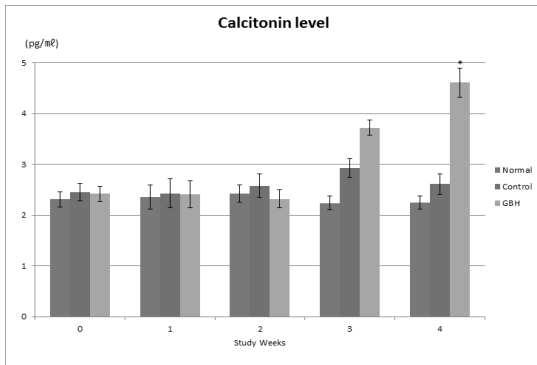


Fig. 3. Effect of YJS on the level of Calcitonin in serum of tibia fractured rat. The result were represent the mean \pm S.D.(Significance of result, *: p (0.05 compared to control).

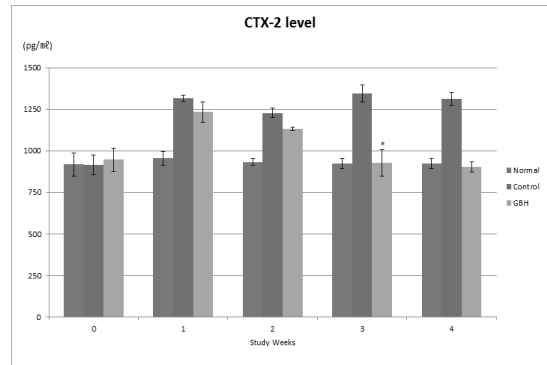


Fig. 4. Effect of YJS on the level of CTX-2 in serum of tibia fractured rat. The result were represent the mean \pm S.D.(Significance of result, *: p (0.05 compared to control)

30.39 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on normal. production rates of 914.37 ± 60.11 pg/ml, 1315.85 ± 20.15 pg/ml, 1227.65 ± 30.56 pg/ml, 1344.04 ± 50.08 pg/ml, and 1312.91 ± 40.91 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on control. production rates of 946.43 ± 70.03 pg/ml, 1232.41 ± 60.54 pg/ml, 1133.28 ± 10.82 pg/ml, 928.40 ± 80.14 pg/ml, and 901.37 ± 30.75 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on YJS at concentration 200 μ g/ml. There was a significant decrease in cells at concentratio at week 3 (Fig. 4).

5. TGF- β Production

Effects on TGF- β production were observed. As a result, the TGF- β production rates of 500.61 ± 1.31 pg/ml, 499.43 ± 1.76 pg/ml, 501.62 ± 1.42 pg/ml, 500.84 ± 1.25 pg/ml, and 499.75 ± 1.89 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on normal. production rates of 500.23 ± 1.50 pg/ml, 1113.12 ± 63.56 pg/ml, 1038.90 ± 22.29 pg/ml, 977.48 ± 53.23 pg/ml, and 845.68 ± 24.51 pg/ml were observed at 0, 1, 2, 3

and 4 weeks respectively on control. production rates of 501.18 ± 34.54 pg/ml, 1224.37 ± 17.42 pg/ml, 1217.58 ± 29.84 pg/ml, 1274.29 ± 41.13 pg/ml, and 923.96 ± 46.51 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on YJS at concentration 200 μ g/ml. There was a significant increase in cells at concentratio at week 3(Fig. 5).

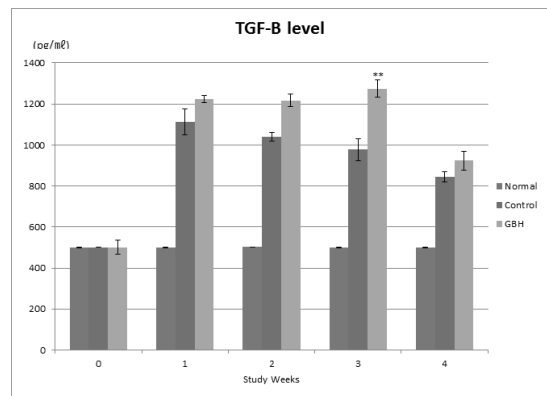


Fig. 5. Effect of YJS on the level of TGF- β in serum of tibia fractured rat. The result were represent the mean \pm S.D.(Significance of result, **: p (0.01 compared to control)

6. BMP-2 production amount

Effects on BMP-2 production were observed. As a result, the BMP-2 production rates of 148.82 ± 1.93 pg/ml, 150.31 ± 2.45 pg/ml, 149.29 ± 2.38 pg/ml, 148.63 ± 1.76 pg/ml, and 150.75 ± 3.21 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on normal. production rates of 150.67 ± 1.14 pg/ml, 181.14 ± 3.29 pg/ml, 197.37 ± 4.04 pg/ml, 184.66 ± 4.04 pg/ml, and 175.23 ± 5.73 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on control. production rates of 148.63 ± 8.09 pg/ml, 180.04 ± 10.34 pg/ml, 171.07 ± 8.29 pg/ml, 157.28 ± 9.94 pg/ml, and 143.96 ± 8.41 pg/ml were observed at 0, 1, 2, 3 and 4 weeks respectively on YJS at concentration $200 \mu\text{g/ml}$. There was a significant decrease in cells at concentratio at week 3 and 4 (Fig. 6).

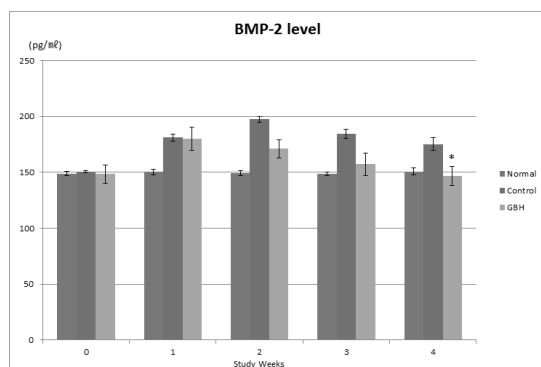


Fig. 6. Effect of YJS on the level of BMP-2 in serum of tibia fractured rat. The result were represent the mean \pm S.D.(Significance of result, *:p<0.05 compared to control)

7. Radiation

To determine the effects of YJS on tibial fracture union, X-rays were taken every two weeks from 0 to 4 weeks after a tibial fracture. Control group has the borderline remained and

fusion was in progress. On the other hand, in the YJS treated group, as the experiment progressed, the boundary line became blurred, the bone outline was clearly visible, and the fracture recover was progressing. (Fig. 7).

Discussion

A fracture is a condition in which bone continuity is lost completely or incompletely. In Korean medicine Uijonggeumgam and Sanggwaboyo, the study of fractures has become more systematic. According to the literature, Cheongeumyobang proposes methods of reduction and fixation. It also describes the use of drug therapy in Taepyeonghyeminhwajegugbang. Treatment involves early wound healing, followed by later reparative and remodeling therapy in reduction¹³⁾.

The fracture-healing process is divided into the inflammatory, reparative, and remodeling phases. In the inflammatory phase, hematomas develop around the damaged bone, soft tissue, and blood vessels. Extravasated blood in Eastern medicine encompasses the meaning of these hematomas¹⁾. In this study, we tried to investigate the effects of YJS on fracture recovery in rats using YJS to remove extravasated blood when the fracture occurred¹⁴⁾.

YJS is effective in removing extravasated blood. YJS can be applied to bruises and fractures¹⁰⁾. YJS has been shown to reduce pain and bruises in traffic accident patients¹²⁾ and to be effective for arthritis¹¹⁾.

Bone formation indicators measure enzymes or proteins produced by osteoblasts or components released during bone formation. Ingredients can be measured in serum osteocalcin, bone alkaline phosphatase (B-ALP), total alkaline phosphatase

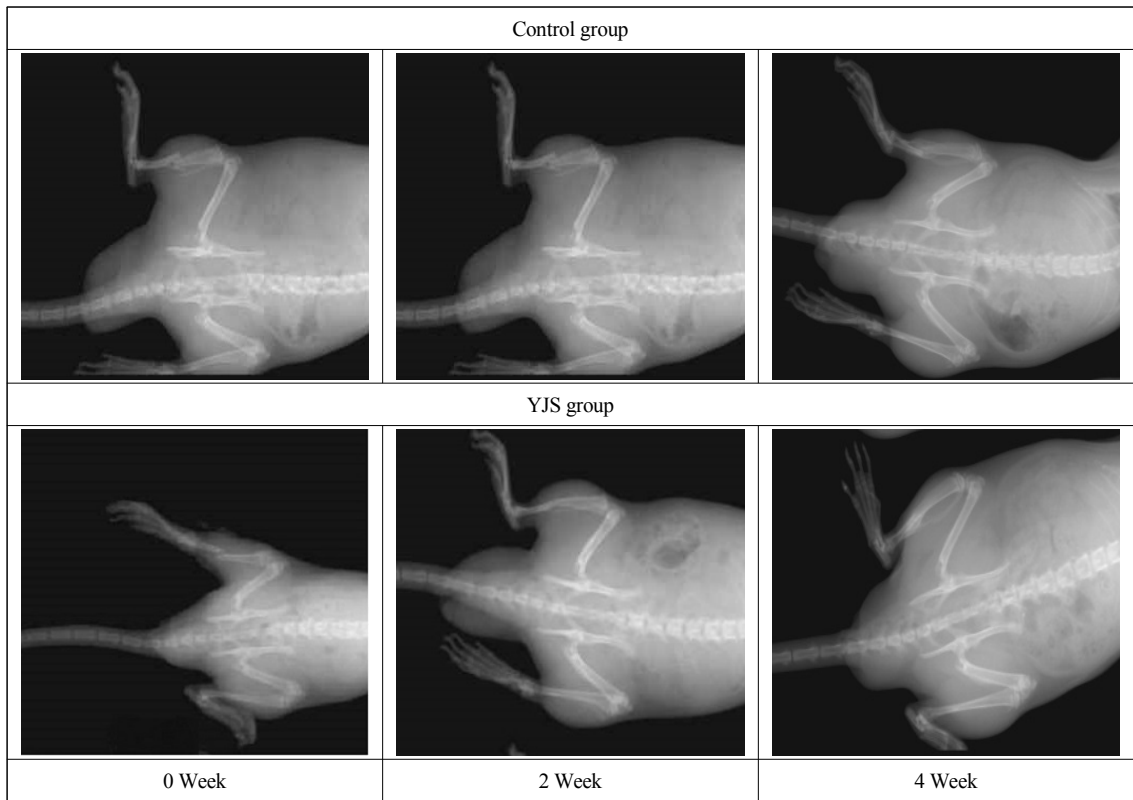


Fig. 7. X-ray Image of YJS and control group on tibia fracture on 0,2 and 4 weeks.

(T-ALP), and procedure I extension peptides (PICP)¹⁵.

The most useful of these is osteocalcin. By measuring its concentration, one can predict the degree of bone formation. This is an indicator reflecting the late differentiation of osteoblasts¹⁶. As a result of measuring the serum osteocalcin change, osteocalcin was found to be significantly increased at 4 weeks.

Calcitonin is a parathyroid hormone, which is important for regulating calcium metabolism. Even if the blood calcium levels rise slightly, calcitonin acts on osteoclasts and inhibits their function. In other words, increased levels of calcitonin reflect the inhibition of bone

resorption¹⁷. At 4 weeks, the serum production of calcitonin was increased compared with that of the control group.

CTX-2 is a collagen substance that is released into the blood when there is bone damage¹⁸. Serum CTX-2 production was significantly decreased at 3 weeks compared with that of the control group.

TGF- β is a growth factor that plays an important role in bone formation. In fracture healing, TGF- β is secreted by platelets. It is synthesized by osteoblasts and chondrocytes and increases bone and cartilage formation¹⁹. In this study, the expression level of TGF- β during fracture healing was significantly increased at 3

weeks.

BMP-2 is a genetic protein that converts fibroblasts into osteoblasts and plays a very important role in bone formation. In addition to BMP-2, BMP-4 and BMP-7 have a wide variety of effects on bone formation and growth. BMP-2 is known to induce strong allogeneic and heterologous bone formation *in vivo*²⁰. In this study, the BMP-2 concentration, which had increased up to 2 weeks, decreased significantly at 4 weeks.

X-rays were taken at 0, 2, and 4 weeks to determine the effects of YJS on fracture restoration. In the control group, fracture boundaries were clearly visible from weeks 0 to 4. However, in the YJS group, fracture union progressed rapidly. In the comparison between the two groups, the YJS-administered group completed restoration faster.

As such, YJS promotes bone formation and suppresses bone resorption in the healing process of fractures and promotes fracture recovery through the regulation of hormones. Based on these findings, YJS could be effective for fracture healing.

Conclusion

The purpose of this study was to investigate the effects of YJS on fracture recovery. As a result, the following conclusions were derived.

1. YJS showed no cytotoxicity up to 200 μ g/ml.
2. Osteocalcin showed a significant increase compared with the control at 4 weeks.
3. Calcitonin showed a significant increase compared with the control at 4 weeks.
4. CTX 2 showed a significant decrease

compared with the control at 3 weeks.

5. TGF- β showed a significant increase compared with the control at 3 weeks.
6. BMP-2 showed a significant decrease compared with the control at 4 weeks.
7. The group treated with YJS on X-ray promoted fracture recovery.

As described above, YJS administration showed significant results for factors related to fracture repair. X-rays were also found to facilitate fracture repair. Thus, YJS could be used to treat fractures in future clinical trials.

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