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Original Article

Effects of *Bojungikgitang-gagambang* on Longitudinal Bone Growth in Adolescent Rats

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Objectives: This study was aimed to investigate the effectof *Bojungikgitang-gagambang* (BJIG) on longitudinal bone growth in rats.
Methods: The BJIG treated group (300 mg/kg) and the control group (vehicle) were administered orally twice daily for 4 days. To investigate the effects of BJIG we measured body weight gain. The bone growth effect was analyzed by measuring between fluorescent lines marked with tetracycline, which plays the role of fluorescent dye on the surface of the tibia. Tetracycline was intraperitoneally injected. The height of growth plates in the epiphyseal plate was measured. The expression of bone morphogenetic protein-2 (BMP-2) and insuline-like growth factor-1 (IGF-1) was investigated by immunohistochemistry.
Results: BJIG caused a significant acceleration of longitudinal bone growth of 349.7 ± 15.9 ㎛/day compared to control (319.8 ± 21.4 ㎛/day). The height of overall growth plate was not significantly more compared to the control, but the size of cells in the proliferative zone and hypertrophic zone were. In the immunohistochemistry, BMP-2 and IGF-1 were expressed markedly in the proliferative or hypertrophic zone, respectively.
Conclusions: BJIG stimulated the chondrocyte hypertrophy and chondrogenesis in the growth plate and directly increased the longitudinal tibia length of rats.

Key Words : *Bojungikgitang-gagambang*, Longitudinal bone growth, Insulin-like growth factor-1, Bone morphogenetic protein-2

Introduction

Idiopathic short stature (ISS) is defined as a condition in which the height of an individual is more than 2 standard deviations score below the corresponding mean height for a given age, sex, and population group without evidence of systemic, endocrine, nutritional, or chromosomal abnormalities[1)]. Growth hormone (GH) replacement therapy is often employed to treat children with ISS. Although GH therapy appears safe, quality-of-life of daily injections, potential short- and long-term adverse effects (including unmet expectations), and treatment costs of GH therapy must be considered[2,3)]. Because of these shortcomings, many investigators have examined the possible efficacy of traditional medicinal herbs as alternative materials instead of GH.

For thousands of years, traditional medicinal herbs had been used to treat children with growth retardation[4,5)]. Five kinds of growth retardation are described in *Dongeuibogam*, the traditional Korean medicinal book; they are retardation in standing up, walking, hair growth, tooth eruption and faculty of speech. The book recommends a number of herbal

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prescriptions for growth retardation; *Bojungikgi-tang* (Bu-Zhong-Yi-Qi-Tang in Chinese) is one. *Bojungikgi-tang* consists of the dried roots of *Astragalusmembranaceus*, *Panax ginseng*, *Glycyrrhizauralensis, Angelica sinensis*, the rhizome of *Atractylodes japonica*, the dried peels of *Citrus unshiu*, the dried whole plants of *Agastacherugosa* and the dried leaves of *Perillafrutescens*. *Bojungikgi-tang* has been reported to possess anti-tumor[6,7], cancer-related fatiguedecreasing[8], radioprotective[9], anti-bacterial[10], anti-nociceptive and anti-depressive activities[11], and to have some effects on impairment of hematopoietic organs[12] and male infertility[13]. Although many functional studies of *Bojungikgi-tang* have been performed, its effect on bone growth has not yet been reported.

In this study, we investigated the effect of BJIG on longitudinal bone growth in adolescent rats. Bone growth was determined by tetracycline staining. Body weight gain and height of growth plates were evaluated. In addition, we analyzed the expression levels of BMP-2 and IGF-1 in growth plates to understand the effects of BJIG on bone growth at the molecular level.

Materials and Methods

1. Plant materials

The dried roots of *Astragalus membranaceus* Bunge, *Glycyrrhiza uralensis* Fisch, *Angelica sinensis* Diels, the dried rhizome of *Atractylodes japonica* Koidz, the dried peel of *Citrus unshiu* Markovich, the dried aerial part of *Agastache rugosa* (Fisch. et Meyer) O. Kuntze and the dried leaf of *Perilla frutescens* (L.) Britt (Yaksoodang Co., Seoul, Korea) were used. *Panax ginseng* C.A. Meyer (KT&G Co., Daejeon, Korea) was used after being steamed. All materials were purchased in 2010. They were identified by Professor Dr. Hocheol Kim and voucher specimens were deposited in the Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, Korea.

2. Preparation of BJIG

The composition of BJIG investigated in this study was made based on *Dongeuisasangsinpyeun* with slight modification. The BJIG in this study was made with the steamed root of *P. ginseng* instead of *P. ginseng*. The dried roots of *A. membranaceus* (96 g), *G. uralensis* (32 g), *A. sinensis* (32 g), the rhizome of *A. japonica* (32 g), the dried peels of *C. unshiu* (32 g), the dried whole plants of *A. rugosa* (16 g), the dried leaves of *P. frutescens* (16 g) were mixed and extracted twice with 70% ethanol for 3 h by reflux heater. The filtrates were evaporated by rotary evaporator and lyophilized by a freeze-dryer (OperonTM, Seoul, Korea). The powder was stored -20 ℃ until used. The yield of the freeze-dried product of mixture extract was 28.8%. The mixture extract powder was dissolved in 10 ml distilled water prior to use. The yield of the steamed root of *P.ginseng* was 65%. Concentration yield was used to calculate from raw material (96 g).

3. Animals

Three-week-old male Sprague-Dawley rats, weighing 60 ± 10 g each, were used (Samtako Co., Osan, Korea). The experimental procedures were performed in accordance with the animal care guidelines of the Kyung Hee University's Institutional Animal Care and Use Committee (KHUASP (SE) - 2009 - 007). Animals were housed under controlled temperature (23 ± 2 ℃ relative humidity (55 ± 10%) and lighting (07:00 - 19:00 h) conditions, with food and water made available ad libitum. After 1 week of acclimatization, BJIG was administered orally.

4. Measurement of longitudinal bone growth

To investigate the effect on longitudinal bone growth, tetracycline was used as a fluorescence marker to label the bone line on the surface of the

tibia. Tetracycline plays the role of fluorescent dye under ultraviolet illumination. The longitudinal bone growth rates were assessed by measuring the length between the fluorescent line formed by tetracycline and the epiphyseal end line of the growth plate. On the third day of the experimentation process, all rats were injected intraperitoneally with tetracycline hydrochloride (20 mg/kg, Sigma Chemicals Co., USA). On the fifth day, all animals were anesthetized using ether and then sacrificed. The control group (vehicle, p.o.) was administered twice daily. The BJIG treated group (300 mg/kg, p.o.) was administered twice daily. This treatment was maintained for 4 consecutive days in each case.

The dissected tibias were fixed in 4% paraformaldehyde for 48 h, and dehydrated by immersion in 30% sucrose for one day. Each bone sample was sectioned longitudinally at a thickness of 40 ㎛ with a sliding microtome (HM440E, Zeiss, Germany). Measurements and calculations of bone growth were obtained by use of a computer program (Optimas 6.5, Optimas Co., Bothell, USA). Focus was placed between the epiphyseal plate and the fluorescent band formed by chelating of tetracycline and calcium on the epiphyseal plate corresponding to injection of tetracycline, which was visible by using a fluorescence microscope (Olympus, Tokyo, Japan). The mean values were from three different sections within the fluorescent band gap.

5. Measurement of height of growth plate

Differential Interference Contrast (DIC) images of dehydrated sections were taken by a confocal microscope and the length of growth plate was measured at three different locations and then an average value was calculated. And the cell of resting, proliferative and hypertrophic zone was observed.

6. Measurement of bone morphogenetic protein-2 and insulin-like growth factor-1 in growth plates

Tissue sections were washed twice in 0.1 M phosphate buffer saline (PBS) and washed twice in 1% triton X-100 (Sigma, U.S.A.) for 15 min, then washed twice with 0.5% of bovine serum albumin (BSA) dissolved in PBS (BSA, Sigma, U.S.A.) for 15 min. The sections were then incubated with goat BMP-2 primary antibody and rabbit IGF-1 primary antibody (1:200, Santa Cruz Biotechnology, CA) over- night at room temperature in a humid chamber. After 24 h, sections were then washed two times with 0.5% BSA in PBS, and then incubated with the biotinylated anti-goat secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and biotinylated anti-rabbit secondary antibody (1:200, Jackson Immuno Research Laboratories, USA) for 1 h respectively. After being washed twice with PBS for 15 min, the sections were incubated with avidin-biotin-peroxidase complex (1:100, Vectastain ABC Kit, Vector Lab- oratories, Burlingame, CA) for 1 h at room temperature. After another washing with PBS, the sections were stained and reacted with 0.05% 3, 3-diaminobenzidine solution containing hydrogen peroxide in PBS. The reaction was stopped by washing with PBS, the slides were then dehydrated with the use of 50%, 75%, 95%, 100% ethanol and xylene in order. The sections were then mounted on glass slides with Permount medium solution (Fisher Scientific, U.S.A.). Micrographs of sections were taken.

7. Statistical analysis

All results were calculated and expressed as mean ± SD. All differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey' multiple comparison test. Values of $p<0.05$ were considered as significant.

Results

1. Effect of body weight

Both experimental groups started with similar mean body weights. Body weights of all groups showed no difference after 4 days treatment (Fig. 1). This result suggests that BJIG treatment does not affect body weight.

2. Effect on longitudinal bone growth

Bone growth effect of BJIG was assessed by taking measurements of the gap between the growth plate and the band formed by tetracycline at three different locations to obtain an average number (Fig. 2)

BJIG caused a significant acceleration of longitudinal bone growth, 349.7 ± 15.9 μm/day compared to the control group 319.8 ± 21.4 μm/day (Fig. 3).

These data imply that BJIG showed a significant increase in the longitudinal bone growth compared to the control.

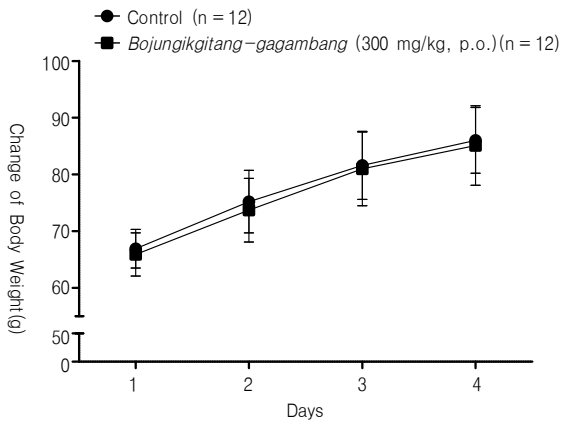

Fig. 1. Change of body weight during *Bojungikgitang-gagambangtreatment* in adolescent rats. Control, control group (vehicle, p.o.); *Bojungikgitang-gagambang*, *Bojungikgitang-gagambang*-treatedgroup (300 mg/kg, p.o.). Each value is shown as mean ± SD (n=12).

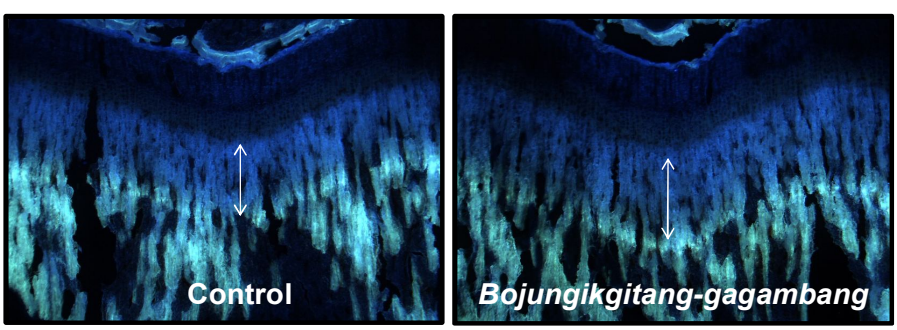

Fig. 2. Fluorescent photomicrographs of longitudinal sections at the growth plate in the proximal tibia. The fluorescent line corresponds to the injection of tetracycline (20 mg/kg), which binds with calcium and can be detected by ultraviolet illumination. The arrow between the fluorescent line formed by tetracycline and the epiphyseal end line of the growth plate indicates the length of bone growth during the 48 h Control, control group (vehicle, p.o.); *Bojungikgitang-gagambang*, *Bojungikgitang-gagambang*-treated group (300 mg/kg, p.o.).

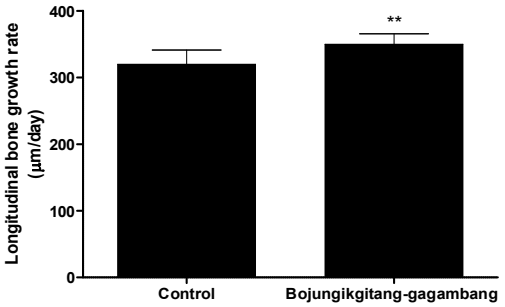

Fig. 3. Longitudinal bone growth rate during *Bojungikgitang-gagambang* treatment in adolescent rats. Control, control group (vehicle, p.o.); *Bojungikgitang-gagambang*, *Bojungikgitang-gagambang*-treated group (300 mg/kg, p.o.). Each value is shown as mean ± SD (n=12). **p<0.01, significant difference compared with control.

3. Effect on growth plate height

Longitudinal bone growth depends on a complex synchronization of the rate of proliferation, advancement and development of hypertrophy, which results in the longitudinal expansion and progression of the growth plate. The heights of the total growth plates were measured at three different locations within the growth plate for each sample and animal in each group by histological study. The overall growth plate zone was divided to the resting zone (RZ, a layer of small, round cells irregularly arranged), the proliferative zone (PZ, wherein the cells divide along the long axis of the bone in regular columns), and the hypertrophic zone (HZ, large, glycogen-filled cells).

The proximal tibia growth plate in the control group was 515.5 ± 39.7 ㎛ thick; the BJIG group was 517.0 ± 23.5 ㎛ Fig. 4). The difference between the control and BJIG group for the growth plate height was not statistically significant, but the size of the cells in the proliferation zone and hypertrophic zone was observed as somewhat larger than the control group (Fig. 5). This result may be related with the proliferation of cartilage cells. However, further studies should be performed to clearly analyze these results.

4. Effects on BMP-2 and IGF-1 expression

Immunohistochemical studies were performed to evaluate the expression of BMP-2 and IGF-1 in the three major principle zones of the growth plate. BMP-2 as well as IGF-1 staining showed the highest change in the cytoplasm of the hypertrophic zone chondrocytes. Treatment of BJIG increased markedly

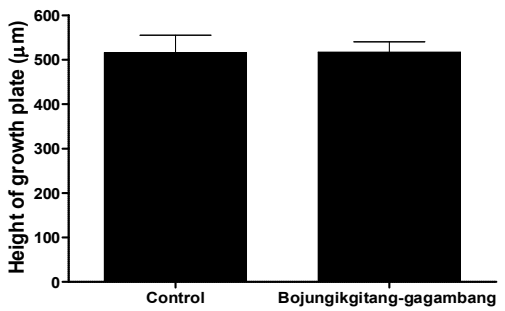

Fig. 4. Height of growth plate. Control, control group (vehicle, p.o.); Bojungikgitang-gagambang, *Bojungikgitang-gagambang*- treated group (300 mg/kg, p.o.). Each value is shown as mean ± SD (n=12).

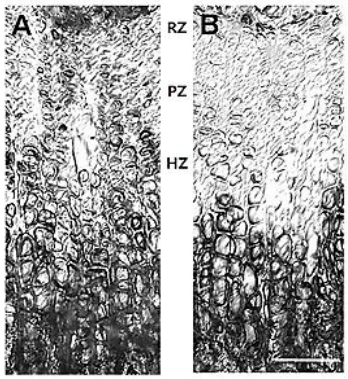

Fig. 5. Photographs of growth plates in a proximal tibia. Representative DIC images of growth plate sections including the three zones in adolescent rat. Sections of tibia growth plates in the control group (A), *Bojungikgitang-gagambang* 300mg/kg group (B). RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone; Scale bar = 100 ㎛

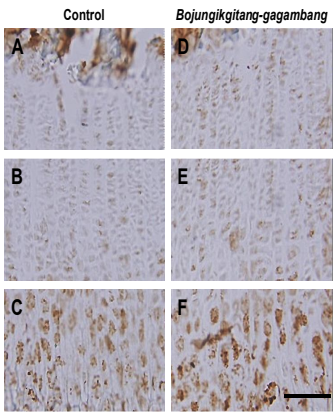

Fig. 6. Immunohistochemical localization of BMP-2 on the growth plate in adolescent rats. Control, control group (vehicle, p.o.); *Bojungikgitang-gagambang*, *Bojungikgitang-gagambang*-treated group (300 mg/kg, p.o.). (A, D) resting zone (RZ) (B, E) proliferative zone (PZ) (C, F) hypertrophic zone (HZ). Scale bar = 50 ㎛

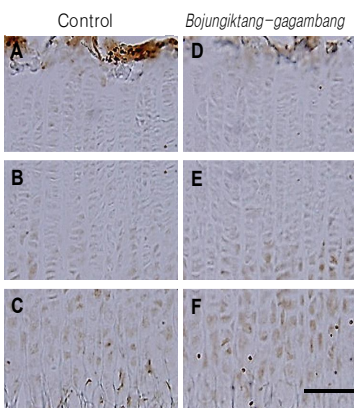

Fig. 7. Immunohistochemical localization of IGF-1 on the growth plate in adolescent rats. Control, control group (vehicle, p.o.); *Bojungikgitang-gagambang*, *Bojungikgitang-gagambang*-treated group (300 mg/kg, p.o.). (A, D) resting zone (RZ) (B, E) proliferative zone (PZ) (C, F) hypertrophic zone (HZ). Scale bar = 50 ㎛

the BMP-2 and IGF-1 expression in hypertrophic zone chondrocytes of the growth plates (Fig. 6 and Fig. 7). Both the number and intensity of BMP-2 and IGF-1 positive cells were increased in the hypertrophic zone. In contrast, staining of BMP-2 was lighter in the proliferative zone chondrocytes. This supportsthat the BMP-2 and IGF-1 expression and chondrogenesis effect of BJIG were correlated.

Discussion

In this study, BJIG significantly increased longitudinal bone growth compared to the control group without significant body weight difference; the height of the overall growth plate was not significantly increased compared to the control group, but the size of cells in the proliferative zone and hypertrophic zoneincreased. In the immunohistochemical study, BMP-2 and IGF-1 were markedly expressed in the proliferative or hypertrophic zone, respectively.

The growth in height is a result of endochondral proliferation in growth plates and conversion of chondrocytes into new bone. The growth plate is a highly organized cartilage structure entrapped between the epiphyseal and metaphyseal bone at the distal ends of the long bones[14]. Longitudinal growth occurs at the growth plate by endochondral ossification[15,16], a process in which cartilage is formed and then remodeled into bone. BJIG caused a significant acceleration of longitudinal bone growth compared to the control group. This result suggests that BJIG enhance the height growth in adolescent rats. This effect is considered to be related GH stimulating effect of *P.ginseng*[17] and *A.membranaceus*[18,19], and we cannot exclude the synergic action of other herbs (Fig. 2 and 3).

Body weight is one of the important growth parameters with height, and infant weight gain is correlated with childhood height[20]. On investigation of body weight change rate, there was no significant difference between the BJIG-treated group and the control group for 4 days. However, it may not be enough to measure the effects of BJIGon body weights for only 4 days (Fig. 1).

In the BJIG-treated group, the height of the overall growth plate was not significantly increased compared to the control group but the cell sizes in the proliferative and hypertrophic zones were observed larger than in the control group. The linear growth of long bones is achieved at the growth plate, a layer of cartilage situated between the epiphysis and metaphysis. The chondrocytes of the growth plate exist within a cartilaginous matrix and are arranged in specific layers, or zones. In order from the epiphysis to the metaphysis, these are the RZ (a layer of small, round cells irregularly arranged), the PZ (wherein the cells divide along the long axis of the bone in regular columns), the prehypertrophic zone, and finally the HZ (large, glycogen-filled cells)[15,21,22]. The proliferative zone contains replicating chondrocytes arranged in columns parallel to the long axis of the bone. The proliferative chondrocytes located farthest from the resting zone stop replicating and enlarge to become hypertrophic chondrocytes[23]. Growth plates in which hypertrophic chondrocytes became larger, grew faster[24]. This result suggests that BJIG increases hypertrophy of chondrocytes at the proliferative and hypertrophic zone in growth plate, and results in increasing longitudinal bone growth (Fig. 4 and 5).

BJIG promoted BMP-2 and IGF-1 generation in bone growth plate, compared to control. BMP is a member of the TGF- family and acts as growth and differentiation factors. BMPs play an important role in skeletal development. These glycoproteins were initially characterized by their ability to induce ectopic cartilage and bone formation in soft tissues[25]. Of the BMP family, BMP-2 is known as a positive regulator of growth plate chondrogenesis. BMP-2 plays an important role in the development of epiphyseal growth plates[26], and stimulates longitudinal bone growth by increasing growth plate

chondrocyte proliferation, hypertrophy, and, at high concentrations, cartilage matrix synthesis[14]. IGF-1 is a growth factor structurally like insulin and induces subsequent cellular activities, particularly on bone growth. The IGF-1 receptor, like the insulin receptor, has intrinsic tyrosine kinase activity[27]. Mechanism of IGF-1 action in promoting statural growth involves 'insulin- like' anabolic effects, supporting the extraordinary biosynthetic activity, somatic growth and matrix production that characterize hypertrophic chondrocytes[28]. The changes in the growth plate organization induced by BJIG may be related to transformation in the production of certain cytokines, including both BMP-2 and IGF-1. This result suggests that BJIG stimulated longitudinal bone growth by the increase levels of local IGF-1 and BMP-2 (Fig. 6 and 7).

Taken together, BJIG increased longitudinal bone growth and stimulated chondrocyte hypertrophy in the growth plates by regulation of IGF-1 and BMP-2 levels. In conclusion, our findings can help to understand the molecular functions of BJIGon bone growth, and suggest that BJIG can be used as alternative candidate to GH for increasing bone growth of children who have ISS or other growth retardation.

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