Amomum villosum induces longitudinal bone growth in adolescent female rats

Sun Haeng Lee, Ji Young Kim, Hocheol Kim, Seul Ki Park, Cho Young Kim, Sun Yong Chung, Gyu Tae Chang

OBJECTIVE: This study was conducted to evaluate the effect of Amomum villosum on longitudinal bone growth.

METHODS: Adolescent female Sprague-Dawley rats were divided into 3 groups and treated for 4 days: control (distilled water, p.o.), recombinant human growth hormone (rhGH; 100 μg/kg, s.c.), and A. villosum (500 mg/kg, p.o.) groups. On day 3, tetracycline (20 g/kg, i.p.) was injected for growth plate identification. On days 2, 3 and 4, 5-bromo-2'-deoxyuridine (BrdU) (50 mg/kg, i.p.) was injected to label proliferating cells. On day 5, tibias were dissected and fixed in 4% paraformaldehyde, dehydrated, and sectioned for immunohistochemistry and histomorphometry.

RESULTS: The rate of bone growth in the A. villosum and rhGH groups increased to (410±44) and (389 ± 46) μm/day (P<0.01), respectively, as compared with the control (330.7±34.7) μm/day. The thickness of the growth plates also increased to (591 ± 37) and (598 ± 32) μm, respectively, as compared with the control (524±89) μm (P<0.001). The number of BrdU-positive cells in the chondrocytes of the A. villosum and rhGH groups was also significantly higher (126±24) and (143±18) cells/mm², respectively) than in the control (109±25) mm² (P<0.05). Insulin-like growth factor-1 and bone morphogenetic protein-2 in the A. villosum and rhGH groups were highly expressed in the growth plate as compared with the control samples, indicating increased bone formation.

CONCLUSIONS: A. villosum could be used to treat growth retardation during adolescence.
ossification. Endochondral ossification in the growth plate leads to longitudinal bone growth, involving replacement of a cartilage model with bone tissue. After birth, much of bony elongation occurs through the activity of chondrocytes within the growth plate, which are continually replaced by bone-forming cells through a combination of proliferation, cartilage extracellular matrix secretion, and hypertrophy. An important stimulator of chondrocyte proliferation in the growth plate is growth hormone (GH), which is activated through stimulation of insulin-like growth factor (IGF)-1. Furthermore, chondrocyte proliferation is also regulated through the activation of bone morphogenetic protein (BMP) signaling in the bone growth plate. 

The Amomum plant is one of the most ancient and highly valued spices in the world. Amomum villosum (Zingiberaceae), native to Guangdong province, is a perennial herb that occurs in the understory of tropical and subtropical forests and is an important medicinal plant for improvement of gastrointestinal motility. Few reports have been published the effects of A. villosum on longitudinal body growth. However, in Dongeuibogam, the traditional book of Korean medicine, malnutrition associated with growth retardation was treated using an herbal mixture containing A. villosum.

In this study, the effects of A. villosum on longitudinal bone growth were determined in the epiphyseal plates of Sprague-Dawley rats. BrdU (5-bromo-2’-deoxyuridine), a thymidine analogue incorporated into DNA during replication, was used to determine proliferation, and tetracycline labeling for growth plate identification. Furthermore, the expressions of BMP-2 and IGF-1 were studied using immunohistochemistry.

METHODS

Animals

Four-week-old female Sprague-Dawley rats, weighing (80±10) g, were used in this study (Samtako Co., Osan, Korea). The experimental procedures were performed in accordance with the animal care guidelines of Kyung Hee University’s Institutional Animal Care and Use Committee (protocol number KHUASP(SE) 2011-008). Animals were housed 5/cage under controlled temperature (23 ± 2)°C, relative humidity [55 ± 10]%), and lighting (lights on 07:00-19:00 h), with food and water made available ad libitum. After 1 week of acclimatization, the rats were split into their respective groups for treatment.

Preparation of amomum villosum water extract

The fruit of Amomum villosum was collected in Guangdong, China (KH Herb Pharm., Seoul, Korea). The dried plant (200 g) was extracted twice with distilled water in 3 h of reflux heating. The filtrates were evaporated by rotary evaporator and lyophilized by a freeze-dryer (Operon ™, Seoul, Korea). The powder was stored at -20° C until it was needed. The yield of the freeze-dried product of A. villosum was about 10.71%.

Treatment groups

Animals were randomly allocated into 3 groups according to supplement regimen. Distilled water (DW; 10 rats) or A. villosum (500 mg/kg; 10 rats) was administered orally twice daily at 8:00 and 20:00 h in a fixed dose volume of 10.0 ml/kg. Recombinant human growth hormone (rhGH) (100 µg/kg; 10 rats) (LG Life Science, Seoul) was subcutaneously injected once daily. Treatment was continued for 4 consecutive days. On day 3, all animals were injected intraperitoneally with tetracycline hydrochloride (20 mg/kg, Sigma, St. Louis, MO, USA) in saline for the measurement of bone growth. On days 2, 3, and 4, 5-bromo-2’-deoxyuridine (BrdU) (50 mg/kg, Sigma) was intraperitoneally injected to label proliferating cells. Twenty-four h after the final injection, animals were sacrificed, and then the tibias were dissected free from the soft tissues.

Tissue preparation and detection of longitudinal bone growth

The dissected tibias were fixed in 4% paraformaldehyde for 48 h and dehydrated by immersion in 30% sucrose for 1 day. Dehydrated bone was longitudinally sectioned at a thickness of 40 µm with a microtome (Leica, Berlin, Germany). The sections were mounted onto gelatinized glass slides and observed by fluorescence microscopy (Olympus, Tokyo, Japan). The longitudinal bone length between the fluorescent line and the epiphyseal end line of the growth plate at three different locations using Image J version 1.43 u (National Institutes of Health, USA).

Data were averaged to calculate the longitudinal bone growth rates.

Detection of chondrocyte proliferation

For the detection of cell proliferation in the growth plate, BrdU-specific immunofluorescence was performed. Dehydrated tibial sections were pretreated in 2 M HCl at 37°C for 1 h and rinsed twice in 0.1 M sodium borate (pH 8.5). The sections were incubated in phosphate-buffered saline (PBS), including 3% bovine serum albumin and 0.3% Triton X-100 for 1 h and incubated with BrdU-specific mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (diluted 1:100) overnight at 4°C. The sections were then washed 3 times with 0.1 M PBS before incubation with fluorescein isothiocyanate-conjugated mouse secondary antibody
Measurement of BMP-2 and IGF-1 in the growth plates
For the detection of BMP-2 and IGF-1 in the growth plate, the dehydrated tibial sections were incubated in 1% Triton X-100 at room temperature and incubated with goat BMP-2 antibody or rabbit IGF-1 antibody (Santa Cruz Biotechnology) (diluted 1:200) for 60 min, respectively, and then stained with 0.05% 3, 3-diaminobenzidine.

Statistical analysis
Results are expressed as the mean±SD of the different experiments under the same conditions. Statistically significant values were compared using Student’s t test for comparisons between groups. P-values<0.05 were considered statistically significant.

RESULTS

Daily body weight change
On days 1-5, the daily body weights of the rats in the control, rhGH and A. villosum group are described in Table 1. From the 1st day to the 5th day, the body weight gain was (18.8±1.5) g (control), (19.1±1.7) g (rhGH), and (17.5±1.7) g (A. villosum). The weight for the rats in the A. villosum treatment group increased slower than the other two groups, but this difference was not statistically significant (Figure 1).

To evaluate the rate of longitudinal bone growth, tetracycline was used to label the newly formed bone (Figure 2). The longitudinal bone growth of the control adolescent female rats was (331±35) μm/day. The rhGH group showed a significantly greater rate of bone growth of (389 ±46) μm/day as compared with the control group (P=0.0050). The A. villosum group also showed a significantly greater rate of bone growth of (410 ± 44) μm/day as compared with the control group (P=0.0003) (Figure 3).

The proximal tibial growth plate in the control group was approximately (524±89) μm thick. Following A. villosum administration, the growth plate thickness increased to (591±37) μm (P=0.0007). For rats in the rhGH group, the growth plate thickness increased to (598±32) μm (P=0.0002) (Figure 4).

BrdU-labeled cells were observed in the chondrocytes to determine the proliferation levels in the growth plates (Figure 5). The number of BrdU-positive cells in the control group was (110 ± 25) cells/mm². In the rhGH group, the number of BrdU-positive cells increased to (143 ± 18) cells/mm² (P<0.0001). The number of BrdU-positive cells in the A. villosum group increased to (126±24) cells/mm² (P=0.02) (Figure 6).

To investigate the expression of BMP-2 and IGF-1,
Lee SH et al. Amomum villosum induces longitudinal bone growth in adolescent female rats

Immunohistochemistry in the growth plate was performed. BMP-2 was highly expressed in the proliferative and hypertrophic zones in the rhGH group but was minimally expressed in the resting zones (Figure 7). IGF-1 expression was relatively higher in the proliferative and hypertrophic zones than in the resting zones (Figure 8). BMP-2 and IGF-1 expression of the A. villosum group was lower than those expressions in the rhGH group, but higher than those expressions in the control group.

DISCUSSION

Amomum villosum increases longitudinal bone growth in adolescent female rats. A. villosum significantly increased the tibial growth rate by 24.07% compared to normal rats \((P<0.001)\). This rate was more than the 17.60% of the rhGH \((P<0.01)\), which has been known to enhance tibial growth in peripubertal female rats. In addition, the daily body weight changes over 5 days were not significantly different among the groups. A. villosum is thought to be effective for bone growth and is not associated with extra weight gain.

The thickness of the growth plate is direct evidence of longitudinal bone growth. In the A. villosum group, the thickness of the growth plate was significantly increased as compared with the controls \((P<0.001)\). A.
Amomum villosum induces longitudinal bone growth in adolescent female rats

Lee SH et al.

BrdU is incorporated into DNA during the S-phase and can be detected by immunohistochemistry. A higher number of BrdU-labeled cells, the greater the number of proliferating chondrocytes. The number of BrdU-positive cells in the rhGH and A. villosum groups was 1.31-fold ($P<0.001$) and 1.15-fold ($P<0.05$) greater than that of the control group. This result suggests that A. villosum induces cell proliferation in chondrocytes.

The growth plate can be divided into three histologically distinct layers—the resting zone, the proliferative zone, and the hypertrophic zone. The topmost layer, the resting zone, contains chondrocytes that are scattered in a cartilage matrix bed and that divide infrequently in postnatal life. The resting zone chondrocytes serve as stem-like cells for the growth plate, with the potential to generate clones of rapidly proliferating chondrocytes, which are located in the proliferative zone in the second layer of the growth plate. In the proliferative zone, these clones of chondrocytes are arranged in columns parallel to the long axis of the bone. Over time, as longitudinal growth proceeds, proliferative cells close to the hypertrophic zone undergo terminal differentiation. During this process, they stop proliferating and physically enlarge to become hypertrophic chondrocytes, composing the third, hypertrophic layer of the growth plate. Simultaneously, the bottom of the hypertrophic zone is invaded by blood vessels, osteoclasts, and differentiating osteoblasts, which remodel the newly formed cartilage into bone tissue.

BMP-2 and IGF-1 were highly expressed in the proliferative and hypertrophic zones after A. villosum treatment. BMP is a member of the transforming growth factor (TGF)-β family, acting as both a growth and differentiation factor. While TGF-β is unable to initiate the entire osteoinduction cascade by itself, BMP-2 uniquely exhibits an ectopic bone formation property. IGF-1 is a growth factor structurally related to insulin that induces subsequent cellular activities, particularly those related to bone growth. Involved in the expression mechanism of growth hormone, IGF-1 regulates cell differentiation, proliferation, and maturation in most parts of the body by autocrine and paracrine activities. The mechanism of IGF-1 action in promoting structural growth involves ‘insulin-like’ anabolic effects, supporting the

Figure 6 Effect on BrdU-positive cells
Control: DW group. rhGH: recombinant human growth hormone (100 μg/kg, s.c.) group. A. villosum: Amomum villosum (500 mg/kg, p.o.) group. Each value is the mean ± SD of 10 rats. Statistical significance was determined using t test: $^aP<0.05$, $^cP<0.001$, compared with the control.

Figure 7 Immunohistochemical localization of bone morphogenetic protein-2 in the growth plate
Control: DW group. rhGH: recombinant human growth hormone (100 μg/kg, s.c.) group. A. villosum: Amomum villosum (500 mg/kg, p.o.) group. RZ: resting zone, PZ: proliferative zone, HZ: hypertrophic zone. Scale bar=100 μm.

Figure 8 Immunohistochemical localization of insulin-like growth factor-1 in the growth plate
Control: DW group. rhGH: recombinant human growth hormone (100 μg/kg, s.c.) group. A. villosum: Amomum villosum (500 mg/kg, p.o.) group. RZ: resting zone, PZ: proliferative zone, HZ: hypertrophic zone. Scale bar=100 μm.
extraordinary biosynthetic activity, somatic growth, and matrix production that characterize hypertrophic chondrocytes.\textsuperscript{20} The expressions of BMP-2 and IGF-1 directly represent the proliferative activity of cartilage. Tibial growth rate of A. villosum was greater than that of rhGH, but the thickness of the growth plate, the number of BrdU-positive cells, and BMP-2 and IGF-1 expressions of A. villosum did not exceed those of rhGH. Thus, A. villosum cannot be an alternative to GH treatment for short stature. However, long-term GH treatment is expensive, and the final height gains in children are small as compared with those of non-GH-deficient children.\textsuperscript{21} GH appears to be relatively safe, but its long-term effects are not yet fully known, and some adverse effects have been reported, including edema and pseudotumor cerebri, gynecomastia, hyperinsulinism or elevated blood glucose level, and a potential increase in nevi.\textsuperscript{22} A. villosum might be an alternative treatment which meets the cost-benefit qualification while avoiding the potential risks of GH therapy. A. villosum stimulated chondrocyte proliferation and chondrocyte hypertrophy in the growth plate through the generation of BMP-2 and IGF-1, as well as directly increasing the longitudinal tibial length. Therefore, A. villosum could be helpful for increasing bone growth in children with growth retardation. Further studies are needed to identify the active components of A. villosum that can provide confirmation of growth promotion, and to better understand the mechanisms in this process.

REFERENCES