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An Herbal Medicine Mixture (HM-10) Induces Longitudinal Bone Growth and Growth Hormone Release in Rats

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Abstract To investigate the growth promoting effects of an herbal medicine formulation (HM-10), Sprague Dawley (SD) male rats (3 weeks old) were divided into 3 groups (8 rats/group). The control group was given a daily oral administration of saline, and the treatment groups, HM-1 and HM-2, were given daily administrations of HM-10 (500 and 1,000 mg/kg BW, respectively). The cumulative tibial bone growth of the HM-1 and HM-2 groups (22.5 and 20.8 mm, respectively), and their cumulative femur bone growth (19.4 and 18.2 mm, respectively), were significantly different compared to the control group (7.5 mm of tibial growth and 7.7 mm of femur growth) ($p<0.05$). Lastly, the growth hormone levels of the HM-1 and HM-2 groups (1.70 and 1.79 ng/mL, respectively), as well as their insulin like growth factor 1 (IGF-1) levels (165.1 and 171.7 ng/mL, respectively) showed significant differences compared to the control (0.93 ng/mL of growth hormone and 125.6 ng/mL of IGF-1) ($p<0.05$).

Keywords: growth hormone, insulin like growth factor 1, herbal medicine, longitudinal bone growth

Introduction

Traditional herbal medicine has been widely used for disease treatment and is recognized as an interesting alternative to conventional medicine (1-5). Multi-herb recipes have frequently been used in traditional medicine and are aimed at collectively exerting therapeutic actions and modulating the ingredients of the constituent herbs. In formulating these traditional herbal medicine recipes, special herb pairs, which are claimed to be unique combinations of traditionally defined medicinal herbal properties (6), are frequently used for achieving mutual enhancement, mutual assistance, mutual restraint, mutual suppression, or mutual antagonism (7).

Herbal medicine, with a history of thousands of years, is the foremost alternative medical system available in the modern world. The major medications for treating diseases in herbal medicine are either a single herb or a combined herbal formula. A major feature of herbal medicine is the unique combinations of multiple herbs for the treatment of various diseases; where there are significant advantages to combining specific multiple medicinal herbs. First, medicinal herbs may act differently to produce synergistic effects. Secondly, the combination of multiple herbs, such as described below, can minimize side-effects by decreasing the amount of each component (8).

Various herbal medicines have been used for centuries in traditional herbal formulations to treat a wide range of diseases, including osteoporosis (9). In the past, the development of herbal anti-osteoporosis formulas was pursued mainly by scientists in Asian countries, including China, Japan, and Korea (10-12). However, as a result of recent evidence that estrogen replacement therapy is associated with increased risk of breast, ovarian, and endometrial cancers in postmenopausal women (13, 14), it is now generally recognized that alternative approaches for the prevention and treatment of osteoporosis may be worth exploring.

In Korea, the growth-promoting effects on humans of specially formulated herb mixtures have been traditionally established. In order to promote their potency, which is perceived to be caused by multiple factors, herbal formulations containing standard plant ingredients are employed. As a whole, traditional medicine in Korea recognizes the importance of herbs in maintaining the well-being of the body. Also, the benefits of consuming herbal mixtures may be due to their synergistic effects (15).

Therefore, the purpose of this study was to investigate the growth promoting effects of an herbal medicine formulation (HM-10) on growing bone. Here we quantified increases of body weight, tibia length, and the height of growth plates in Sprague Dawley (SD) rats through X-ray morphology examination.

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Materials and Methods

Herbal medicine (HM-10) extract The ingredients for

the herbal formulation are as follows, by descending order of weight: *Zizyphi fructus* (20 g); *Polygonatum odoratum and Dioscorea oppostifolia* L. (7.5 g each); *Acanthopanax senticosus*, seed of *Carthamus tinctorius* L, *Fallopia multiflora (Thunb. ex Murray) Haraldson* var. *multiflora*, *Astragalus membranaceus Bunge* var. *membranaceus*, peel of *Citrus unshiu*, barley sprout, fruit of *Schizandra chinensis*, root of *Glycyrrhiza glabra*, *Lycium chnense* Miller, fruit of *Morus alba* L., and *Taraxacum platycarpum* Dahlst. (5 g each); *Dongchughacho* (*Cordyceps millitaris*), *Chrysanthemum morifolium* Ramat., *Angelicae gigantis Radix*, and *Eucommia ulmoides* (2.5 g each); *Rubus coreanus* and *Zingiber officinale* Roscoe (1.25 g each).

The air dried herbal medicine material (500 g) was extracted with water (2,000 mL, repeated 3 times) in a percolator at 100°C for 4 hr. The extracted tea was then filtered using a filter (200 mesh) and concentrated by a vacuum falling filter evaporator. The concentrate was dried by a spray-drier using dextrin as the stabilizer. The yield was approximately 32 g per 500 g of the herbal medicine material.

Animals and housing The experimental protocol was reviewed and approved by the Korea University Animal Care Committee. A total of 24 male SD rats of 3 weeks old (Central Lab. Animal Inc., Seoul, Korea) were used. They were individually housed in plastic cages with grated stainless steel floors. The colony room was maintained at 24±1°C with 60% atmospheric humidity, and a 12-hr light/12-hr dark cycle. Before the experiment, the rats had *ad libitum* access to water and to commercial diet (Samyang Co., Seoul, Korea) containing the following (g/kg of diet): moisture, 80; protein, 230; fat, 35; fiber, 50; carbohydrate, 600; and water.

Experimental design After an adaptation period, the rats were divided into 3 groups (8 rats/group). The control group was given a daily oral administration of saline, and the HM-1 and HM-2 groups were given daily administrations of HM-10 (500 and 1,000 mg/kg of BW, respectively). Each group was fed the commercial diet *ad libitum* for 6 weeks. During the experiment, the rats were weighed (±1 g) every other day before the lights were turned off, and during the *ad libitum* consumption period, food intake was recorded and corrected for spillage (±0.1 g) every day.

Nutritional and biochemical analysis Food intake and body weight were monitored every other day for 6 weeks. The food was removed for 12 hr at the end of the experimental term, and the rats were anesthetized with ethyl ether and dissected. Blood was collected from the heart with a heparinized syringe. The plasma was separated by centrifugation at 2,900×g for 15 min at 4°C and was then stored at -70°C until the analysis. Blood plasma levels of total cholesterol (TC), HDL cholesterol, LDL cholesterol, and TG were measured using enzymatic kits (Wako Chemical Co., Osaka, Japan).

Measurements of tibial, femur, and proximal epiphisis (growth plate) length Radiographs of the tibia, femur, and proximal epiphysis lengths (growth plate) were obtained on day 0 (before the first administration of HM-10), and then repeated every 2 weeks. After 6 weeks, the tibial, femur, and proximal epiphysis lengths were assessed radiographically on dorsoventral films. The vertical distance from the X-ray tube to the platform was always 25 cm. The radiographs were taken at 25 kV and 15 msec. Measurements were made directly on the radiographs using a microfilm reader (Model 605-0070 837; NCR, Dayton, OH, USA).

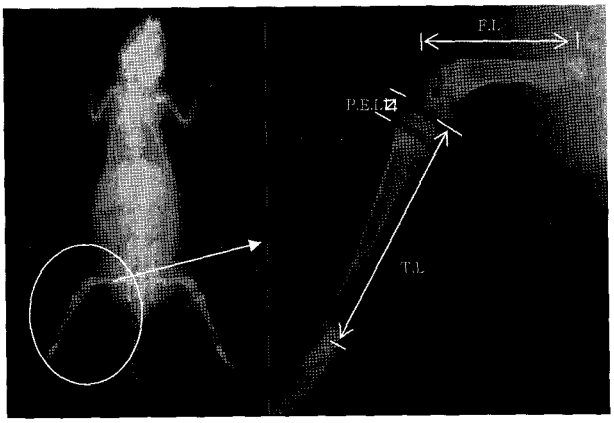

Fig. 1. Radiograph of a longitudinal bone showing femur and tibial morphometric parameters. TL, total tibial length; PEL, proximal epiphysis length; FL, femur length.

Total tibial length was measured as the long axis of the bone between the proximal articular line and the distal articular line (Fig. 1). The femur length was measured as the long axis of the bone in the leg that extends from the hip to the knee. Proximal epiphysis length was defined by the growth plate height (Fig. 1).

Measurements of insulin like growth factor 1 (IGF-1) and growth hormone (GH) At the end of 6 weeks, plasma IGF-1 was measured using a mouse/rat IGF-1 kit (DSL-2900; DSL Webster, TX, USA) after acid ethanol extraction, according to the manufacturer's recommendations (16). The assay included quality controls provided by the manufacturer, and the standard curve of the assay was performed in accordance to the manufacturer's provided samples. Each sample was assayed in duplicate. The average coefficient of variance was 5.96%, in keeping with the manufacturer's reported intra-assay variability (3.8-5.9%).

Plasma growth hormone levels were determined by the enzyme-linked immunosorbent assay (ELISA) method, using the protocol provided in the kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and previously described (17). Each sample was assayed in duplicate.

Statistical analysis The data were subjected to analysis of variance and are expressed as the mean ± standard deviation (SD). The significance of the differences was compared using Duncan's multiple range tests. Values of $p<0.05$ were considered statistically significant. The calculations were made with the SPSS software package (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Body weight, food intake, and food efficiency ratio (FER) The body weight gains after daily oral administration of saline and HM-10 (92.4% carbohydrate, 0.2% crude protein, 0.1% crude fat, 2.7% crude ash, and 4.7% moisture) for the 6 week experimental period are shown in Table 1. The rats administrated HM-10 showed a tendency for increased body weight gain, and there were significant differences ($p<0.05$) between the body weight gains of the control and treated groups (HM-1 and HM-2). The body weight gain in HM-1 was significantly ($p<0.05$) higher than that in HM-2; however, there were no significant differences ($p>0.05$) between the groups. As shown in Table 1, the amount of food intake and the FER were not different among all 3 groups.

Relative organ weights and plasma lipids The changes in the liver and other internal organ weights of the rats after 6 weeks of feeding each diet are presented in Table 2. No significant ($p>0.05$) differences were observed in the weights of the liver, kidneys, and spleen, in relation to body weight, among the 3 groups.

The changes in plasma lipid levels for the control and treated groups (HM-1 and HM-2) are summarized in Table 3. The plasma lipid (TG, total cholesterol, HDL-cholesterol, and LDL-cholesterol) levels were not significantly different ($p>0.05$) between the control and treated groups (HM-1 and HM-2). However, the glucose levels between the control and HM-1 group were significantly different ($p<0.05$). There was no significant difference between HM-1 and HM-2, but HM-10 treatment had a tendency to reduce plasma glucose levels.

Table 1. Body weight gain, food intake, and food efficiency ratio (FER) in rats fed experimental diets[1)]

Parameter	Control[3)]	HM-1	HM-2
Body weight gain (g/day)	6.27±0.45[a]	8.21±0.50[b]	7.74±0.61[b]
Food intake (g/day)	15.84±2.22	15.47±2.40	16.53±3.09
FER[2)]	0.34±0.06	0.35±0.02	0.34±0.02

[1)]Values are mean±SD for 8 rats; Means with different superscript letters within a row are significantly different at $p<0.05$ by Duncan's multiple range tests.
[2)]Food efficiency ratio = body weight gain/food intake.
[3)]Control, no administration of HM-10; HM-1, administration of HM-10 (0.5 g/100 g); HM-2, administration of HM-10 (1 g/100 g).

Table 2. Weights of liver, spleen, and kidneys after 6 weeks of treatment[1)]

(weight/100 g of BW)

Organ	Control	HM-1	HM-2
Liver	3.07 ± 0.42	2.97 ± 0.25	3.06 ± 0.15
Kidney	0.82 ± 0.05	0.76 ± 0.04	0.75 ± 0.02
Spleen	0.20 ± 0.01	0.22 ± 0.03	0.22 ± 0.03

[1)]Values are mean±SD for 8 rats; Means with different superscript letters within a row are significantly different at $p<0.05$ by Duncan's multiple range tests. Control, no administration of HM-10; HM-1, administration of HM-10 (0.5 g/100 g); HM-2, administration of HM-10 (1 g/100 g).

Table 3. Plasma triglyceride and cholesterol levels after 6 weeks of treatment[1)] (mg/dL)

Parameter	Control	HM-1	HM-2
Glucose	141.50±16.94[b]	110.83±10.42[a]	122.14±22.59[ab]
Triacylglycerol	67.75±4.79	72.17±5.15	73.00±8.49
Total cholesterol	53.00±7.62	57.00±11.42	60.43±5.68
HDL-cholesterol	15.75±2.50	18.67±4.84	19.86±2.54
LDL-cholesterol	23.70±4.65	23.90±8.33	25.97±2.96

[1)]Values are mean±SD for 8 rats; Means with different superscript letters within a row are significantly different at $p<0.05$ by Duncan's multiple range tests. Control, no administration of HM-10; HM-1, administration of HM-10 (0.5 g/100 g); HM-2, administration of HM-10 (1 g/100 g BW).

It was reported that 4 main flavonoids with 1 or 2 isoprenoid groups, as well as a 2-arylbenzofuran, were isolated and identified in *Morus alba*. These compounds may attribute to the fact that in one study, the phenolics in *M. alba* root bark extract acted as scavengers of free radicals (18) that arose as a result of STZ-intoxication, and resulted in alleviating the state of diabetes mellitus found in STZ-induced diabetic rats (19). A similar antihyperglycemic fraction containing 2-arylbenzofurans (moracin M and glycosides of 2-arylbenzofuran with an isoprenoid group) was prepared from the leaves of the Argentine mulberry tree, *Morus insignis* (20).

Lee *et al.* (21) that the water extract of *Eucommia ulmoides* leaves improved hyperglycemia in type 1 diabetic rats by increasing plasma insulin levels through enhanced pancreatic cell function. The results also demonstrated that supplementation with the *E. ulmoides* leaf water extract significantly improved fasting blood glucose levels and intraperitoneal glucose tolerance tests in the type 2 diabetic model of *db/db* mice (22).

The reductions of plasma glucose in the HM-10 treated groups might have been caused by the hypoglycemic effects of *M. alba* and *E. ulmoides*. Therefore, this reduced glucose effect by HM-10 should be investigated in detail through further studies.

Increases in body growth and longitudinal bone growth The rats given 500 and 1,000 mg/kg of HM-10 daily for 6 weeks gained significantly more weight than the control rats (control: 175.1±9.1 g, HM-1: 229.9±11.6 g, and HM-2: 216.9±12.9 g). At the beginning of the experimental period, the mean body weights of the control and treated groups were not statistically different. After 6 weeks, HM-10 caused a significant increase of the cumulative body growth (Fig. 2) and longitudinal bone growth (Fig. 3). The cumulative tibial bone growths of HM-1 and HM-2 (22.5±11.7 and 20.8±10.9 mm, respectively) were significantly different when compared to the control group (7.5±1.2 mm) ($p<0.05$). And the cumulative femur bone growths of HM-1 and HM-2 (19.4±8.5 and 18.2±7.3 mm, respectively) also showed significant differences compared to the control (7.7±0.6 mm) ($p<0.05$). However, there were no significant differences between HM-1 and HM-2 for cumulative

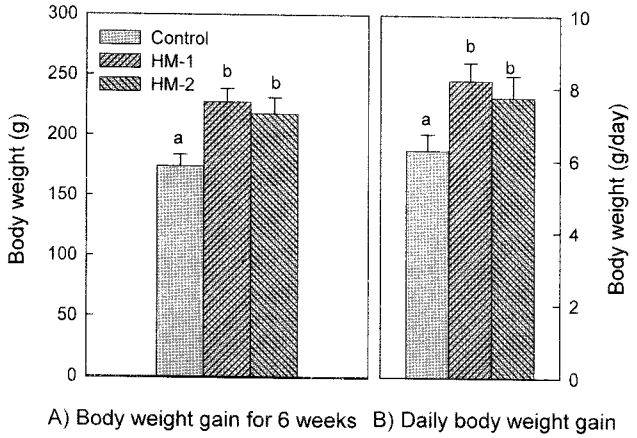

Fig. 2. Body weight and body weight gain in rats treated with HM-10.

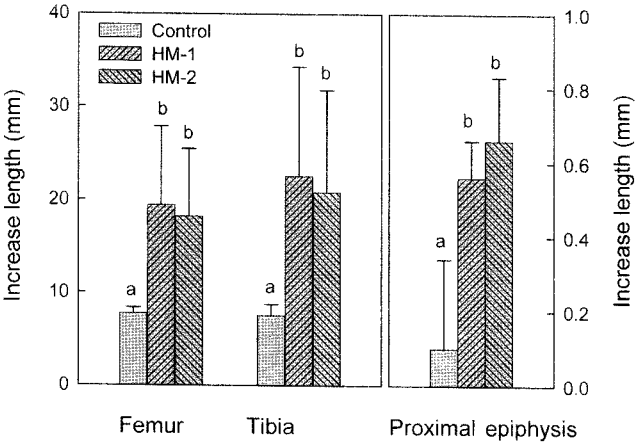

Fig. 3. Effects of HM-10 on femur, tibia, and proximal epiphysis length.

longitudinal bone growth. Longitudinal bone growth occurs through the transformation of the growth plate chondrocytes into bone, by transformation from cells to calcified bone matrix.

Since the height of the growth plate is correlated with body growth rate (23), we measured the heights of the growth plate. The growth plates of the HM-10 treated rats appeared thicker than those of the control rats (Fig. 3). The whole growth plates (proximal epiphysis) of HM-1 and HM-2 showed heights of 0.56±0.10 and 0.66±0.17 mm, respectively. The height for the control group was 0.10± 0.24 mm. The cumulative heights of the growth plates in the HM treated groups (HM-1 and HM-2) showed significant differences when compared to the control group ($p<0.05$). However, there was not a significant difference between the HM-1 and HM-2 groups for height. Overall, the results show that HM-10 promoted bone growth.

Normal longitudinal growth depends on a well-defined process of maturational stages that occur within the epiphyseal growth plate. The process involves a population of stem cells in the resting zone, which proceed through proliferation, maturation, and hypertrophy, finally giving way to bone (24). Longitudinal bone growth results from a

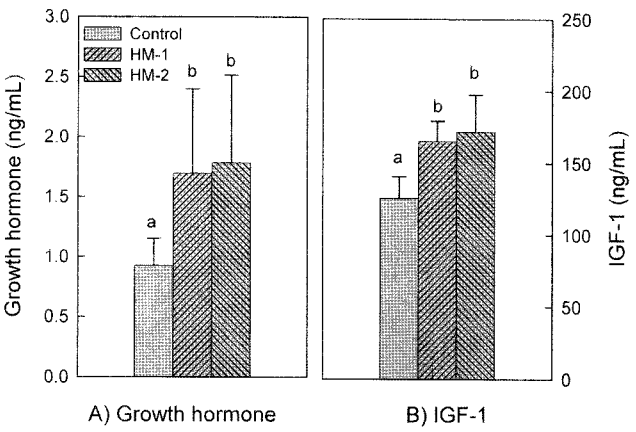

Fig. 4. Effects on HM-10 on IGF-1 and growth hormone levels.

sequence of cellular events occurring in the growth plate, and metaphysis beginning with the proliferation of chondrocytes, and progressing through chondrocyte maturation, matrix synthesis, chondrocyte hypertrophy, matrix mineralization, and the vascular invasion of hypertrophic zone II (25, 26).

Since longitudinal bone growth is equal to the production rate of new cells per cartilage column multiplied by the average size of hypertrophic cells (26), it seems from the present study that HM-10 promoted the maturation and multiplication of chondrocytes. However, the mechanism by which it mediates its affect on bone formation remains unclear.

IGF-1 and GH plasma levels After 6 weeks, HM-10 caused significant increases in growth hormone and IGF-1 levels (Fig. 4). The growth hormone levels of HM-1 and HM-2 (1.70±0.70 and 1.79±0.73 ng/mL, respectively) were significantly different compared to the control (0.93± 0.23 ng/mL) ($p<0.05$). And the IGF-1 levels of the HM-1 and HM-2 groups (165.1±14.2 and 171.7±25.8 ng/mL, respectively) also showed significant differences when compared to the control (125.6±15.1 ng/mL, respectively) ($p<0.05$). However, there were no significant differences between HM-1 and HM-2 for their growth hormone and IGF-1 levels.

IGF-I is synthesized and secreted in the liver and other organs, following stimulation by growth hormones. It accelerates growth, differentiation, and substrate synthesis activity in osteoblasts and chondroblasts (27-29). Furthermore, IGF-I has been reported to play important roles in the growth of long bone as well as the growth of mandibular condyle (30, 31). Itoh *et al.* (32) noted that the histological changes induced in the mandibular condyle following local administration of IGF-I, differed between 3 and 12 week-old rats. Interestingly, after the local administration of IGF-I, the histological structure of the mature condyle in the 12 week-old rats changed to resemble that of a younger growth stage (33).

The effects of GH on the longitudinal bone growth rate (LGR) are well established, and have been intensively investigated in hypophysectomized rats, where systemic as well as local administration of small doses of GH into the epiphyseal growth plate (34, 35), or into the femoral artery

(36), increase LGR. Because endogenous release of GH can be accomplished by IGF-1 increases, HM-10 should inherently have the potential to act at the growth plate, inducing LGR.

In conclusion, HM-10 appears to promote longitudinal bone growth, the height of growth plates, and the endogenous release of GH and IGF-1. Thus, HM-10 may be useful in treating children who suffer from growth delays.

Acknowledgments

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