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Cynanchum wilfordii and *Phlomis umbrosa* Extract (IPLUS-CWPU) Stimulated Bone Growth and Differentiation by Enhancing Growth-Related Factors in Young Sprague Dawley Rats

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Abstract

The purpose of this study was to investigate the effects of extract mixture of *C. wilfordii* and *P. umbrosa* (IPLUS-CWPU) on bone growth in 4-week old young male SD rats. To confirm the effect of IPLUS-CWPU, we measured the length of bone growth plate, the ratio of proliferative zone to the length of growth plate and the expression level of insulin-like growth factor, IGF-1. The IPLUS-CWPU treatment shows a significant increase of tibial and femoral growth plate and the ratio of proliferative zone in growth plate. Especially, the length increased by 13.9% and 25.3% in the tibia and femur, respectively, in the high-dose group compared to the normal group. Moreover, the expression of IGF-1 gene in liver was upregulated in IPLUS-CWPU treated groups. These results indicated that IPLUS-CWPU administration could increase the proliferative zone of bone growth plate in early developmental stage by upregulation of IGF-1 gene.

Key words: *Cynanchum wilfordii*, *Phlomis umbrosa*, growth plate, bone differentiation, proliferative zone, insulin like growth factor-1

Introduction

The growth is complexly regulated by external factors such as nutrition and environment and internal factors such as heredity, growth factors and hormones (Racine & Serrat 2020). Recently, due to westernized diet and economic growth, the average height of South Korea has increased according to the improvement of nutrition supply compared to the past (Kim et al. 2017).

Growth of skeletal muscle and bone can be influenced by a variety of factors, including heredity, nutrition, and hormones (Estívariz & Ziegler 1997; Gat-Yablonski & De Luca 2017). The growth plate, which is a layer of hyaline cartilage where ossification occurs in immature bones, adds length and width to the bone. Height will not increase after age 18 to 20 due to the complete fusion of the growth plates (Patt & Maloney 1972; Abad et al. 1999; Crowder & Austin 2005). The plate is replaced by an epiphyseal line in adults.

Various growth factors and hormones are strongly related to bone metabolism during the growth phase (Canalis E 1983; Sharan & Yadav 2014; Bachagol et al. 2018). Ossification of the growth plate is regulated by the factors and horomes such as insulin-like growth factor (IGF), sex hormones, thyroid hormones, and glucocorticoid (Florini JR 1987; Giustina et al. 2008).

Growth hormone stimulates protein anabolism and bone growth in children, which indirectly acts on tissues through IGF (Tanner et al. 1977; Healy et al. 2003). It plays a central role not only in children but also in adults, because it regulates protein metabolism and body composition (Umpleby & Russell-Jones 1996; Carroll et al. 1998). Especially, patients with growth hormone deficiency (GHD) can develop problems such as decreased muscle mass and bone density, high cholesterol levels, or cardiovascular disorders (Lombardi et al. 2012; Xia et al. 2015; Tavares & Collett-Solberg 2021).

Depending on the state of growth, growth hormone therapy

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is conducted to patients with GHD, but the therapy can cause various side effects such as leukemia, central nervous system tumors, hypothyroidism, epilepsy and diabetes (Ranke MB 1993; Bienkowski RS 1995; Kristensen et al. 2000; Hertel et al. 2002; Darendeliler F 2018).

Cynanchum wilfordii, which is used as an treatment of geriatric and musculoskeletal disease, is distributed in East Asia including Korea (Lee & Lee 2015; Lee et al. 2018). Several studies have reported that it also has a significant improvement in bone metabolism, such as blood levels of IGF-1 (Lim et al. 2014). *Phlomis umbrosa* is known to be effective in anti-inflammatory, bone density and recovering bone damage caused by fractures (Wong et al. 2007; Khan et al. 2014).

It has been reported in previous studies that bone growth differs according to sex and growth period. Moreover Thickness of cartilage in middle and posterior region were significant differences between 2-week experimental groups (Yu et al. 2012; Salahi et al. 2018; Lee et al. 2019; Kim et al. 2020). In this study, the growth-promoting effect of *Cynanchum wilfordii-Phlomis umbrosa* mixture (IPLUS-CWPU) was evaluated in 4-week-old young SD rats by measuring the length of growth plate and the expression level of IGF-1 gene.

Materials and Methods

1. Preparation of the extracts

Cynanchum wilfordii and *Phlomis umbrosa* extracts (IPLUS-CWPU) were used for manufacturing after verification that *Cynanchum auriculatum* Royle ex was not used in preparing the IPLUS-CWPU by the specified Institute according to the inspection standards of Ministry of Food and Drug Safety. Dried *Cynanchum wilfordii* was extracted by adding 10 times of purified water and heating for 10 hrs. After adding α-amylase (3%, based on extracted weight) and incubating for 6 hrs at 70℃, the mixtures were heated at 95℃ for 15 min to stop the α-amylase activity. The extracts were filtered, concentrated under reduced pressure at 10 brix, and freeze-dried. *Phlomis umbrosa* was extracted in the same way.

Finally, IPLUS-CWPU was prepared by mixing the same amount of the extract of *Cynanchum wilfordii* and *Phlomis umbrosa* at a ratio of 1:1.

2. Animal experiments

Three-week-old male Sprague–Dawley rats weighing approximately 50~65 g were purchased from Orientbio (Korea). The experimental procedures were conducted in according to the protocol approved by the Osong KBIO Health Institutional Animal Care and Use Committees (KBIO-IACUC-2021-007). One week before the experiment, rats were acclimatized under constant temperature, humidity and light-dark cycles (20±4℃; 50±10%; 08:00–20:00 light cycle). After 1 week of acclimation, the rats were randomly divided into five groups (n=8). Group 1 was an untreated Normal control. Group 2 received IPLUS-CWPU (200 mg/kg/day). Group 3 received IPLUS-CWPU (600 mg/kg/day). Group 4 received IPLUS-CWPU (1,800 mg/kg/ day). Group 5 was a positive control only received Eutropin $PLUS_{Inj}$ (Human growth hormone, somatropin 0.5 mg/kg/day, LG Chem). All experimental group except positive control group were orally administrated every day for 2 weeks. The normal control group was administrated vehicle (deionized water). The positive control group was subcutaneously injected a total of 7 times once every two days for 2 weeks. Body weight were recorded twice a week until the end of experiment. All animals were sacrified on 15 days. The liver was excised, washed with cold saline, and put into RNA later (Thermo Fisher, USA) for the RT-qPCR. Tibia and femur of the right hind limb were removed and measured immediately after extraction using a Vernier caliper and photographed.

3. Histological analysis

The extracted tibia and femur were fixed in 10% neutral formalin. After demineralization, the samples were embedded in paraffin and sectioned with thickness of 40 μm. H&E (Hematoxylin & Eosin) staining was performed, observed under a microscope, and photographed. The growth plate length and differentiation zones for bone formation was analyzed by Image J (Image J version 1.52p, National Institutes of Health, U.S.A.).

4. RT-qPCR

Total RNAs from the rat liver tissues were isolated using the RNeasy Mini Kit (QIAGEN, USA). Total RNA was quantified by measuring absorbance at 260 and 280nm using Infinite 200 PRO (Tecan, Austria). The iScript™ cDNA Synthesis Kit was used for cDNA synthesis (BIORAD, Hercules, CA, USA). RT-qPCR analysis was performed using TOPreal™ One-step RT-qPCR Mix (Enzynomics, Korea). The Reverse transcription reaction and amplification were carried out the following conditions; reverse transcription (50℃ for 30 min, 1 cycle),

pre-denaturation (95℃ for 15 min), denaturation (95℃ for 5 s), annealing and extension (60℃ for 30 s, 45 cycles).

Information of primer sequence is Table 1.

Target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, which is a relative quantification method.

5. Statistical analysis

Statistical analyses of all results were performed using student's *t*-test using SPSS (SPSS Version 21.0 Inc. U.S.A.). Data are presented as the means±standard error of the means. Student's t-tests for pairwise comparisons were used for data analysis. *p*-values<0.05 were considered statistically significant.

Results and Discussion

1. Body weight, and tibial and femoral bone length

The results of measuring body weight during the two-week experimental period are shown in Fig. 1. There was no significant difference in body weight increment between experimental groups. At final day of experiment, the animals were sacrificed, the tibia and femur were removed, and the lengths of each were measured (data not shown). The length of tibia and femur of normal control group were 34.25±0.22 and 34.25±0.22 mm, respectively. The lengths of tibia and femur of IPLUS-CWPU 200, 600 and 1,800 mg/kg administrated group were did not show any differences to that of normal control group. The lengths of the tibia and femur of the positive control group (EutropinPLUS$_{Inj}$) were 34.03±0.14 and 30.88±0.1 mm, respectively.

These results did not show any differences in weight and tibia and femur lengths between the experimental groups, and is thought to be due to the short duration of the two-week experiment period.

Table 1. Information of qPCR primer sequence

Gene		Primer sequence (5'-3')
IGF-1 (Yu et al. 2012)	Forward	TGCTTGCTCACCTTTACCAGC
	Reverse	TAAAAGCCCCTTGGTCCACAC
GAPDH	Forward	ACTCCCATTCTTCCACCTTTG
	Reverse	ACTCCCATTCTTCCACCTTTG

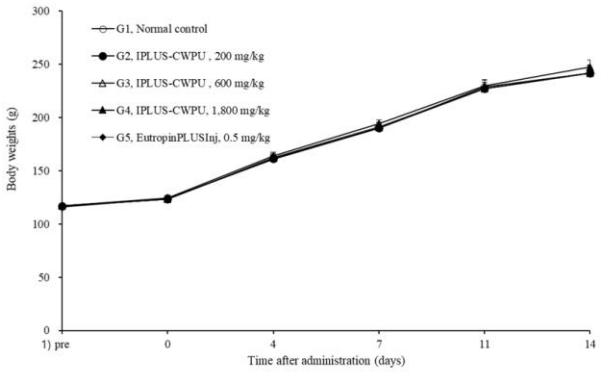

Fig. 1. Changes of body weight during the 2 weeks experimental period. Each data represented the mean± S.E.(n=8). No statistically significant differences was noted in the substance groups from the Normal control group (G1) (p>0.05, Independent *t*-test). [1)]Pre means group assignment day.

2. Tibial and femoral growth plate

Bone growth occurs at the growth plate, a thin layer situated at both ends between the diaphysis and the epiphysis of all long bones (Kronenberg HM 2003).

In order to confirm the effect of the IPLUS-CWPU administration on the bone growth plate, the length of growth plate of tibia and femur was measured, and the result is shown in Fig. 2.

The length of tibial growth plate of normal control group was 466.15±14.96 μm. The tibial growth plate lengths of the IPLUS-CWPU 200 and 600 mg/kg group were 488.31±18.13 and 502.37±14.40 μm, respectively, which did not show any significant difference from the normal control group. However, in IPLUS-CWPU 1,800 mg/kg group the length was 530.99±17.97 μm, which shows statistically significant increment compared to normal control group (p<0.05).

The length of the femoral growth plate of normal control group was 508.34±22.99 μm. The femoral growth plate length of the IPLUS-CWPU 200, 600 mg/kg and 1,800 group were 508.34±22.9, 624.51±41.81 and 637.04±20.26 μm, respectively. This result shows statistically significant increments in the 600 and 1,800 mg/kg groups (p<0.05 and p<0.01, respectively).

The lengths of tibial and femoral growth plates in positive control group (EutropinPLUS$_{Inj}$) were 515.4±11.37 and 604.44± 28.34 μm, respectively, which showed statistically significant increments (p<0.05).

A system of growth hormone (GH) is controlled bone growth by chondrocyte proliferation and ensuring hypertrophy in growth plates (Nilsson et al. 2005). It was reported that the administration of EutropinPLUS$_{Inj}$ increase the growth plate growth, which was consistent with our result (Lee et al. 2017).

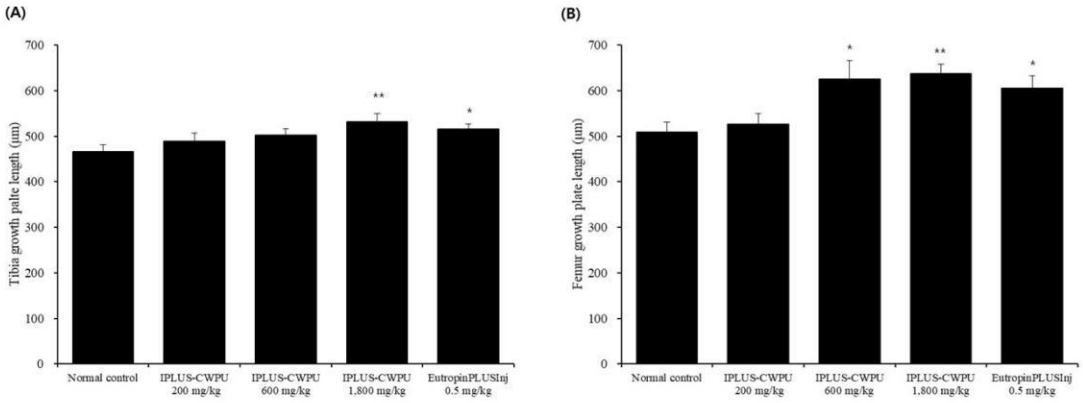

Fig. 2. Effect of IPLUS-CWPU administration on growth plate. (A) Tibial and (B) femoral growth plate length. Extracted tibia and femur was fixed in 10% neutral formalin. After demineralization, embedded in paraffin and sectioned with thickness of 40 μm. H&E (Hematoxylin & Eosin) staining was performed, observed under a microscope, and photographed (×400). Tibial and femoral growth plate length were calculated using Image J program. Each data represented the mean±S.E.(n=8). Significant difference from the Normal control group (G1) by Independent *t*-test ($^{*}p<0.05$, $^{**}p<0.01$).

3. Longitudinal bone growth

Longitudinal bone growth is the end result of metabolic processes taking place during the cell cycle of chondrocytes in the epiphyseal growth plate.

The growth plate consists of several distinct zones that reflect the gradual transition of cells through different stages of differentiation (Leach & Twal 1994). The resting zone is located at the top of the growth plate and acts as a reserve of precursor cells for the proliferating chondrocytes. In the proliferative zone, chondrocytes undergo rapid division, forming columns that serve as scaffolds for bone formation. Chondrocyte hypertrophy occurs in the hypertrophic zone, triggering bone formation (Leach & Twal 1994; Kronenberg HM 2003).

To confirm the effect of IPLUS-CWPU administration on the longitudinal bone growth, we performed the H&E staining and measured the height of 3 parts of proliferative zone as an indicator of longitudinal growth. In addition, the ratio of proliferative zone compared to total chondrocyte zone was calculated (Fig. 3A).

The proportion of tibial proliferative zone in the normal control group was 54.82±1.5%, and IPLUS-CWPU 200, 600 and 1,800 mg/kg administration groups were 53.11±1.68, 58.73±1.34 and 59.01±1.2%, respectively, in which the values were increased in a dose-dependent manner and were statistically significant.

The proportion of femoral proliferative zone in the normal control group was 44.98±4.19%, and IPLUS-CWPU 200, 600 and 1,800 mg/kg administration group were 42.50±3.23, 50.97±2.05 and 50.85±1.53%, respectively, in which the values were increased in a dose-dependent manner (Fig. 3B).

In case of positive control group, the proportion of tibial and femoral proliferative zone were 57.52±1.43 and 49.02±1.72%, respectively, which the vlaues were increased compared to the normal control group, but not statically significant.

This result suggests that the IPLUS-CWPU administration promotes bone growth by increasing the proliferative zone of the chondrocytes in bone growth plate.

4. IGF-1 gene expression in liver tissue

Growth hormone, a representative physiological active substance involved in growth, is produced in the pituitary gland. The hormone mainly targets the liver and regulates the expression level of IGF-1 through the JAK/STAT signaling pathway (Laron Z 2001; Cummings & Merriam 2003; Räz et al. 2008; Guevara-Aguirre et al. 2018; Zaidi et al. 2018). IGF-1 has a growth hormone independent growth stimulating effect and synergistic actions with the hormone (Yu et al. 2012; Bikle et al. 2015).

Therefore, we, evaluated the expression level of hepatic of IGF-1 by performing RT-qPCR and the results are shown in Fig. 4.

The IGF-1 gene expression level of the normal control group was 1.00±0.03, the expression levels of the IPLUS-CWPU 200, 600 and 1,800 mg/kg administration group were 1.12±0.12, 1.07±0.07 and 1.34±0.14, respectively. At 200 and 600 mg/kg

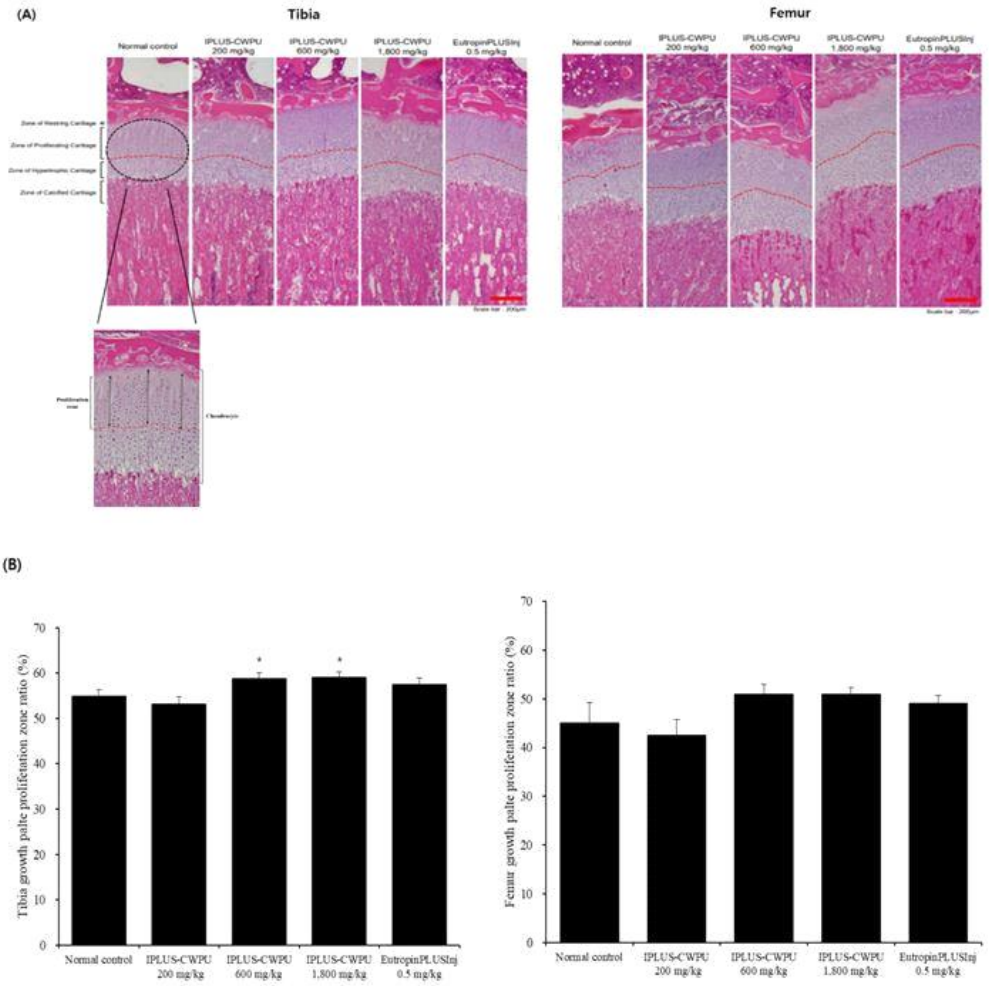

Fig. 3. Effect of IPLUS-CWPU administration on longitudinal bone growth. (A) Representative images of proximal end of the tibia and femur stained with H&E. The growth plate consists of several distinct zones that reflect the gradual transition of cells through different stages of differentiation. The resting zone, proliferative zone, hypertrophic zone and calcified zone. (B) Each proliferation height was measured three parts of chondrocytes at ramdomly, and then calculated the ratio of proliferation zone to the chondrocytes. Each data represented the mean±S.E.(n=8). Significant difference from the Normal control group (G1) by Independent *t*-test ($^{*}p<0.05$).

group there was not found any increase of mRNA level, but at 1,800 mg/kg group there was significant increase ($p<.0.05$).

The expression levels of IGF-1 in the positive control group were 1.35±0.1 and the level of IGF expression was statistically increased.

IGF-1 is mainly produced in liver by growth hormones and are most often expressed during the growth phase. IGF-1 binds to IGFBP-3 and migrates through the blood, is then separated from IGFBP-3 and binds to IGFR in the cell membrane, moves into the cell, and is enhanced the gene expression involved in growth (Emons et al. 2011).

This result suggests that the IPLUS-CWPU administration promotes bone growth by enhancing gene expression level of the hepatic IGF gene.

Conclusion

The purpose of this study was to evaluated the growth-promoting effect of *Cynanchum wilfordii-Phlomis umbrosa* mixture (IPLUS-CWPU) in 4-week-old young SD rats by measuring the length of growth plate and the expression level of IGF-1 gene.

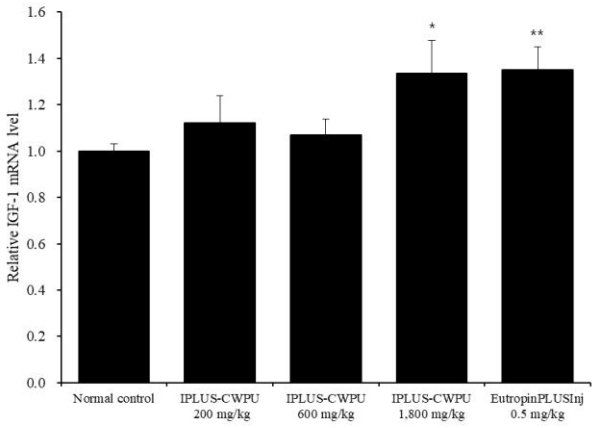

Fig. 4. Effect of IPLUS-CWPU administration on IGF-1 gene expression. Expression of IGF-1 gene wasere confirmed by RT-qPCR. After total RNA extraction, cDNA synthesis and amplification were performed. The expression level of genes were calculated using a relative quantitative method, and GAPDH was used as an internal control. Each data represented the mean±S.E.(n=8). Significant difference from the Normal control group (G1) by Independent *t*-test ($^{*}p<0.05$).

In this study, growth promoting efficacy was evaluated using a natural mixture extract IPLUS-CWPU, which is expected to have fewer side effects compared to growth hormone therapy. Previous studies have shown that the extract have the effects of improving muscular dystrophy, increasing bone density and regenerating bone tissue (Wong et al. 2007; Kim et al. 2017).

As a result, it was confirmed that the rats administered orally with IPLUS-CWPU of 1,800 mg/kg for 14 days increased the expression of IGF-1 by about 34% compared to the normal rats. This result was similar to the positive control group that injected with Somatropin (growth hormone). The length of the bone growth plates in tibia and femur increased by 13.9 and 25.3%, respectively, compared to normal rats. However, there was no significant difference in bone length of the tibia and femur. It is estimated that the administration period of the test substance should be designed to be longer than 2 weeks in order to confirm a significant difference in bone length due to osteogenesis.

In conclusion, administration of *Cynanchum wilfordii-Phlomis umbrosa* mixture (IPLUS-CWPU) significantly increased tibial and femoral growth plate length and hepatic IGF-1 expression level, indicating that the test substance has a growth promoting effect. In further studies, we plan to investigate whether IPLUS-CWPU is involved in the classical signaling model of IGF, involving ligand-receptor interaction of IGF via downstream signaling transduction such as PI3K-AKT and ERK pathways, or is involved in other mechanisms.

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