大韓本草學會誌 제32권 제1호(2017년 1월)
Kor. J. Herbol. 2017 ; 32(1) : 69-74

ISSN 1229-1765(Print), ISSN 2288-7199(Online)
http://dx.doi.org/10.6116/kjh.2017.32.1.69.

Effects of *Angelica sinensis* Root on Longitudinal Bone Growth Rate in Adolescent Female Rats

Donghun Lee[#], Hocheol Kim[*]

Kyung Hee University, College of Korean Medicine, Department of Herbal Pharmacology

ABSTRACT

Objectives : This study aimed to investigate the effects of Angelicae sinensis Radix on longitudinal bone growth rate in rats. We have screened traditional medicinal herbs to develop the longitudinal bone growth stimulator by well-established rat model. *A. sinensis* was identified as one of the effective herbs in the screening process.

Methods : Adolescent female rats were administered *A. sinensis* at doses of 30 ㎎/㎏ and 300 ㎎/㎏ for 10 consecutive days. To observe the rate of longitudinal bone growth, tetracycline was injected intraperitoneally on day 8 to stain a fluorescent band on the anew formed bone. To elucidate the mode of action, we observed insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) expression after *A. sinensis* administration in growth plate.

Results : In the 300 ㎎/㎏ *A. sinensis* group, the length between the proximal endpoint of the tetracycline label and the division line between growth plate and bone was significantly increased compared with vehicle-treated control group. Height of the proximal tibial growth plate was higher in the *A. sinensis* group compared with control group. *A. sinensis* also upregulated the expressions of IGF-1 and BMP-2 in the proliferative zone and hypertrophic zone of the proximal tibial growth plate.

Conclusions : *A. sinensis* increases longitudinal bone growth rate in rats. According to immunohistochemistry, *A. sinensis* increases local IGF-1 and BMP-2 expressions in the growth plate which can be considered as direct stimulation of GH on the local growth plate.

Key words : *Angelica sinensis*, longitudinal bone growth, IGF-1

I. Introduction

Short stature is defined as the height that is -2.0 standard deviations below the average height for a given age and gender[1]. Children with short stature might have a behavioral and psychosocial disorders including social immature, lower self-esteem, social immaturity, or being teased[2-5]. People with shorter height tend to have a lower health-related quality of life[6] and higher morbidity of cardiovascular diseases, probably because of small diameter of blood vessels[7,8].

Only about 20% of all short children have certain disorders including growth hormone (GH) deficiency, but the remaining 80% have short stature without determined reason, so-called idiopathic short stature (ISS)[1]. In case of GH deficiency, mean increase in final height due to GH therapy is about 30 cm compared to predicted adult height[9,10]. In case of ISS, mean increase in final height attributable to GH therapy is just 3.5-7.5 ㎝ (4-7 years)[11,13-16]. High cost of treatment, estimated 10,000-20,000 dollars/㎝[1,15], injection pain, and limitations of use that it can only be used for short stature patients are also considered controversial[17,18].

We have screened traditional medicinal herbs to develop the longitudinal bone growth stimulator by well-established rat model which can evaluate the bone growth rate with tetracycline[19]. The root of

*Corresponding author : Hocheol Kim, Kyung Hee University, College of Korean Medicine, Department of Herbal Pharmacology, Seoul, 130-701, Republic of Korea.
· Tel : +82-2-961-0419 · E-mail : hckim@khu.ac.kr
#First author : Donghun Lee, Kyung Hee University, College of Korean Medicine, Department of Herbal Pharmacology, Seoul, 130-701, Republic of Korea.
· Tel : +82-2-961-0419 · E-mail : allstart2925@naver.com
· Received : 17 November 2016 · Revised : 5 January 2017 · Accepted : 15 January 2017

Angelica sinensis (Oliv.) Diels (Apiaceae) was identified as one of the effective herbs in the screening process. *A. sinensis* is widely used to tonify blood, alleviate pain and regulate menstruation. *A. sinensis* was reported to have anti-inflammatory, anticoagulative, hepatoprotective and neuroprotecitve effects[20]. *A. sinensis* contains ferulic acid, butylphathlide, butylidenephthalide, Z-ligustilide, linoleic acid known to be responsible for its varied biological activities[21]. Aqueous extract of *A. sinensis* has been reported to stimulate proliferation of osteoprecursor cells[22].

To evaluate the effects of *A. sinensis* on longitudinal bone growth rate, tetracycline labeling was performed to stain anew formed bone in the growth plate. We have also analyzed the effects of *A. sinensis* on zonal height in growth plate of proximal tibia. Height growth is the consequence of proliferation and hypertrophy of chondrocytes in the growth plates caused by direct stimulation of GH or circulating insulin-like growth factor-1 (IGF-1)[23,24]. To elucidate the mode of action whereby *A. sinensis* exerts its growth-stimulating effect, we evaluated the IGF-1, and bone morphogenetic protein-2 (BMP-2) expression after *A. sinensis* administration in growth plate.

Ⅱ. Materials and Methods

1. Material

The dried root of *A. sinensis* was purchased from Yaksudang in Seoul, Korea. *A. sinensis* was imported from China. *A. sinensis* was identified Dr Hocheol Kim, Department of Herbal Pharmacology, Kyung Hee University, where the voucher specimen (#HP114) was deposited.

2. Sample Preparation

The dried root of *A. sinensis* was extracted in a reflux apparatus (30% aqueous ethanol, 3 h at 90℃) and lyophilized after concentration under reduced pressure. The yield was 46.8%.

3. Animals

Female 25-day-old Sprague-Dawley rats were used (Samtako Inc, Korea). This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP[SE]-13-028). All rats were housed under controlled conditions (22 ± 1℃, 12 h of light starting at 07:00) in an isolated ventilated chamber with food and water available *ad libitum*.

4. Treatment

After 7 days of acclimatization, rats were randomly divided into four groups: control, recombinant human GH (rhGH) 20 ㎍/kg. *A. sinensis* 30 ㎎/kg and *A. sinensis* 300 ㎎/kg, which are equivalent to 150 mg/day and 1.5 g /day for 30 kg children. Vehicle, *A. sinensis* 30 ㎎/kg or 300 ㎎/kg were orally administered twice daily (8:00 am; 8:00 pm) and rhGH 20 ㎍/kg (Eutropin, LG, Korea) was subcutaneously injected once daily (8:00 am) for 10 consecutive days. On the 11th day, rats were killed for analysis.

5. Longitudinal Bone Growth Rate

Tetracycline hydrochloride 20 ㎎/kg was injected intraperitoneally 72 h prior to sacrifice (Sigma Aldrich, USA). Tibias were dissected, fixed in 4% paraformaldehyde and underwent decalcification by immersion in 50 mM ethylenediaminetetraacetic acid solution (Sigma Aldrich). After dehydration by immersion in 30% sucrose, tibias were cut at sagittal sections of the proximal part with thickness of 40 ㎛ using a cryostat (CM3050S, Leica Microsystems, Germany). Bone growth rate per day was assessed by measuring length between the proximal endpoint of the tetracycline label and the division line between growth plate and dividing the distance into three. Fluorescent band was observed with a fluorescent microscope (BX50, Olympus, Japan) and the length was blind measured with Image J software (NIH, USA) by three different researchers to avoid the possible distinction among individuals.

6. Growth Plate Height

Cresyl violet (CV) staining of the chondrocytes was used to measure the height of growth plate (Sigma Aldrich). Overall growth plate height was measured at three different locations by using Image J software.

7. Immunohistochemistry

For detecting IGF-1 and BMP-2 expression in the growth plate, dehydrated sections of tibia were prepared as described above and reacted with rabbit IGF-1 antibody and goat BMP-2 primary antibody diluted 1/200 overnight (Santa Cruz, USA). After washing, sections were reacted with biotinylated rabbit antibody diluted 1/200 (Jackson Immunoresearch Laboratories), incubated with avidin-

biotin complex reagent diluted 1/100 (Vectastain ABC Kit, Vector Laboratories, USA) for 1 h and developed with 0.05% 3,3-diaminobenzidine solution (Sigma Aldrich) containing hydrogen peroxide.

8. Statistics

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, USA). Differences with $p<0.05$ between groups were considered statistically significant. All results were expressed as mean values ± SD.

Ⅲ. Results

1. Effect on Longitudinal Bone Growth Rate

To evaluate the effect of *A. sinensis* on longitudinal bone growth rate, tetracycline was used as an intravital marker to label the anew formed bone of proximal tibia. The double-headed arrow indicates the length of bone growth in proximal tibial growth plate during 72 h period (Figure 1A). The distance was significantly increased by oral administration of *A. sinensis* at doses of 30 or 300 ㎎/㎏ compared with the control group. Longitudinal bone growth rate in the control group was 352.8 ± 19.4 ㎛/day, and in rhGH treated group was 373.6 ± 17.2 ㎛/day. Oral administrated *A. sinensis* 30 and 300 ㎎/㎏ significantly increased the longitudinal bone growth rate by 364.7 ± 22.3 and 373.9 ± 25.5 ㎛/day.

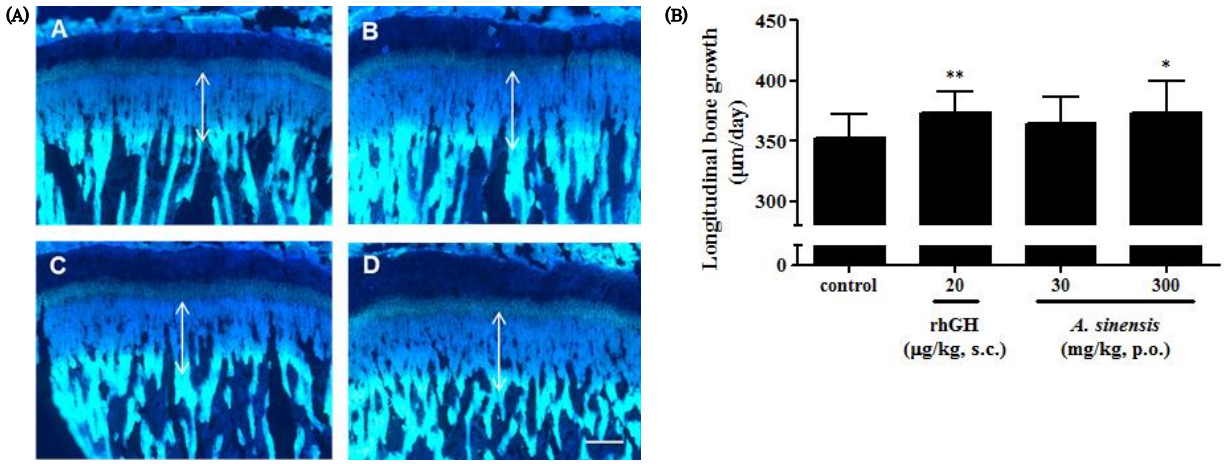
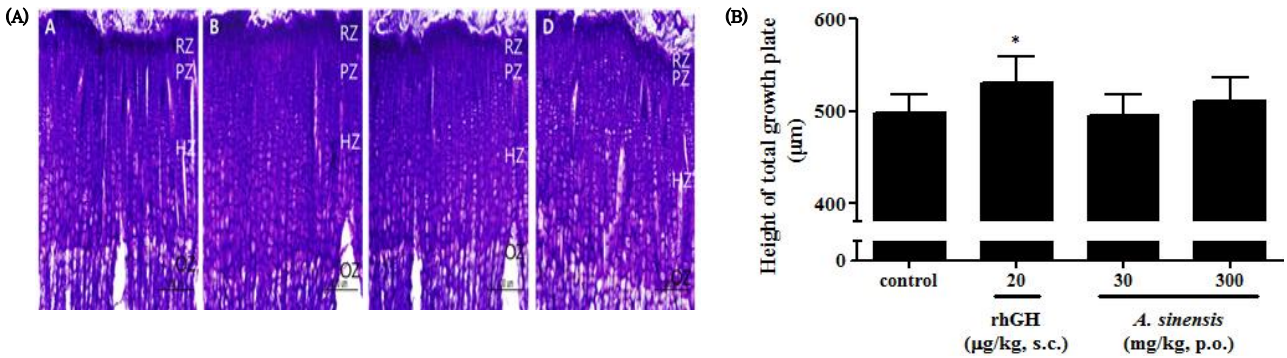


Figure 1. (A) Representative fluorescence photomicrographs of sagittal sections of proximal tibial growth plate in rats. The double-headed arrow shows the length between the proximal endpoint of the tetracycline label and the division line between growth plate and bone which indicates the length of bone growth in proximal tibial growth plate during 72 h period. a: vehicle treated control group, b: rhGH 20 ㎍/㎏ (s.c.) treated group, c: *A. sinensis* 30 ㎎/㎏ (p.o.) treated group, d: *A. sinensis* 300 ㎎/㎏ (p.o.) treated group. The scale bar is 200 ㎛ (B) Effects of *A. sinensis* on longitudinal bone growth rate in proximal tibial growth plate. Each value is the mean ±SD.

2. Effect on Growth Plate Height

Height of growth plate was measured by CV staining. Typical images of stained sections are shown in Figure 2. The overall height of growth plate was 497.4 ± 20.8 ㎛ in the control group and in rhGH treated group was 531.3 ± 27.7 ㎛. Oral administration of *A. sinensis* increased growth plate height exhibiting 510.9 ± 25.2 ㎛ at dose of 300 ㎎/㎏ but the difference was not statistically significant compared with the control group.



photomicrographs of cresyl violet-stained chondrocytes of the proximal tibial growth plate in rats. a: vehicle treated control group, b: rhGH 200 ㎍/㎏ (s.c.) treated group, c: *A. sinensis* 30 ㎎/㎏ (p.o.) treated group, d: *A. sinensis* 300 ㎎/㎏ (p.o.) treated group. The scale bar is 200 ㎛. (B) Effects of *A. sinensis* on overall height of proximal tibial growth plate. Each value is the mean ±SD.

3. Effects on IGF-1 and BMP-2 Expression

Protein expressions of IGF-1 and BMP-2 were assessed with antigenspecific immunohistochemistry in the proximal tibial growth plate. IGF-1 expression was higher in hypertrophic zones than in resting and proliferative zones. Administration of *A. sinensis* or rhGH remarkably increased the IGF-1 expression intensity in the proliferative and hypertrophic zone compared with control. BMP-2 was more highly expressed in the hypertrophic zones and markedly increased by administration of *A. sinensis* or rhGH particularly.

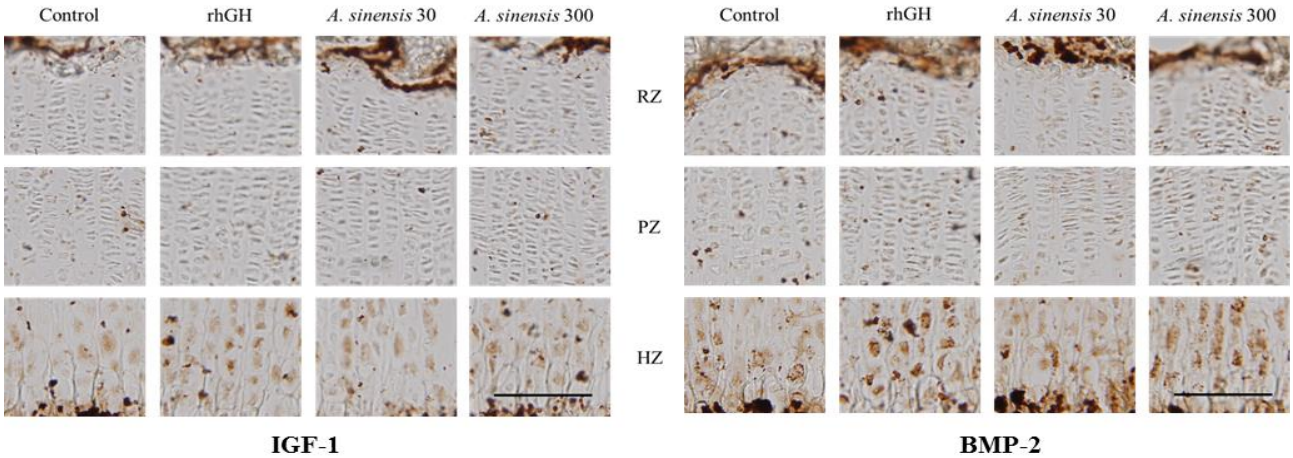


Figure 5. Immunohistochemical localization of insulin-like growth factor-1 and bone morphogenetic protein-2 on the proximal tibial growth plate in rats. control: vehicle treated control group, rhGH: rhGH 200 μg/kg (s.c.) treated group, A. sinensis 30: A. sinensis 30 mg/kg (p.o.) treated group, A. sinensis 300: A. sinensis 300 mg/kg (p.o.) treated group, RZ: resting zone, PZ: proliferative zone, HZ: hypertrophic zone. The scale bar is 100 μm.

Ⅳ. Discussion

Oral administration of 30% EtOH extracts of *A. sinensis* significantly increased longitudinal bone growth rate, height of the proximal tibial growth plate compared to control. *A. sinensis* also upregulated the expressions of IGF-1 and BMP-2 in hypertrophic zone of the growth plate.

A. sinensis at doses of 30 and 300 ㎎/㎏ was shown to increase the bone growth rate to 3.4% and 6.0% in the proximal tibial growth plate, respectively. Tetracycline gets deposited in anew formed bones and led to fluorescence band corresponding to the injection, and the length between the proximal endpoint of the tetracycline label and the division line between growth plate and bone indicates the longitudinal bone growth rate, the amount of bone growth during specified period of time[25]. The result suggests that *A. sinensis* increases longitudinal bone growth rate.

A. sinensis at dose of 300 ㎎/㎏ increased overall height of growth plate by 2.7% compared to the control group in the proximal tibial growth plate. Growth plate is composed of chondrocytes with three characteristic histological regions, resting zone, proliferative zone and hypertrophic zone[18]. Brisk chondrocyte division in proliferative zone and following chondrocyte enlargement in hypertrophic zone cause rise in bone growth rate and growth plate height[26]. Former studies have found high correlations between bone growth rate and growth plate height regardless of bone location or animal age[27-29].

A. sinensis increased the expressions of local IGF-1 and BMP-2 in the proliferative zone and hypertrophic zone of tibial growth plate. Bone growth is the consequence of chondrocyte proliferation and hypertrophy of the growth plates stimulated by either circulating IGF-1 mainly produced in the liver by GH or local IGF-1 produced by direct stimulation of GH. Production of local IGF-1 is mainly dependent on circulating GH and local IGF-1 is combined with IGF-1 receptor of the surface of chondrocytes in the growth plate, like systemic IGF-1[17]. Local IGF-1 in the growth plate is the main mediator of the direct GH effects on the longitudinal bone growth[18,30].

Local BMP-2 expression has been known to stimulate the bone growth by accelerating proliferation and hypertrophy of chondrocytes[31]. Lately, it was reported that circulating GH increases local BMP-2 production in the growth plate unlike circulating IGF-1[32]. These findings suggest that GH-dependent increase of local BMP-2 expression in the growth plate may be due to direct growth-promoting effects of GH. Taken together, these results suggest that growth stimulating effects of *A. sinensis* might be due to local IGF-1 and BMP-2 expression by direct stimulation of GH on the local growth plate.

Ⅴ. Conclusion

This study addressed the effects of *A. sinensis* on longitudinal bone grwth rate in adolescent female rats. The summary of results and conclusion are as follows:

1. Oral administration of *A. sinensis* increased the longitudinal bone growth rate in adolescent female rats.
2. *A. sinensis* increased growth plate height but the difference was not statistically significant.
3. *A. sinensis* increased the intensity of IGF-1 expression in proliferative and hypertrophic zone of growth plate.
4. BMP-2 expression was also markedly increased by administration of *A. sinensis*.

Taken together, *A. sinensis* increases longitudinal bone growth rate. According to the immunohistochemistry, growth-stimulating effect of *A. sinensis* may also be due to local IGF-1 and BMP-2 expressions in the growth plate, which can be considered as direct stimulation of GH on the local growth plate.

Acknowledgments

This study was supported by a grant of the Korean Health Technology R & D Project, Ministry of Health & Welfare, Korea (HI14C0976).

References

1. Cohen P, Rogol AD, Deal CL, Saenger P, Reiter EO, Ross JL, Chernausek, SD, Savage MO, Wit JM. Consensus statement on the diagnosis and treatment of children with idiopathic short stature: A summary of the growth hormone research society, the lawson wilkins pediatric endocrine society, and the european society for paediatric endocrinology workshop. J Clin Endocrinol Metabol. 2008; 93: 4210-7.
2. Downie AB, Mulligan J, Stratford RJ, Betts PR, Voss LD. Are short normal children at a disadvantage? The wessex growth study. Bmj. 1997; 314: 97-100.
3. Stabler B, Clopper RR, Siegel PT, Stoppani C, Compton PG, Underwood LE. Academic achievement and psychological adjustment in short children. The national cooperative growth study. J Dev Behav Pediatr. 1994; 15: 1-6.
4. Voss LD, Sandberg DE. The psychological burden of short stature: Evidence against. Eur J Endocrinol. 2004; 151: Suppl 1 S29-33.
5. Zimet GD, Owens R, Dahms W, Cutler M, Litvene M, Cuttler L. Psychosocial outcome of children evaluated for short stature. Arch Pediatr Adolesc Med. 1997; 151: 1017-23.
6. Christensen TL, Djurhuus CB, Clayton P, Christiansen JS. An evaluation of the relationship between adult height and health-related quality of life in the general uk population. Clin Endocrinol. 2007; 67: 407-12.
7. Paajanen TA, Oksala NK, Kuukasjarvi P, Karhunen PJ. Short stature is associated with coronary heart disease: A systematic review of the literature and a meta-analysis. Eur Heart J. 2010; 31: 1802-9.
8. Wannamethee SG, Shaper AG, Whincup PH, Walker M. Adult height stroke and coronary heart disease. Am J Epidemiol. 1998; 148: 1069-76.
9. Hindmarsh PC, Dattani MT. Use of growth hormone in children. Nat Clin Pract Endocrinol Metab. 2006; 2: 260-8.
10. Wit JM, Kamp GA, Rikken B. Spontaneous growth and response to growth hormone treatment in children with growth hormone deficiency and idiopathic short stature. Pediatr Res. 1996; 39 295-302.
11. Hintz RL, Attie KM, Baptista J, Roche A. Effect of growth hormone treatment on adult height of children with idiopathic short stature Genentech collaborative group. N Engl J Med. 1999; 340: 502-7.
12. Leschek EW, Rose SR, Yanovski JA, Troendle JF, Quigley CA, Chipman JJ, Crowe BJ, Ross JL, Cassorla FG, Blum WF. Effect of growth hormone treatment on adult height in peripubertal children with idiopathic short stature: A randomized double-blind placebo-controlled trial. J Clin Endocrinol Metab. 2004; 89: 3140-8.
13. August GP, Julius JR, Blethen SL. Adult height in children with growth hormone deficiency who are treated with biosynthetic growth hormone: The national cooperative growth study experience. Pediatrics. 1998; 102: 512-6.
14. Bajpai A, Menon PS. Growth hormone therapy. Ind J Ped. 2005; 72: 139-44.
15. Bryant J, Baxter L, Cave CB, Milne R. Recombinant growth hormone for idiopathic short stature in children and adolescents. Cochrane Database Syst Rev. 2007; CD004440.
16. Finkelstein BS, Imperiale TF, Speroff T, Marrero U, Radcliffe DJ, Cuttler L. Effect of growth hormone therapy on height in children with idiopathic short stature: A meta-analysis. Arch Pediatr Adolesc

Med. 2002; 156: 230-40.
17. Hintz RL. Growth hormone: Uses and abuses. Bmj. 2004; 328: 907-8.
18. Silverman BL, Blethen SL, Reiter EO, Attie KM, Neuwirth RB, Ford KM. A long-acting human growth hormone (nutropin depot): Efficacy and safety following two years of treatment in children with growth hormone deficiency. J Pediatr Endocrinol. 2002; 15: Suppl 2 715-22.
19. Leem K, Park SY, Lee DH, Kim H. Lovastatin increases longitudinal bone growth and bone morphogenetic protein-2 levels in the growth plate of sprague-dawley rats. Eur J Pediatr. 2002; 161: 406-7.
20. Wen LW, Rui Z, Cai MG, Yan Q, Ling FH. Angelica sinensis in China-A review of botanical profile ethnopharmacology phytochemistry and chemical analysis. J Ethnopharmacol. 2016; 190: 116-41.
21. Gu ZR, Wang YL, Sun YJ, Ding JX. Simultaneous determination of five constituents in Angelica Sinensis from different areas and the quality evaluation. Chin Tradit Patent Med. 2014; 36: 2135-9.
22. Yang Q, Populo SM, Zhang J, Yang G, Kodama H. Effect of Angelica sinensis on the proliferation of human bone cells. Clin Chim Acta. 2002; 324: 89-97.
23. Sims NA, Clement-Lacroix P, Da Ponte F, Bouali Y, Binart N, Moriggl R, Goffin V, Coschigano K, Gaillard-Kelly M, Kopchick J. Bone homeostasis in growth hormone receptor-null mice is restored by igf-i but independent of stat5. J Clin Invest. 2000; 106: 1095-103.
24. Yakar S, Rosen CJ. From mouse to man: Redefining the role of insulin-like growth factor-i in the acquisition of bone mass. Exp Biol Med. 2003; 228: 245-52.
25. Hansson LI, Stenstrom A, Thorngren K. Skeletal deposition and toxicity of methacycline. Nature. 1968; 219: 624-5.
26. Hunziker EB. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech. 1994; 28: 505-19.
27. Breur GJ, VanEnkevort BA, Farnum CE, Wilsman NJ. Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. J Orthop Res. 1991; 9: 348-59.
28. Hansson LI. Daily growth in length of diaphysis measured by oxytetracycline in rabbit normally and after medullary plugging. Acta Orthop Scand. 1967; Suppl 101: 101+.
29. Kuhn JL, DeLacey JH, Leenellett EE. Relationship between bone growth rate and hypertrophic chondrocyte volume in new zealand white rabbits of varying ages. J Orthop Res. 1996; 14: 706-11.
30. Hero M, Wickman S, Dunkel L. Treatment with the aromatase inhibitor letrozole during adolescence increases near-final height in boys with constitutional delay of puberty. Clin Endocrinol. 2006; 64: 510-3.
31. De Luca F, Barnes KM, Uyeda JA, De-Levi S, Abad V, Palese T, Mericq V, Baron J. Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. Endocrinology. 2001; 142: 430-6.
32. Wu S, Yang W, De Luca F. Insulin-like growth factor-independent effects of growth hormone on growth plate chondrogenesis and longitudinal bone growth. Endocrinology. 2015; 156: 2541-51.