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원저

Effects of *Ulmus davidiana* Planch(Ulmaceae) herbal acupuncture solution on the proliferation of human bone cells

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국문초록

유근피 약침액이 인체의 골세포 증식에 미치는 영향

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유근피는 혈액청정작용과 혈액순환에 영향을 주는 성분으로서 골 손상의 처방전으로 자주 사용된다. 현재까지 유근피가 골 재형성에 미치는 영향은 약리학적으로 불확실하였다. 이에 저자들은 본 연구에서 유근피를 약침액으로 제조하여 유근피 약침액이 골세포에 미치는 영향을 in vitro에서 연구하였다. 방법으로 인체의 골아전구세포osteoprecursor cells (OPC-1)를 각각의 다른 유근피 농도를 함유한 매체내에서 부화시키고 그에 따른 세포증식을 연구하였으며, 유근피 약침액의 농도가 100 μg/ml 미만이었을 때 OPC-1의 증식량은 증가되었다. 그러나 농도가 180 μg/ml을 초과하였을 때는 약물의 독성에 의해서 OPC-1의 증식량이 확연히 억제되었다. 대부분의 처치에서 세포들이 cyclooxygenase-2 (Cox 2) 단백질에 대해서 매우 명백한 발현을 보여줬다. 배양과정 중에 유근피 약침액 농도 최소치인 1.0μg/ml에서 최대치인 500μg/ml까지 경미하게 강화된 띠를 나타내었다. 이와 같은 실험의 결과로 볼 때 유근피 약침액은 골세포의 증식활동, alkaline phosphatase (ALP) 활동 및 total protein 분비의 증가와 골세포내에서

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의 농도의존적 약침액 투여량에 따른 OPC-1의 독특한 type I collagen 합성에 직접적인 억제작용을 주는 것을 관찰할 수 있으므로 추후 이와 유사한 실험을 통한 보다 발전적인 연구가 이루어져야 한다고 사료되었다.

Abbreviations : ALP, alkaline phosphatase; OPC-1, osteoprecursor cell line 1; PBS, phosphate buffer solution

Key words : Ulmus davidiana Planch (Ulmaceae) Herbal acupuncture solution; Human osteoprecursor cells; Alkaline phosphatase; Type- I Collagen

I. Introduction

Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models. Estrogen [1-2], bisphosphonates [1], calcitonin [1], calcium products [1, 3], ipriflavone [4] and anabolic steroids [5] are clinically employed as effective medications. Oriental medicines have been reevaluated by clinicians [6], because these medicines have fewer side effects and because they are more suitable for long-term use as compared to chemically synthesized medicines. About forty kinds of oriental medicines are claimed to be effective for gynecological diseases such as climacteric psychosis, feeling of cold, menstrual disorders, dysmenorrhea, and low back pain. It has been suggested that the effectiveness of oriental medicines on low back pain seems to correspond to their efficacy in curing osteoporosis [7].

From ancient times in China, Korea and Japan, women who have had low back pain in climacteric and senescent periods have been treated with oriental medicines. For example, some formula have been used in treating ovary function failure, used in treating low back pain during the climacteric period, and also used after oophorectomies because of malignant tumors [7-8]. However, no data are available as to the recovery of bone mass by any of these oriental medicines. *Ulmus davidiana* Planch (Ulmaceae) (UD), is effective for the treatment of deficient kidneys manifested as lower back pain, weakness of the legs, tinnitus or toothache by function of tonifing the kidney, and invigorating blood and stop bleeding according to the Korean and Chinese medicinal literature [9]. Since a large decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis [2]. UD has been known for a long time for its effects of cleansing blood and increasing circulation and utilized as a valuable remedy for anemia, menstrual irregularities, and constipation in traditional Korean and Chinese medicine. To treat

the osteoporosis, a herbal formula containing UD is being used in Korean medicine [10]. Clinical data has shown that these prescriptions had significant effect in reducing the time needed for the injured bones to heal.

Ulmus davidiana Planch (Ulmaceae) is a deciduous tree, which is widely distributed in Korea. The barks of the stem and the root of this plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation [11-13]. As a part of our search for new biologically active substances from traditional medicines, we evaluated whether UD-HAS with extracts of *U. davidiana* stem barks (UD) could modulate the induction of RA in mice. UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Chinese and Korean medicine [14-15]. UD is known for their functions in maintaining or assisting blood circulation. UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue [16]. UD has long been used by Korean traditional medicine physicians in Korea. However, little is still known about the mode of action of this traditional medication on bone metabolism.

However, because these prescriptions were produced in centuries by trial and error and their effects were confirmed only through repeated clinical applications, it is unclear how the herbs pharmacologically influence the bone tissue to prompt healings. Possibly, the impact of UD on the circulation and immune systems has improved nutrition supply and immunity of the injured site. Nevertheless, especially in the case of pasting medicine in contact with the injured tissue, UD is likely to have direct stimulations on bone formation. Currently, no scientific research has been done on this subject.

The present research focuses on the direct cellular-level effect of UD-HAS on bone cells. It tests whether this herbal acupuncture stimulation in the form of aqueous extract has the ability to stimulate the proliferation and protein production, particularly type I collagen synthesis, of human osteoprecursor cells, a cell line of osteoblast suited for in vitro culture. Whether different concentrations of UD can induce cytotoxicity during the course of culture was also examined.

II. Materials and Methods

1. UD materials and chemicals

UD is from Korean Herbs and Traditional Korean Medicine Hospital, Kyungju, Korea. The voucher specimen (No. UD-W-57) is deposited in the herbarium of this college. Its identity was confirmed by comparison with descriptions of characteristics and appropriate monograph in Korea Pharmacopoeia [14]. *UD* extracts was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University (Kyungju, Korea). The traditional method for the clinical preparation of herbal treatment was employed. Briefly, finely cut UD 10 g was added to distilled water (100 ml) in a flask with a condensation apparatus on the top allowing evaporated steam to reenter the system and heated at 100℃ for 24 hrs in an oil bath, using an electric hot plate as heat source. After the solution cooled,

residue precipitation was filtered off and put into water for secondary extraction. The aqueous extracts were mixed and evaporated to dryness under reduced pressure with a rotary evaporator at 40 °C. The dried residue was dissolved in distilled water and 1% DR aqueous extract was used for cell culture].

The herb had a moisture content of <10% by weight, and was air-dried. Air-dried barks (totalling 70 g dry weight) were mixed, minced with a grinder, and extracted by storing in 1 litre of boiling water for 3 hours. The supernatant was filtered with 10 μm cartridge paper and ethanol was removed by rotary evaporation (Eyela, Tokyo, Japan), and concentrated extracts were freeze-dried. This process generally produced 15 g of brown powder. A voucher specimen has been deposited at the Kyungju Oriental Medical Hospital, Dongguk University, Kyungju city, Kyungbuk, Korea under acquisition number UD-13.

All chemicals and laboratory materials were from Sigma (St. Louis, MO) or Gibco BRL (Grand Island, NY) unless otherwise stated. Tissue culture media and reagents, Fetal bovine serum (FBS) were from Gibco (Chagrin Falls, OH). Human osteoprecursor cells (OPC-1) were obtained as described by Winn et al. [15].

2. Osteoblasts isolation and culture

Cells were grown to confluent in 75㎠ culture flasks (Falcon) in Dulbesso's modification of Eagel's medium (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10 % fetal calf serum (FCS; Gibco, BRL, Bethesda, MD, USA). Incubations were carried out at 37℃ in a humidified atmosphere of 5 % CO_2/95 % air; the medium was changed every 2-3 days. Cells were grown to confluence at 37℃ and cultured in duplicate or triplicate wells for an additional 24 hrs in serum-free medium supplemented with Polymixin B sulfate to prevent endotoxin effects prior to treatment. Initially, human osteoprecursor cell line (OPC-1) was cultured in 250 ml tissue culture flask (Becton Dickinson, England) without the interference of herbal extract using 15 ml F12 tissue culture medium with 10% fetal calf serum and antibiotics (1% penicillin and streptomycin) at 37℃ with 5% CO_2 and 95% air. The medium was changed on the third day. The cells showed complete adhesion to the bottom of the flask after 2 days. On the seventh day of culture, the cells were harvested with 5 ml Trypsin-EDTA (Gibco, USA) and diluted with 35 ml of fresh medium.

Newly harvested OPC-1 cells were split into culture dishes (j 8.5 mm) for subculture under the influence of UD-HAS . Each dish contained 2 ml of harvested cells and 0 -800 m l of 1% UD-HAS with corresponding amount of F12 medium so the total volume of culture medium was 8 ml with a plating cell density 10x104 cells/ml. For the control group, no DR and 800 ml of distilled water and 5.2 ml of F12 medium were added since the aqueous extract contained considerable amount of water. Three sets of eight samples with final DR concentrations 0, 12.5, 31.3, 62.5, 125, 250, 500, 750 and 1000 μg/ml were obtained.

The cells were cultured in the conditions described above for 5 days. The growth of cells was monitored under a light microscope every 12 h and the cell numbers in each dish were counted as cell proliferation. The cells were fixed in 2.5% paraformaldehyde and 2% glutaraldehyde in PBS for 30 min and microscopic pictures were taken on

the fourth day.

3. Evaluation of alkaline phosphatase (ALP) activity

Because OPC-1 cells produce strongly ALP-positive secretions, the activity of alkaline phosphatase was evaluated as another mark of bone cell proliferation. The activities of alkaline phosphatase were measured by the method of Ishaug et al. [16]. Briefly, on the fifth day of culture, 100 ml medium from each dish was taken out and mixed with 1 ml of p-nitrophenyl phosphate solution (16 mmol/l, Diagnostic Kit 245, Sigma) at 30℃ for up to 5 min.

The light absorbance at 405 nm of p-nitrophenol product formed as a result of ALP-p-nitrophenyl phosphate substrate complex was measured on a microplate reader (Bio-Tek Elx 800, Fisher Scientific, USA) and compared with serially diluted standards. The activity of the enzyme was expressed as nanomole of p-nitrophenol per minute per dish.

4. Harvest cells and protein extraction

After 5 days of culture (approximately 120 hrs), the cells in each dish were harvested with 2.5 ml trypsin. After the cells completely detached from the bottom of the dish, the mixture was diluted with 6.5 ml of F12 medium and transferred to 10 ml tubes to be centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were rinsed once with 1X phosphate buffer solution (PBS: 8 g NaCl, 0.2 g KCl, 0.14 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l H_2O; pH 7.3). Each tube was added 200 ml of lysis buffer/CLAP solution (Lysis buffer: 0.187 g HEPES, 0.4235 g NaCl, 0.001 g $MgCl_2$ and 0.19 g EGTA dissolved in 50 ml PBS. CLAP solution: 4 l each of chymostatin, leupeptin, antipain and pepstatin A in 100 ml PBS. Lysis buffer/CLAP solution: 100 ml CLAP solution added to 6.6 ml Lysis buffer). To assure complete rupture of the cells, the tubes were stored in -20℃ for 12 hrs. Lysed cells for each UD or control treatment were pipetted into a tube.

Total protein concentration was quantified using the bovine serum albumin (BCA) protein assay kit (Fisher Scientific), which measured the light absorbance at 562 nm versus a standard curve on a microplate reader. Four 15 ml of 1:5 diluted samples were drawn for each treatment.

5. Western blot analysis for type I collagen and cyclooxygenase-2 (Cox 2)

The protein was obtained as described above and the sample volume that would contain 15 g total protein was calculated according to the protein concentration. The samples with 15 ml loading buffer (loading buffer: 2.4 ml of 1 mol/l Tris-HCl; pH 6.8, 3 ml of 20% SDS, 3 ml of 10% glycerol, 1.6 ml -mercaptoethanol and 6 mg bromophenol blue) each were then boiled at 100 ℃ for 3 min and subjected to gel electrophoresis with pre-prepared 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 110 min at 125 V [17-18]. Electrophoresed proteins were transferred onto Imobilon-P membrane (Nippon Millipore) using a semidry blotting apparatus (Sartorius, USA) for 60 min at 2.0 mA/cm^2. The membrane was rinsed with deionized water, placed into 5% fat-free milk, 1% fetal bovine serum (FBS) and 1X PBS-Tween to shake overnight.

To ensure that equal amount of total protein was loaded to the membrane, glycerealdehyde-3-phosphate dehydrogenase (GAPDH) was detected using rat-anti-human GAPDH antibody (ICN Biochemicals, USA) followed by peroxidase-conjugated rabbit-anti-rat IgG antibody (E.Y. Laboratories, USA). Next, Western blotting membranes were prepared in the same method for detection of type I collagen and Cox 2 (Cyclooxygenase-2), a dioxygen and peroxidative enzyme acting as the inflammatory factor of cells which was tested here for the cytotoxicity of UD extract. Type I collagen was detected using goat-anti-human collagen I antibody (ICN) followed by peroxidase-conjugated rabbit-anti-goat IgG antibody (E.Y. Laboratories). Cox 2 was detected using mouse-anti-human Cox 2 antibody (ICN) and goat-anti-mouse IgG antibody (E.Y. Laboratories). Molecular weights of the proteins were determined using prestained molecular weight standards (14,300-200,000 molecular weight range; Gibco BRL). The lanes were scanned by Epson GT 8000 (Seiko Epson, Japan) and the intensity of the protein bands were analyzed using NIH Image software (Wayne Rasband, National Institute of Health, USA).

6. Statistics

Data were obtained from 3-5 measurements and were expressed as the means ± standard deviations. The calculation for ALP activity and total protein concentration from optical density was performed on SPFT max Pro program (Molecular Devices, USA). Other statistical analyses were carried out on Microsoft Excel program. All quantitative data reported here are expressed as means of samples for each treatment with or without UD aqueous extract. Statistical analyses also included a student t-test, with significance established at p0.05.

Ⅲ. Results

1. The effect of UD-HAS on human osteoprecursor cells

The growth of human osteoprecursor cells (OPC-1) over the 5-days culture period are shown in Table 1. The control group cultured without UD-HAS expressed steady increase and was confluent by the fifth day. At concentrations of 10.0, 30.0, 60.0 and 120 μg/ml, UD-HAS accelerated cell growth over the control group ($p<0.05$). The best concentration of UD-HAS in the present investigation was 120 μg/ml ($p<0.01$). On the other hand, at higher concentrations, UD-HAS had suppressing effect on OPC-1 cells. Cell growth started to slow down compared with the control group from the second day of culture. This was most evident at the highest concentration 1.0 mg/ml ($p<0.05$).

Table 1. The effect of UD-HAS on OPC-1 growth over the 5-day culture period, expressed here as the cell numbers in the culture dish covered by OPC-1

	Concentration of UD (μg/ml)					
	0	10	20	50	100	200
Cell proliferation (X 10^5 cells/well)						
Start	0	0	0	0	0	0
Day-1	21.4±2.3	22.4±3.6	25.6±1.6	28.5±4.2	31.3±2.3	24.4±1.4
Day-2	40.5±5.3	43.6±5.7	46.6±3.4	51.2±5.6	55.2±4.3	42.3±3.7
Day-3	60.3±8.2	64.4±3.5	71.3±9.3	76.5±6.7	81.6±6.3	68.2±3.5
Day-4	80.5±6.7	85.7±7.7	91.3±9.6	94.3±5.6	97.3±5.7	87.4±6.3
Day-5	100.5±15.4	101.1±9.5	102.2±8.6	106.6±9.5	107.4±7.9	91.2±7.4

The cells were treated as described in Materials and methods and the values are mean±standard deviation of three samples.

2. Effect of UD-HAS on the alkaline phosphatase secreted by OPC-1 cells

OPC-1 cells grown in different culture mediums on the fourth day of culture were observed at different proliferation under light microscope. In the control dish, the cells held high density and well-developed inter-cellular collagen networks. Cell density increased as the concentration of UD-HAS increased in the medium at first (0 to 120 μg/ml), but decreased as the concentration of UD-HAS increased in the medium when the concentration of UD-HAS was higher than 120 μg/ml. Precipitations of chemical component crystals from UD-HAS were also observed at higher concentrations. The cell growth was often unbalanced in higher concentrations of UD -medium, having crowded and completely empty places on the dishes. When water was added to the medium instead of UD-HAS extract, the cells demonstrated less development of matrix connection compared to the dishes with the lower concentrations of UD-HAS (not shown)

Table 2 showed the effect of UD-HAS aqueous extract on the activity of alkaline phosphatase secreted by OPC-1 cells. The regression line for ALP was y=0.0135x+0.010; here y represented optical absorbance (OD) at 405 nm and x was the activity of alkaline phosphatase (nanomole of p-nitrophenol/min). The correlation coefficient of the standard curve was 0.99 (Table 2A). ALP activity decreased significantly in the control medium where 800 ml of water was added in place of UD-HAS extract. At concentrations <120 μg/ml, Angelica sinensis stimulated the activity of ALP enzyme to approximately 2.5 times higher than that in control medium ($p<0.05$ for concentrations 20, 100 and 200 μg/ml; $p<0.01$ for concentrations 10.0, 50.0, 100 and 200 μg/ml).

Table 2. Effect of UD-HAS on the alkaline phosphatase secreted by OPC-1 cells

(A)

	p-Nitrophenyl phosphate (nM/min)					
	0	10	20	40	80	160
OD at 405 nm						
Start	0	0.15±0.01	0.26±0.03	0.45±0.02	0.9±0.01	1.7±0.1

(B)

	Concentration of UD-HAS (μg/ml)					
	0	10	20	50	100	2000
Alkaline phosphatase activity (nmol p-nitrophenol/min/dish)						
	6.5±0.3	9.5±1.3	10.2±2.1	15.3±1.6	16.4±1.4	11.3±1.5

(A) Standard curve of alkaline phosphatase showed that the regression line for ALP was y=0.0135x+0.010, here y represented optical absorbance (OD) at 405 nm and x was the activity of alkaline phosphatase (nano mole of p-nitrophenol per minute). The correlation coefficient of the standard curve was 0.99. (B) Effect of UD-HAS on the alkaline phosphatase secreted by human osteoprecursor cells (OPC-1) at the end of a 5-day culture period, expressed as amount of p-nitrophenol produced per minute per dish (8 ml of medium). The samples were treated as described in Materials and methods. The values are mean±standard deviation of five samples.

3. Effect of UD-HAS on protein production of human osteoprecursor cells at the 5-day culture

Table 3 showed the concentration of total protein produced by OPC-1 cells at the end of 5-day culture period. When UD-HAS aqueous extract was added to the medium, protein production in the bone cells was increased. However, at the highest concentration of UD-HAS, 1 mg/ml, protein production was reduced to 30% of that in control medium ($p<0.01$). On another hand, the cells grown in the medium with lower concentration of UD-HAS aqueous extract synthesized and secreted more proteins than that in control medium ($p<0.05$ for concentrations

33.5 and 61.4 g/ml). The highest protein production was observed in the cells grown in the medium with 100 ug/ml aqueous extract of UD-HAS ($p<0.01$).

Table 3. Effect of UD-HAS on protein production of human osteoprecursor cells at the end of the 5-day culture period

	Concentration of UD-HAS (μg/ml)					
	0	10	20	50	100	2000
Total proteins (μg/ml)	823.5±43.2	860.6±96.3	885.5±65.5	960.4±76.2	1364±73.3	1053±132.2

It's expressed as the concentration of total protein(㎍/㎖).
The values are meanstandard deviation of four samples.

4. Western blot analysis for type I collagen and Cox-2 protein in human osteopercursor

Western blot analysis results showed the influence of OPC-1 grown in the medium with different concentrations of UD-HAS aqueous extract. GAPDH showed an equal protein expression of the cells grown in all dishes (Fig. 1B). Type I collagen expressed an increasing trend accompanying the increase of UD-HAS concentration in medium with the peak at 100 μg/ ml (Fig. 1A). Under most treatments, the cells presented very pale expression for Cox 2 protein; slightly intensified band showed at the highest UD-HAS concentration, 500 μg /ml(Fig. 1C)

Fig. 1. Western blot analysis for GAPDH, type I collagen and Cox-2 protein expression in human osteopercursor cells at the end of 5-day culture period affected by the concentration of UD-HAS in medium. (A) Collagen type I; (B) GAPDH; (C) Cox-2. The result was one of three independent experiments.

IV. Discussion

Oriental medicines, which have been developed over some 3000 years [6] and are known to have low toxicity, may offer advantages over the longer term over synthetic agent medication. Although the preventive mechanism of these agents remains to be explained, this initial study does show that UD have traditionally been effective for the gynecological diseases [7-8] may also be administered for the prevention of osteoporosis.

Plants used in folk medicine have been accepted as one of the main sources of drug discovery and development. In Korea, there is a rich treasury of ethnobotanical knowledge and over the past decade [19-20]. During our field studies, we have coincided following Oriental and herbal remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. A literature survey on the plant UD-HAS revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. Previously, it was shown that the UD-HAS extract could prevent the development of bone loss induced by ovariectomy in rats. UD-HAS extract was useful for preventing postmenopausal osteoporosis and osteoporosis associated with both the ovary function failure. It was also demonstrated that interaction between PGE2 and its cell surface receptor results in activation of the PKA signaling pathway. Treatment and pretreatment of the UD-HAS extract to the cells strongly inhibited inflammatory LPS-stimulated IL-1b production and IL-1b mRNA transcription.

UD-HAS is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional oriental medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. The UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support [21]. Although the barks of UD stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-inflammatory action in vivo. To evaluate the role of UD on inflammatory diseases, we studied the effect of UD-HAS the production of collagen-induced RA in rats in vivo. Our results showed that the UD-HAS clearly reduced this inflammatory disease in a dose-dependent manner.

From quantitative and morphological observation on the human osteoprecursor cells during the 5-day culture period, this experiment suggests that low concentrations of UD-HAS aqueous extract had an effect in accelerating the proliferation of bone cells. In contrast, at high concentrations of 0.5 to 250 μg/ml, the addition of UD-HAS to culture medium had suppressive effect on the cell proliferation and differentiation. The results of this experiment also suggests that lower concentration supplement of UD-HAS increase the total amount of protein produced in OPC-1 cells and stimulated the activity of alkaline phosphatase and the production of type I collagen, two important proteins synthesized by bone cells, particularly osteoblasts, during osteogenesis, the formation of

bone.

Human bone is composed of mineralized organic matrix and bone cells. Osteoblasts are the active mature bone cells that synthesize the organic matrix and regulate its mineralization. Osteogenesis starts with osteoblasts producing and secreting type I collagen, which makes about 90% of the organic bone matrix, or the osteoid. Osteoblasts also become high in alkaline phosphatase, a phosphate-splitting enzyme. Alkaline phosphatase is released into the osteoid to initiate the deposit of minerals. Calcium hydroxyapatite Ca10(PO4) 6(OH) 2, which comprises 70% of the bone mass, crystallizes along the cavities in the three-dimensional collagen fibrils. After mineralization, the complete bone becomes hard and rigid with necessary mechanical properties to withstand the external forces to support the body and protect the internal organs [22].

Three components are thus essential to bone formation: bone cells, type I collagen production, and sufficiency of mineral deposits. Therefore, on the micro scale, in an environment with adequate supply of calcium, phosphorus and other mineral elements, the proliferation and activity of osteoblasts control the speed of bone formation. Accelerated osteoblast growth and protein synthesis are the key factors for efficient bone repair. Evidently from the depletion of cell density, shrinkage of cell body, low total protein concentration and Cox 2 expression, the highest concentration of UD-HAS used in this experiment, 1.0 mg/ml, caused slight cytotoxicity to OPC-1 cells during in vitro culture. Probably, some chemical components of UD-HAS dissolved in the aqueous extract reached the maximum limit of safe concentration and damaged the cells. UD-HAS contains several organic compound reported to be toxic to cell development, mainly phenol and furocoumarine groups which were present in the aqueous extract used in this experiment as indicated by the infrared spectra. It is then a contradictory phenomenon that the activity of alkaline phosphatase and the production of type I collagen were the strongest at 120 μg/ml UD-HAS concentration. One possible explanation is that UD-HAS in the medium became a stimulus for the cells to generate the specific proteins. This stimulation was too trivial to detect at high concentrations, so the protein productions were close to those expressed by the cells cultured in control medium. High UD-HAS concentration may inhibit the proliferation of the cells.

Another factor that must be taken into consideration in evaluating the results is that the aqueous extract of UD-HAS contained 99% distilled water. It occupied 0.125% to 10% of the total volume of culture medium and might have changed the osmotic gradient in the cells' environment compared to the culture medium. For this reason, a control group to which 800 ml of distilled water was added instead of UD-HAS extract was created. The results suggest that water was indeed another stimulus for the cells. It significantly reduced the protein production, collagen type I formation and ALP activity in bone cells. It also had prompting effect on collagen I synthesis. Because at higher UD-HAS concentrations, the cells behaved in a very different, sometimes even the opposite, manner compared to the cells cultured in the medium with water, possibly UD-HAS had overcome the effect of water and acted upon the cells by the stimulation factors it had. To clarify this point,

extraction of UD root should be completed with PBS that is isoosmotic to the cells when this experiment is repeated in the future.

This research introduced some insights to the subject of the effect of UD-HAS on human bone cells. Since aqueous extract of UD-HAS improved the activity of alkaline phosphatase, the messenger initiating calcification, it is possible that the herb accelerated mineralization of the organic matrix, thus speeding up bone formation. UD-HAS not only enhanced total protein production significantly but also increased type I collagen synthesis, increasing the portion of collagen in total protein. Usually, traditional prescriptions combine three to ten herbal and mineral medicines; although only one or two are responsible for the central effect, the supplemental ingredients are also important in achieve the goal of remedy. Therefore, some aspect of UD-HAS may only be present in combination with other medicines, which may simultaneously lessen its cytotoxicity. Typical length of clinical treatment for bone injuries with UD-HAS ranges from a week to a couple months. Some long-term effects of UD-HAS may not have been revealed in the short culture period of this experiment. In addition, the concentration of UD-HAS may change once the medicine is taken into the body because of protein-binding compounds. The concentrations used in this research were only a standard in vitro. Whether the effect of UD-HAS will change in vivo is not known. Further investigation is planned to examine these possibilities.

Therefore, the need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study. This result also suggested that the UD-HAS extracts is effective for anti-bone resorptive action in bone cells.

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