

Original Articles

Effects of *Drynariae Rhizoma* on the Proliferation of Human Bone Cells

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Drynariae Rhizoma (DR), an herbal medicine known for its effect to purify blood quality and improve circulation, frequently appears as the main ingredient in prescriptions for bone injuries. Currently, how pharmacologically it contributes to the reformation of bone is unclear. In the present study, the effect of the aqueous extract of DR on bone cells was investigated *in vitro* for the first time. The human osteoprecursor cells (OPC-1) were incubated in the medium with different concentrations of the aqueous extract of DR and the cell proliferation was studied. When the concentration of DR aqueous extract was <120 μ g/ml, the proliferation of OPC-1 was enhanced. However, the proliferation of OPC-1 was inhibited by DR extract with the concentrations >250 μ g/ml. Under most treatments, the cells presented very pale expression for cyclooxygenase-2 (Cox 2) protein; a slightly intensified band showed at the highest DR concentration, 1.0 mg/ml during the course of culture. From the results, it was concluded that the aqueous extract of DR was found to directly stimulate the proliferation, alkaline phosphatase (ALP) activity, protein secretion and particularly type I collagen synthesis of OPC-1 at dose-dependent manner. (*Korean J of Oriental Med* 2003;24(4):43-53)

Key Words: *Drynariae Rhizoma*, human osteoprecursor cells, alkaline phosphatase, collagen type I

Introduction

The primary cause of postmenopausal osteoporosis is estrogen deficiency resulting in a decrease in bone mass. An ovariectomized rat model, which artificially produces a depleted state of estrogen, has been used for the study of postmenopausal osteoporosis. Both aged rats and mature rats have been used as animal models to study experimentally-induced osteoporosis, and the

mature rat model has characteristics that are comparable with those of early postmenopausal trabecular bone loss¹⁾.

Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models. Estrogen^{1,2)}, bisphosphonates¹⁾, calcitonin¹⁾, calcium products^{1,3)}, ipriflavone⁴⁾ and anabolic steroids⁵⁾ are clinically employed as effective medications.

Traditional Chinese medicines have been reevaluated by clinicians⁶⁾, because these medicines have fewer side effects and because they are more suitable for long-term use as compared with chemically synthesized medicines. About forty kinds of oriental medicines are claimed to be effective for gynecological diseases such

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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 osteoporosis⁷.

From ancient times in Korea, 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. *Drynariae Rhizoma* (*DR*) is effective for the treatment of deficient kidneys manifested as lower back pain, weakness of the legs, tinnitus or toothache by function of tonifying the kidney, invigorating blood and stopping bleeding according to the traditional Chinese medicinal literature⁹. Since a notable decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis².

DR, sometimes referred to by its Korean name as Gol-se-bo, is the root of *Drynariae Rhizoma* (*Oliv.*), a herbaceous perennial plant belonging to the *Drynaria* family². To treat osteoporosis, an herbal formula containing *DR* is used in Korea¹⁰.

Besides the illnesses discussed above, *DR* frequently appears in traditional prescriptions for bone and tendon injuries. For example, 56 of the 73 fracture prescriptions collected in the Encyclopedia of Esoteric Prescriptions in Traditional Chinese Medicine contain *DR* as one of the main ingredients¹¹. Clinical data has shown that these prescriptions had significant effect in reducing the time needed for the injured bones to heal.

Golsebo-tang, mainly consisting of *DR*, crab shells and several pain-killing herbs, when applied as a pasting medicine to 112 closed fracture cases of people

raging from age 1 to 40 years, on average the patients regained health in 31.6 days, much shorter than the normal healing time of 8-10 weeks. Medicines prepared with water or wine stir-baking technique for oral intake also yielded similar results. X-ray images presented the formation of new bone tissue at fracture sites within 7-10 days of injury. This further proved the advantage of traditional prescriptions over conventional Western surgical treatment¹².

In order to evaluate the effectiveness of traditional Chinese medicines on osteoporosis, Lee et al. examined whether *DR* could prevent the progression of bone loss induced by ovariectomy in rats¹³. The assays for the inhibition of bone resorption and collagenolysis were composed of *in vitro* PGE2-stimulated IL-1 β production and cAMP-PKA signaling pathway to regulate IL-1 β . The *DR* showed inhibitory effects against the increase of the PGE2-stimulation¹³. The preventive effects by *DR* on the progress of bone loss induced by ovariectomy in rats were investigated for a period of 6 weeks. The bone mineral density of tibia in ovariectomized rats decreased by 22% from those in sham-operated rats, with the decrease completely inhibited by the administration of the *DR* or 17beta-estradiol. The administration of the *DR* and 17beta-estradiol to ovariectomized rats preserved the fine particle surface of the trabecular bone. The *DR* extract strongly inhibited PGE2- and LPS-stimulated IL-1 β production. Pretreatment of the *DR* after 1h and 24h of treatment also suppressed the IL-1 β production. The *DR* extract strongly inhibited the PGE2-stimulated IL-1 β transcription. *DR* was as effective as 17beta-estradiol in preventing the development of bone loss induced by ovariectomized in rat and *DR* is effective for anti-bone resorptive action in bone cells¹³.

However, because these prescriptions were produced over 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 healing.

Possibly, the impact of *DR* 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, *DR* is likely to have direct stimulation on bone formation. Currently, no scientific research has been done on this subject.

The present research focuses on the direct cellular-level effect of *DR* on bone cells. It tests whether *DR* medication 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 osteoblasts suited for *in vitro* culture.

Whether different concentrations of *DR* can induce cytotoxicity during the course of culture was also examined.

Materials and Methods

1. Materials and chemicals

Drynariae Rhizoma (*DR*) extracts, mass produced 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, 10 g of finely cut *DR* root 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 °C for 24 h in an oil bath, using an electric hot plate as a 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.

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 and 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 cm² culture flasks (Falcon) in Dulbesso's modification of Egel's medium (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco BRL, Bethesda, MD). Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO₂/95% air; the medium was changed every 2-3 days. Cells were grown to confluence at 37 °C and cultured in duplicate or triplicate wells for an additional 24 h 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 °C with 5% CO₂ 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 (ø 8.5 mm) for subculture under the influence of *DR* aqueous extract. Each dish contained 2 ml of harvested cells and 0 - 800 µl of 1% *DR* aqueous extract with corresponding amount of F12 medium so the total volume of culture medium was 8 ml with a plating cell density 10 × 10⁴ cells/ml. For the control

group, no DR and 800 μ l 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, 10, 30, 60, 125, 250, 500, 750 and 1000 μ g/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 μ l 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 °C 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 nanomoles of p-nitrophenol per minute per dish.

4. Harvest cells and protein extraction

After 5 days of culture (approximately 120 h), 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 1 \times phosphate buffer solution (PBS: 8 g NaCl, 0.2 g KCl, 0.14 g Na₂HPO₄, 0.24 g KH₂PO₄ in 1 l H₂O; pH 7.3). To each tube was added 200 μ l of lysis buffer/CLAP solution (Lysis buffer: 0.187 g HEPES, 0.4235 g NaCl, 0.001 g MgCl₂ and 0.19 g EGTA dissolved in 50 ml PBS. CLAP solution: 4 L each of chymostatin, leupeptin, antipain and pepstatin A in 100 μ l PBS. Lysis buffer/CLAP solution: 100 μ l CLAP solution added to 6.6 ml Lysis buffer).

To assure complete rupture of the cells, the tubes were stored in -20 °C for 12 h. Lysed cells for each DR 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 μ l 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 μ g total protein was calculated according to the protein concentration. The samples with 15 μ l 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 °C 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/cm². The membrane was rinsed with deionized water, placed into 5% fat-free milk, 1% fetal

bovine serum (FBS) and $1 \times$ PBS-Tween to shake overnight.

To ensure that equal amounts of total protein were loaded to the membrane, glyceraldehyde-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 by 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 *DR* 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 mean \pm standard deviation. 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 *DR* aqueous extract. Statistical analyses also included a Student's *t*-test, with significance established at $p < 0.05$.

Results

1. The effect of *DR* on human osteoprecursor cells

The growth of human osteoprecursor cells (OPC-1) over the 5-day culture period are shown in Table 1. The control group cultured without *DR* expressed steady increase and was confluent by the fifth day. At concentrations of 10.0, 30.0, 60.0 and 120 $\mu\text{g/ml}$, *DR* accelerated cell growth over the control group ($p < 0.05$). The best concentration of *DR* in the present investigation was 120 $\mu\text{g/ml}$ ($p < 0.01$). On the other hand, at higher concentrations, *DR* had suppressing effect on OPC-1 cells. Cell growth started to slow down compared to 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 *Drynariae Rhizoma* (*DR*) on Human Osteoprecursor Cells (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 <i>DR</i> ($\mu\text{g/ml}$)						
	0	10	30	60	120	250	500
	Cell proliferation ($\times 10^5$ cells/well)						
Start	0	0	0	0	0	0	0
Day-1	20.4 \pm 2.3	21.4 \pm 3.6	24.6 \pm 1.6	27.5 \pm 4.2	30.3 \pm 2.3	23.4 \pm 1.4	20.2 \pm 1.6
Day-2	40.5 \pm 5.3	43.5 \pm 5.7	45.6 \pm 3.4	50.3 \pm 3.6	54.3 \pm 6.3	41.4 \pm 3.6	38.4 \pm 4.3
Day-3	60.3 \pm 8.2	64.6 \pm 3.7	70.3 \pm 9.3	75.6 \pm 8.3	80.3 \pm 5.7	67.8 \pm 7.8	55.3 \pm 6.4
Day-4	80.5 \pm 6.7	85.5 \pm 8.2	90.3 \pm 10.8	93.2 \pm 5.7	96.4 \pm 7.2	86.3 \pm 9.6	75.4 \pm 8.2
Day-5	100.5 \pm 15.4	102.1 \pm 9.3	103.2 \pm 8.3	105.3 \pm 9.3	106.3 \pm 8.9	90.4 \pm 9.5	81.2 \pm 4.6

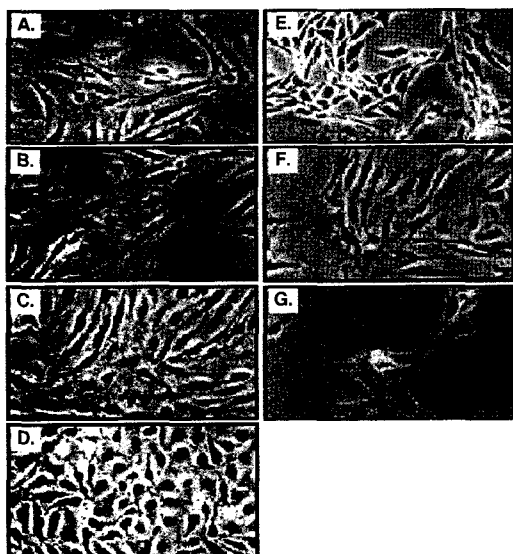


Fig. 1. Human osteoprecursor cells (OPC-1) on the fourth day of culture affected by the *Drynariae Rhizoma* concentration in the medium. The cells were treated and the figures taken as described in Materials and Methods. Magnification: original $\times 40$ under light microscope. A. Control, B. 10 $\mu\text{g/ml}$, C. 30 $\mu\text{g/ml}$, D. 60 $\mu\text{g/ml}$, E. 120 $\mu\text{g/ml}$, F. 250 $\mu\text{g/ml}$, G. 500 $\mu\text{g/ml}$.

2. Human osteoprecursor cells (OPC-1) on the fourth day of culture affected by the DR concentration in the medium

As shown in Fig. 1, 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 DR increased in the medium at first (0 to 120 $\mu\text{g/ml}$), but decreased as the concentration of DR increased in the medium when the concentration of DR was higher than 120 $\mu\text{g/ml}$. Well-developed matrix connections were also observed in the DR concentration of 60 $\mu\text{g/ml}$ (Fig. 1D).

Occasionally, overextended matrix networks were present in DR medium (Fig. 1E).

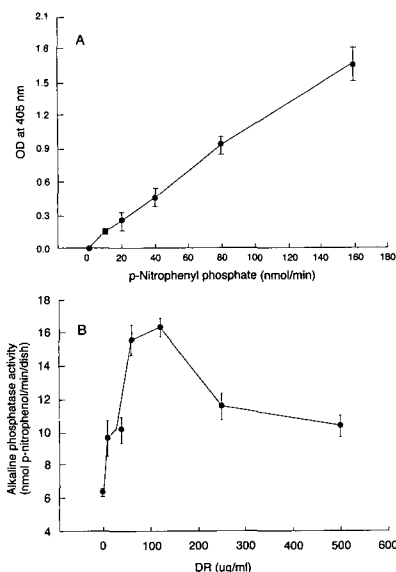


Fig. 2. A. Standard curve of alkaline phosphatase showed that the regression line for ALP was $y=0.0149x+0.0118$, here y represented optical absorbance (OD) at 405 nm and x was the activity of alkaline phosphatase (nanomoles of p-nitrophenol per minute). The correlation coefficient of the standard curve was 0.9989.

B. Effect of *Drynariae Rhizoma* (DR) 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 \pm standard deviation of five samples.

However, generally, shrinkage of the cell body and large number of dead cells, as indicated by the bright spots, were evident in Fig. 1E, F and G. Precipitations of chemical component crystals from DR extract were also observed at higher concentrations. The cell growth was often unbalanced in higher concentrations of DR medium, having crowded and completely empty places on the dishes. When water was added to the medium instead of DR extract, the cells demonstrated less development of matrix connection compared to the dishes with the lower concentrations of DR aqueous extract (Fig. 1A, C and D), but had a greater density than the cells

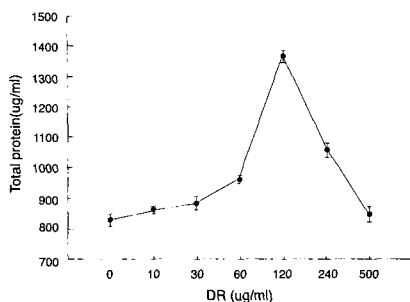


Fig. 3. Effect of *Drynariae Rhizoma* (DR) on protein production of human osteoprecursor cells at the end of the 5-day culture period, expressed as the concentration of total protein ($\mu\text{g/ml}$). The values are mean \pm standard deviation of four samples.

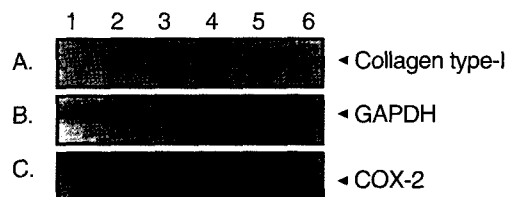
cultured in the medium with higher concentration of DR aqueous extract (Fig. 1A, E, F and G).

3. Effect of DR aqueous extract on the activity of alkaline phosphatase secreted by OPC-1 cells

Fig. 2 shows the effect of DR aqueous extract on the activity of alkaline phosphatase secreted by OPC-1 cells. The regression line for ALP was $y=0.0149x+0.0118$; here y represented optical absorbance (OD) at 405 nm and x was the activity of alkaline phosphatase (nanomoles of p-nitrophenol/min). The correlation coefficient of the standard curve was 0.9989 (Fig. 2A). ALP activity decreased significantly in the control medium where 800 ml of water was added in place of DR extract. At concentrations $<120 \mu\text{g/ml}$, DR stimulated the activity of ALP enzyme to approximately 2.5 times higher than that in control medium ($p<0.05$ for concentrations 31.3, 250 and $1000 \mu\text{g/ml}$; $p<0.01$ for concentrations 10.0, 60.0, 120, 250 and $500 \mu\text{g/ml}$).

4. Effect of DR on protein production of human osteoprecursor cells at the end of the 5-day culture period

Fig. 3 shows the concentration of total protein



A.

Lane	DR ($\mu\text{g/ml}$)	Intensity	% of control
1	0	12	100
2	30	13	103
3	60	134	1023
4	120	245	2043
5	250	121	978
6	500	23	189

B.

Lane	DR ($\mu\text{g/ml}$)	Intensity	% of control
1	0	365	100
2	30	375	105
3	60	356	98
4	120	366	101
5	250	365	100
6	500	368	101

C.

Lane	DR ($\mu\text{g/ml}$)	Intensity	% of control
1	0	3	100
2	30	3	100
3	60	3	99
4	120	4	120
5	250	56	1540
6	500	59	1578

Fig. 4. Western blot analysis for GAPDH, type I collagen and Cox-2 protein expression in human osteoprecursor cells at the end of 5-day culture period affected by the concentration of *Drynariae Rhizoma* (DR) in medium. A. Collagen type I, B. GAPDH, C. Cox-2. The result was one of three independent experiments.

produced by OPC-1 cells at the end of the 5-day culture period. When DR aqueous extract was added to the medium, protein production in the bone cells increased. However, at the highest concentration of DR, 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 DR

aqueous extract synthesized and secreted more proteins than that in control medium ($p < 0.05$ for concentrations 31.3 and 62.5 g/ml). The highest protein production was observed in the cells grown in the medium with 125 g/ml aqueous extract of *DR* ($p < 0.01$).

5. Western blot analysis for type I collagen and Cox-2 protein expression in human osteoprecursors

Western blot analysis results showed the influence *DR* aqueous extract on OPC-1 grown in the medium with different concentrations. GAPDH showed an equal protein expression of the cells grown in all dishes (Fig. 4B). Type I collagen expressed an increasing trend accompanying the increase of *DR* concentration in medium with the peak at 120 $\mu\text{g/ml}$ (Fig. 4A). Under most treatments, the cells presented very pale expression for Cox 2 protein; a slightly intensified band showed at the highest *DR* concentration, 500 $\mu\text{g/ml}$ (Fig. 4C).

The cells were treated as described in materials and methods and the values are mean \pm standard deviation of three samples.

Discussion

Traditional Chinese medicines, which have been developed over some 3000 years⁶⁾, are known to have low toxicity and 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 *DR*, which has traditionally been effective for gynecological diseases^{7,8)}, may also be administered for the prevention of osteoporosis.

In Korea, there is a rich treasury of ethnobotanical knowledge and over the past decade^{19,20)} about traditional Chinese medicines. I have selected the

following herbal remedy claimed to be useful in the treatment of rheumatism, bone resorption and related inflammatory diseases. A literature survey on the plant *DR* revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. Previously, it was shown that the *DR* extract could prevent the development of bone loss induced by ovariectomy in rats. *DR* extract was useful for preventing postmenopausal osteoporosis and osteoporosis associated with both the ovary function failures. 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 *DR* extract to the cells strongly inhibited inflammatory LPS-stimulated IL-1 β production and IL-1 β mRNA transcription.

From quantitative and morphological observation on the human osteoprecursor cells during the 5-day culture period, this experiment suggests that low concentrations of *DR* aqueous extract had an effect in accelerating the proliferation of bone cells. In contrast, at high concentration of 500 $\mu\text{g/ml}$, the addition of *DR* to culture medium had suppressive effect on the cell proliferation and differentiation. The results of this experiment also suggest that a lower concentration supplement of increased 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 $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, 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 organs²¹.

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 *DR* used in this experiment, 1.0 mg/ml, caused slight cytotoxicity to OPC-1 cells during *in vitro* culture. Probably, some chemical components of *DR* root dissolved in the aqueous extract reached the maximum limit of safe concentration and damaged the cells. *DR* contains several organic compounds reported to be toxic to cell development, mainly phenol and furocoumarin 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 $\mu\text{g/ml}$ *DR* concentration. One possible explanation is that *DR* 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 *DR* 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 *DR* 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 *DR* 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 *DR* concentrations, the cells behaved in a very different, sometimes even the opposite, manner compared to the cells cultured in the medium with water, possibly *DR* had overcome the effect of water and acted upon the cells by the stimulation factors it had. To clarify this point, extraction of *DR* 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 *DR* on human bone cells. Since aqueous extract of *DR* improved the activity of alkaline phosphatase, the messenger initiating calcification, it is possible that *DR* accelerated mineralization of the organic matrix, thus speeding up bone formation. *DR* 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 achieving the goal of

remedy. Therefore, some aspect of *DR* may only be present in combination with other medicines, which may simultaneously lessen its cytotoxicity. Typical length of clinical treatment for bone injuries with *DR* ranges from a week to a couple of months. Some long-term effects of *DR* may not have been revealed in the short culture period of this experiment. In addition, the concentration of *DR* may be changed 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 *DR* 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 *DR* extracts is effective for anti-bone resorptive action in bone cells.

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