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Effects of Achyranthes Radix Extracts on Osteoblasts and Osteocalsts

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The present study was performed to investigate whether *Achyranthes* Radix extracts play roles in the bone metabolism. Three kinds of *Achyranthes* Radix extracts (methylene chloride (MC), ethylacetate (Ea), and water (W)) were used for bioassay. We examined cellular activities of osteoblasts by measurement of cell proliferation rate, alkaline phosphatase (ALP) activity, and calcified nodule formation. Osteoclast generation was assayed by measuring the number of tartrate-resistant acid phosphatase (TRAP) (+) multinucleated cells after culture of osteoclast precursor cells. There was a maximum 20% increase in proliferation rate of osteoblastic cells after treatment with MC. First and second subfraction of MC layer increased proliferation of osteoblast. Ea layer and second subfraction of MC layer increased ALP activity. Also MC layer and second subfraction of MC layer from *Achyranthes* Radix extracts increased the calcified nodule. MC layer and second subfraction of MC layer from *Achyranthes* Radix extracts significantly decreased in the number of TRAP (+) multinucleated cells. Taken together, *Achyranthes* Radix stimulates the proliferation and bioactivities of bone-forming osteoblasts, and inhibits activities of bone-resorbing osteoclasts.

Keywords: Achyranthes Radix, Osteoblast, Osteoclast

Introduction

Bone is highly specialized, dynamic organ that undergoes continuous regeneration. Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone with new at the same location (Frost, 1963). This process, called remodeling, is a coupled process in which bone resorption is normally followed by new bone formation. During early life, bone formation exceeds bone resorption with a net increase in bone mass, while late in life, bone resorption exceeds bone formation with net loss of bone. Excessive bone resorption that overcomes bone formation results in bone abnormalities such as osteoporosis, which is characterized by a reduction in bone mass and a higher incidence of bone fractures (Weinreb *et al.*, 1989).

Osteoporosis is the most frequently occurring metabolic bone disease and is particularly common in elderly woman. Although a gradual decline in bone mass occurs with aging in both men and women, osteoporosis results from an exaggeration of the imbalance between resorption and formation. In most cases, the diseases are characterized by back pain from recurrent vertebral compressions, although fractures of the distal tibia, hip, ribs, or wrist can be the initial presentation. Therapeutic modalities for prevention and treatment of osteoporosis must increase bone mass or at the very least, prevent further bone loss. Several therapies are currently being investigated for their potential to increase bone mass in postmenopausal women, including calcitonin, fluoride salts, vitamin D analogs, parathyroid hormone, several bisphosphonates, and estrogen (Genant *et al.*, 1989; Recker, 1993; Rodan, 1994; Heaney, 1996; Nelson *et al.*, 2002; Lemay, 2002; Cryer and Bauer, 2002). Traditional hormonal replacement and other antiresorptive therapy have beneficial effects on osteoporosis in postmenopausal women. Despite these beneficial effects, problem remains about the risk/benefit ratio of therapy.

There has been continuous need of the development of new drugs that have new mechanism and noble structure, less toxicity and side effects and are effective in prevention and treatment of osteoporosis. For many years, it has been recognized that some compounds in several natural products

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have beneficial medicinal effects to health. There are many efforts to develop new antiosteoporotic drug from these traditional natural products. Among these, *Achyranthes bidentata* has known to have various physiological and pharmacological properties such as antiarthritic, antibacterial and antiviral effect (Desta, 1993; Gessler *et al.*, 1994). According to Shen Nung pen tsao ching, *Achyranthes bidentata* has a protect effect on liver and kidney and a diuretic effect, alleviates pain and strengthens joints.

Achyranthes bidentata (family Amaranthaceae), a genus of herbs or small shrubs, is distributed throughout the tropical and subtropical regions (Kirtikar and Basu, 1935). *Achyranthes* radix is from dried root of *Achyranthes bidentata*. The plant is used as an antiarthritic, purgative, diuretic, antimalarial, estrogenic, antileprotic, antispasmodic, antibacterial, analgesic, anti-inflammatory, and antiviral agent (Ojha and Singh, 1968; Khurana and Bhagva, 1970; Wadhwa *et al.*, 1986; Khanna *et al.*, 1992; Desta, 1993; Gessler *et al.*, 1994; Tian *et al.*, 1995; Lu *et al.*, 1997). It is also used for asthmatic cough, snakebite, hydrophobia, urinary calculi, rabies, influenza, piles, bronchitis, diarrhea, renal dropsies, gonorrhea and abdominal pain (Jain and Puri, 1984; John, 1984; Singh, 1986; Reddy *et al.*, 1989; Bhattari, 1993).

Phytochemical work on this herb showed that it contains saponins, betaines, achyranthine, amino acids, β-ecdysone, lauric acid, myristic acid, β-sitosterol, stigmasterol, oleanolic acid and flavonoids (Kapoor and Singh, 1966; Kong *et al.*, 1976; Ratra, 1979; Seshatri *et al.*, 1981; Raman and Faroque, 1996).

Xiang and Li (1993) demonstrated that *Achyranthes bidentata* polysaccharide induces the synthesis of both interleukin-1 and tumor necrosis factor-α from thioglycolate-primed mouse peritoneal macrophages *in vitro*. These cytokines are well-known regulators of bone metabolism. Recently, Gao *et al.* (2000) reported that ecdysone in *Achyranthes bidentata* Bl. promotes the proliferation of osteoblast-like cells. Ecdysone is one of the major component of *Achyranthes bidentata*. From above reports, it is hypothesized that *Achyranthes bidentata* might have potential effects in regulating bone metabolism. In the present study, the *in vitro* effect of a *Achyranthes* radix extracts on the proliferation and activity of both osteoblasts and osteoclasts was investigated in order to determine the possible bioactivities of *Achyranthes* radix on bone metabolism.

Materials and Methods

Reagents

Achyranthes radix was obtained from the Pung San pharmaceutical company (Korea). β-Ecdysone was purchased from Sigma-Aldrich Korea. Media and fetal bovine serum (FBS) and other cultural reagents were purchased from Gibco laboratories (Grand Island, USA). Recombinant human receptor activator of NF-kB ligand (RANKL) and recombinant human transforming growth factor-β (TGF-β) were purchased from PeproTech EC Ltd (London, UK). Tartrate-resistant acid phosphatase (TRAP) stain kit and all the other reagents were purchased from Sigma-Aldrich Korea.

Preparation of extraction of *Achyranthes* radix

Achyranthes radix 300 g was heated in 2 L of 70% methanol for four hours two times and the extracts were filtered after cooling at room temperature. Concentration was carried out under decompression at 40°C or less in order to remove methanol. This resultant was put into 1 L separatory funnel with 400 mL methylene chloride or ethylacetate to remove internal pressure. It was repeated three times and the lower layer was used as a MC or Ea layer, respectively. The rest of them was separated as a water layer (W layer). Thus, three kinds of extracts (MC, Ea, and W) were obtained and each extract was used for bioassay.

Subfraction of MC layer (MC-1, MC-2, MC-3)

MC layer were divided into three fractions (MC-1, MC-2 and MC-3) by column chromatography using silica gel and the solvent (methylene chloride : methanol = 30 : 1).

Osteoblastic cell proliferation

HOS cells were plated at a density of 2×10^4 cells/well in 24-well plate and cultured for 48 hrs with Dulbecco's modified Eagle medium (DMEM) containing 10% FBS in the presence of extracts. After culture, adherent cells were detached by trypsinization and resuspended in phosphate buffered saline. Cells were counted in a hemocytometer under the light microscope. Results were expressed as percent proliferation rate [100×(number of cells in treated wells/number of cells in control wells)].

Alkaline phosphatase activity

HOS cells were plated at a density of 2×10^4 cells/well in 24-well plate and cultured for 48 hours. When cells reached about nearly confluence, cells were treated with media containing various concentrations of extracts for additional 48 hours. After washing with phosphate buffered saline (PBS), cells were lysated with 0.1% Triton X-100/saline and ALP activity was measured by spectrophotometry using p-nitrophenyl phosphate (pNPP, 100 mM) as a substrate. Protein concentration of each sample was measured using a BCA protein assay reagent.

Calcified nodule formation *in vitro*

Effect of *Achyranthes* radix extracts on calcified nodule formation was determined in MC3T3-E1 cell cultures. Cells were plated in 24-well plates at a density of 2×10^4 cells/well and cultured until reached confluency. After reached

confluency, media were changed with DMEM containing 10% FBS supplemented 50 μg/ml ascorbic acid and 10 mM â-glycerophosphate. Media were changed every second or third day and cultures were maintained up to 17-19 days in the presence of extracts. To observe the produced calcified nodule, cell layer was fixed with 10% neutral buffered formalin and stained in situ by the von Kossa technique for mineral deposits. Number of calcified nodules was counted at ×100 magnification using a light microscope.

RAW264.7 cell culture

RAW264.7 cells were maintained with DMEM containing 10% FBS. RAW264.7 cells were cultured in 100 mm culture dish and maintained at CO_2 incubator. When reached confluency, cells were subcultured at a ratio of 1 : 10. RAW264.7 cells were plated at a density of 5×10^3 cells/well in 96-well plate with α-MEM containing 10% FBS. Media were changed at third day and cultures were maintained up to 5 days in the presence of 50 ng/ml RANKL, 1 ng/ml TGF-β and various concentrations of extracts with α-MEM containing 10% FBS.

Enzyme histochemistry for TRAP

After culture, adherent cells were rinsed with a PBS, fixed with citrate-acetone-formaldehyde for 5 mins, and stained for TRAP by incubating the cells for 1 hr at 37°C in acetate buffer (pH 5), containing naphthol AS-BI phosphate, fast Garnet GBC solution, and 7 mM tartrate. TRAP (+) cells appeared dark violet. TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclast-like cells by light microscope.

Statistical analysis

All data represent the mean values obtained from at least five replicates in each experiment. Data were analyzed using Student's *t*-test. A *P* value less than 0.05 was taken as a significant difference between the pairs. The values are presented as mean ±S.E.

Results

In this study, the *in vitro* effect of an *Achyranthes* radix extracts on the proliferation and activity of both osteoblasts and osteoclasts was investigated in order to determine the possible bioactivities of *Achyranthes* radix on bone metabolism.

Effects *Achyranthes* radix extracts on the cell proliferation of osteoblasts

To observe the effects of *Achyranthes* radix extracts on osteoblast proliferation, the cell number was evaluated after the extracts and ecdysone treatment. The proliferation rate of the HOS increased after the extracts treatment (Fig. 1).

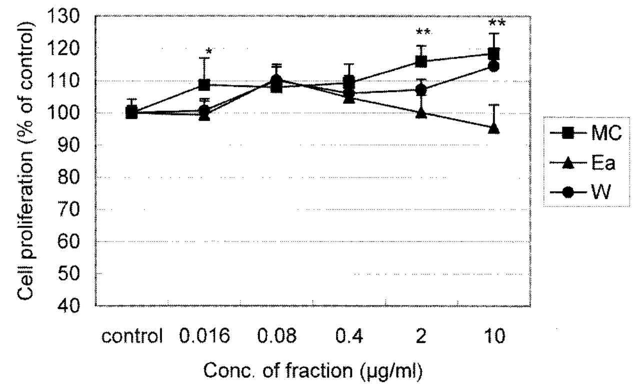

Fig. 1. Effects of *Achyranthes* radix extracts on the osteoblastic cell proliferation.
Osteoblasts were plated at a density of 2×10^4 cells/well in 24-well plates and treated with extracts for 48 hrs. After culture, cells were collected and counted in a hemo-cytometer under the light microscope. The data represent a mean±S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, Ea: ethylacetate layer, W: water layer. *: $P<0.05$, **: $P<0.01$.

The cells treated with MC layer and W layer showed a maximal increase of 20% cell proliferation compared to the control group. MC layer promoted significantly the proliferation of osteoblast under all the concentrations (0.016 10 μg/ml) dose dependently. In case of treatment of Ea layer, osteoblastic cell proliferation was not changed (Fig. 1).

Effects of *Achyranthes* radix extracts on the ALP activities and calcified nodule formation

The ALP activity and calcified nodule formation were measured to observe the effects on osteoblast differentiation. Even though, MC layer does not increase of ALP activity, they increased significantly calcified nodule formation. Ea layer showed increased ALP activity (Fig. 2 and 3).

Effects of *Achyranthes* radix extracts on the osteoclast generation

To observe the effects of *Achyranthes* radix extracts on osteoclast generation, the TRAP (+) multinucleated cells number was evaluated after the extracts and ecdysone treatment. MC layer significantly decreased the number of TRAP (+) multinucleated cells dose-dependent and most significant at 10 μg/ml. Ea and W layers does not alter osteoclast generation (Fig. 4).

Effects of MC layer subfractions

Because MC layer showed the most predominant effects on the osteoblasts and osteoclast, MC layer was separated by column chromatography and repeated the above experiments.

First and second subfraction increased proliferation of osteoblast at concentrations of 0.016 2 μg/ml, but third subfraction decreased proliferation of osteoblast (Fig. 5). As

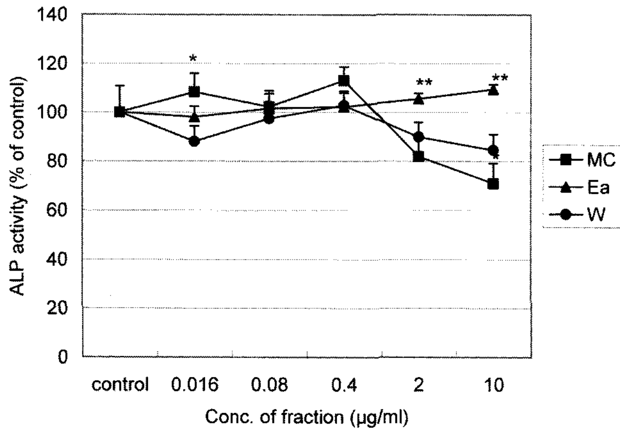

Fig. 2. Effects of *Achyranthes* radix extracts on the ALP activity of osteoblastic cells.
The osteoblastic cells were plated at a density of 2×10^4 cells/well in 24-well plates and treated with extracts for 48 hrs. Enzyme activity was measured by spectro-photometric method using p-nitrophenyl phosphate as a substrate. The data represent a mean ±S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, Ea: ethylacetate layer, W: water layer. *: P<0.05, **: P<0.01.

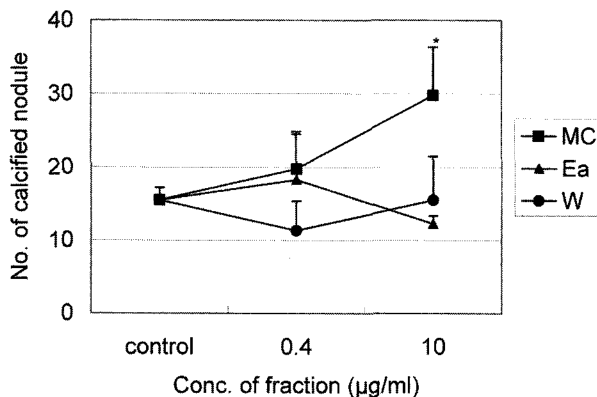

Fig. 3. Effects of *Achyranthes* radix extracts on the calcified nodule formation.
The osteoblastic cells were plated at a density 2×10^4 cells/well in a 24-well plate, cultured for 19 days in the presence of ascorbic acid, β-glycerophosphate and various extracts. After culturing, the silver nitrate stained spots were counted as calcified nodule. The data represent a mean ±S.E. of 5 experiments. MC: methylene chloride layer, Ea: ethylacetate layer, W: water layer. *: P<0.05.

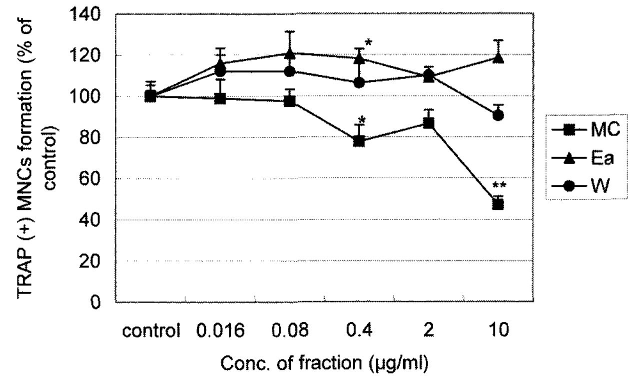

Fig. 4. Effects of *Achyranthes* radix extracts on the osteoclast generation.
The RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 50 ng/ml RANKL and 1 ng/ml TGF-β and various extracts concentrations. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteo-clasts. The data represent a mean±S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, Ea: ethylacetate layer, W: water layer. *: P<0.05, **: P<0.01.

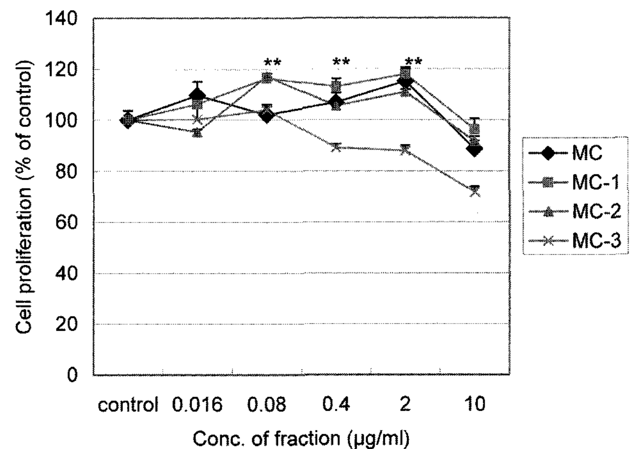

Fig. 5. Effects of *Achyranthes* radix MC subfractions on the osteoblastic cell proliferation.
Osteoblasts were plated at a density of 2×10^4 cells/well in 24-well plates and treated with *Achyranthes* radix MC subfractions for 48 hours. After culture, cells were removed and counted for dye exclusion in a hemocytometer under the light microscope. The data represent a mean ± S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, MC-1: first subfraction of MC layer; MC-2: second subfraction of MC layer, MC-3: third subfraction of MC layer. *: P<0.05, **: P<0.01.

a result of measuring ALP activity in order to observe the activity of osteoblast, second subfraction increased ALP activity (Fig. 6). Also, second and third subfraction promoted the formation of calcified nodule (Fig. 7).

As a result of TRAP stain in order to observe the effect on the osteoclast generation, MC layer decreased of the osteoclast generation at the all concentration. In contrast to the inhibitory effects of MC layer on osteoclast generation, all the subfraction increased osteoclast generation at concentrations less than 10 μg/ml (Fig. 8).

Discussion

Bone loss occurs in the postmenopausal woman as a result of an increase in the rate of bone remodeling and an imbalance between the activity of osteoclasts and osteoblasts. Osteoporosis is a clinical syndrome of increased fracture susceptibility. Fractures are the complications of osteoporosis that result in the patient's symptoms, and their physical,

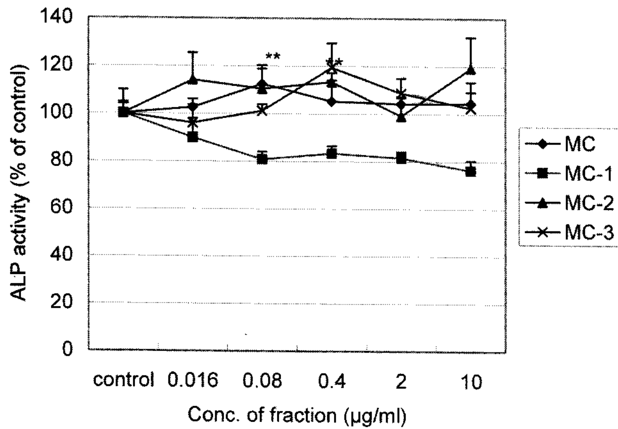

Fig. 6. Effects of *Achyranthes* radix MC subfractions on the ALP activity of osteoblastic cells.
The osteoblastic cells were plated at a density of 2×10^4 cells/well in 24-well plates and treated with *Achyranthes* radix MC subfractions for 48 hours. Enzyme activity was measured by spectrophotometric method using p-nitrophenyl phosphate as a substrate. The data represent a mean ± S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, MC-1: first subfraction of MC layer; MC-2: second subfraction of MC layer, MC-3: third subfraction of MC layer. *: P<0.05, **: P<0.01.

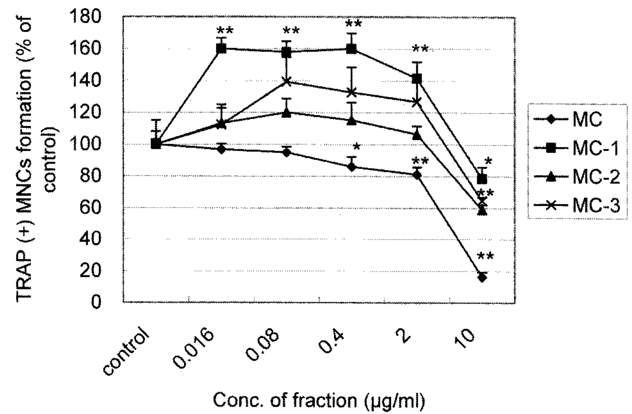

Fig. 8. Effects of *Achyranthes* radix MC subfractions on the osteoclast generation.
The RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 50 ng/ml RANKL and 1 ng/ml TGF-β and various *Achyranthes* radix MC subfractions. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean±S.E. of 4 experiments and are expressed as a ratio to the control. MC: methylene chloride layer, MC-1: first subfraction of MC layer; MC-2: second subfraction of MC layer, MC-3: third subfraction of MC layer. *: P<0.05, **: P<0.01.

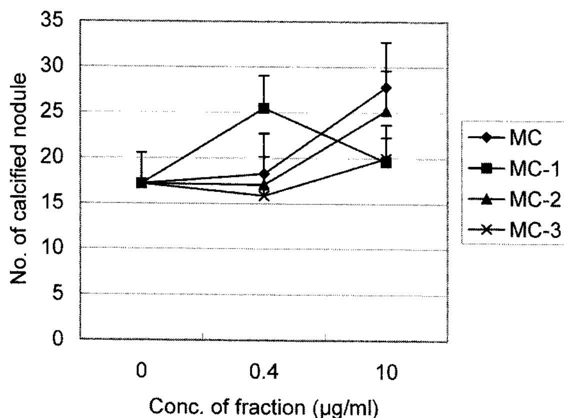

Fig. 7. Effects of *Achyranthes* radix MC subfractions on the calcified nodule formation.
The osteoblastic cells were plated at a density 2×10^4 cells/well in a 24-well plate, cultured for 19 days in the presence of ascorbic acid, β-glycerophosphate and various *Achyranthes* radix MC subfractions. After culturing, the silver nitrate stained spots were counted as calcified nodule. The data represent a mean ±S.E. of 5 experiments. MC: methylene chloride layer, MC-1: first subfraction of MC layer; MC-2: second subfraction of MC layer, MC-3: third subfraction of MC layer.

functional and psychosocial problems. The primary objective in the management of patients with established osteoporosis is to reduce the risk of fracture by increasing the bone mass to a normal level. Estrogen replacement therapy is indeed effective in preventing postmenopausal bone loss. However, there have been some of adverse effects, such as uterine bleeding and increased risk of cancer (Genant *et al.*, 1989; Nelson *et al.*, 2002; Lemay, 2002). Calcium supplement has been widely used for osteoporosis prevention and/or treatment. While calcium supplement represses secretion of parathyroid hormone and prevents bone loss, there are many individual differences in maintaining bone mass (Heaney, 1996). Bisphosphates have been used as therapeutic agents in osteoporosis, however, burning sensation at the upper respiratory system is reported in the case of inadequate oral administration (Reginster, 1995; Fleisch, 1996; Cryer and Bauer, 2002).

In traditional medicine, many herbal drugs have been used to treat bone and joint diseases for over two thousand years. However, the precise mechanism of their action is not yet known. *Achyranthes bidentata*, commonly known as Rough chaff tree in English, is an erect, annual herb, commonly found in India, Ceylon, Tropical asia, Africa, Australia and America (Kirtikar and Basu, 1935, Nadkarni and Nadkarni, 1976). This herb has been attributed with cardiac stimulant, astringent, diuretic, alterative, purgative and antiinflammatory properties (Satyavati *et al.*, 1976; Akhtar and Iqbal, 1991, Vetrichelvan and Jegadeesan, 2003).

In the Korean traditional medicine encyclopedia, Dong-eui-bo-gam, it is documented that *Achyranthes* radix alleviates the symptoms of joint diseases. Recently, Gao *et al.* (2000), reported that *Achyranthes bidentata* promotes proliferation of osteoblasts. All these reports attracted our interest to the therapeutic utility of this herb in treating bone diseases.

The increase in bone mass can be achieved by increasing the osteoblastic bone formation, decreasing osteoclastic bone resorption, or both. Therefore, in this study, the effects of *Achyranthes* radix extracts on osteoblast and osteoclast

proliferation were examined. Osteoblast proliferation and activity were investigated by the following measurements: cell proliferation, ALP activity and calcified nodule formation. The generation of osteoclasts was studied by measuring TRAP (+) multinucleated cells formation.

The enhancement in new bone formation was obtained by the increase in the number of osteoblasts and/or the degree of osteoblast differentiation. There was an increase in the proliferation rate of osteoblastic cells after the *Achyranthes* radix extracts treatment. The cells treated with MC layer and W layer showed a maximal increase of 15~20% in their proliferation rate compared to the control cells (Fig. 1). MC layer promoted dose dependently the proliferation of osteoblast under all the concentrations (0.016~10 μg/ml). Among the subfraction of MC layer, first and second subfraction showed tendency of promoting proliferation of osteoblast (Fig. 5). Ea layer did not alter osteoblastic cell proliferation. These results were not in accordance with the report of Gao *et al.* (2000) which showed that ecdysterone in *Achyranthes bidentata* Bl. promoted proliferation of UMR106 cells.

ALP is a widely accepted phenotypic marker of differentiated osteoblastic cells (Farley and Baylink, 1986). Ea layer increased ALP activity, MC layer does not increase (Fig. 2). However, second subfraction of MC layer increased ALP activity (Fig. 6). When the osteoblasts were plated at near the confluent density, they proliferated and differentiated in the culture over a 14-16 day period to form multilayered sheets of cells that included prominent mineralized nodules (Stein *et al.*, 1996). To determine whether *Achyranthes* radix extracts also alters the differentiation of the osteoblasts, formation of calcified nodule was observed. In this study, the osteoblastic cells formed confluent monolayers within 3 days. After 17-19 days, calcified nodule was confirmed by von Kossa stain. MC layer increased the calcified nodule from osteoblasts. Also, second and third subfraction of MC layer promoted the formation of calcified nodule (Fig. 7). These results suggested that the admini-stration of *Achyranthes* radix extracts enhances new bone formation *in vivo*.

The primary cells responsible for bone resorption are the osteoclasts. Osteoclasts, which are present only in bone, are multinucleated giant cells (Nijweide *et al.*, 1986; Mundy and Roodman, 1987). Their hemopoietic precursors proliferate and differentiate into osteoclasts through interaction with osteoblastic stromal cells. Hemopoietic precursor cells differentiate into osteoclasts at bone-resorbing sites under the control of 1,25-dihydroxycholecalciferol, parathyroid hormone, prostaglandin E_2 and local factors produced in the microenvironment (Takahashi *et al.*, 1988). Mature osteoclasts express intense TRAP activity and abundant calcitonin receptors. Osteoclast generation was measured by examining the number of TRAP (+) multinucleated cells after culturing osteoclast precursor cells on 96-well plates. It is well known that TRAP is a phenotypic marker of osteoclasts (Minkin, 1982). Treating the precursor cells with MC layer from *Achyranthes* radix extracts significantly decreased the number of TRAP (+) multinucleated cells. Ea and W layers did not alter osteoclast generation (Fig. 4). In contrast to the inhibitory effects of MC layer on osteoclast generation, all the subfraction increased osteoclast generation at concent-rations less than 10 μg/ml (Fig. 8). These results showed that MC layer of *Achyranthes* radix extracts inhibited osteo-clast generation.

In conclusion, *Achyranthes* radix extracts stimulated the proliferation and activity of bone forming osteoblasts, while inhibiting generation of bone resorbing osteoclasts. Although the active substances have not yet been identified, it is suggested that the MC layer from *Achyranthes* radix contains effective substances that have the potential to enhance the bone metabolism in osteoporosis. However, it was not clear which subfraction had active substance. Further studies are needed to elucidate the active substance.

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