Beneficial Effects of Ajuga decumbens on Osteoporosis and Arthritis

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Extract of the whole plant, Ajuga decumbens (KE) has long been used in China as a medication for the relief of joint pain. Previously, we proved that KE up-regulated the synthesis of collagen in false aged model rats. In this paper we examined the effects of KE on nitric oxide (NO) production, expression of inducible nitric oxide synthase (iNOS), osteoblast and osteoclast activity. We also investigated whether KE had any anti-osteoporosis or anti-arthritis activity by using ovariec-tomized mice and adjuvant induced arthritic rats. KE exhibited down-regulation of differentiation into osteoclast and up-regulation of mineralization in osteoblast-like MC3T3-E1 cells in a concentration-dependent manner. NO synthesized by iNOS plays important roles in inflammatory disease and imbalance between bone resorption and bone formation caused by estrogen depletion. KE inhibited expression of iNOS which caused concentration dependent inhibition of NO production. Furthermore, KE prevented brittle bones in ovariec-tomized mice and swelling of the left hind ankle in adjuvant induced arthritic rats. Therefore, KE improved the balance of bone resorption and bone formation, showing anti-inflammatory effects. Consequently, KE is beneficial for sufferers of bone and joint disease.

Key words osteoporosis; arthritis; Ajuga decumbens; inducible nitric oxide synthase; osteoclast; osteoblast


discussion

Ajuga decumbens is a naturally occurring herb in Japan and China. The whole flowering plant of A. decumbens has been used in treatment of hypertension, hemoptysis, carbuncles and joint pain, etc.1,2) Many compounds have been isolated and identified from the whole plant, A. decumbens,3—7) several of which show anti-tumor activity.8) Recently, the number of cases of age related illnesses, e.g. osteoporosis and osteoarthritis have been trending upwards. These illnesses seriously affect people’s daily lives because bones and joints play an important role in supporting the body during everyday movement.

Collagen is one of the important bone and joint composition, and decreases naturally with age. Calcium resorption is caused by this effect. It was reported that A. decumbens up-regulates the synthesis of collagen in false aged model rats.9) Accordingly, we focused on the effects of A. decumbens on bone and joint disease.

Post-menopausal osteoporosis is a metabolic bone disease characterized by a decrease of bone mass after menopause.10) Many factors contribute to the development and maintenance of skeletal mass before and after menopause.11,12) In fact, bone mass is the end result of a dynamic and complex process called remodeling. This process is characterized by a balance between osteoclast bone resorption and osteoblast bone formation. Estrogen is one of the factors that regulates bone metabolism, therefore, estrogen depletion after menopause causes an imbalance between bone resorption and bone formation.11) In addition, it is reported that NO, synthesized by iNOS, is related to the imbalance between osteoclast bone resorption and osteoblast bone formation.13—15) It is widely known that NO is involved in a diverse array of physiological processes. Furthermore, NO, especially synthesized by iNOS, is related to inflammatory diseases like osteoarthritis and rheumatoid arthritis.14,16) Based on this knowledge, we hypothesized that the inhibition or down-regulation of NO production by iNOS would provide a new form of treatment for the prevention of inflammatory bone diseases and bone metabolic disease.

MATERIALS AND METHODS

Animals Eight to ten-week-old female ddY mice and eight-week-old male LEW/SsN rats were obtained from SLC (Shizuoka, Japan). The mice and rats were housed under a 12 h/12 h light/dark cycle in a temperature and humidity-controlled room. They were allowed food and water ad libitum. After a week to adapt to the lighting conditions, healthy animals were used in a variety of experiments. All experimental procedures were approved by the Committee for Ethics and Animal Experimentation, Matsuura Yakugyo Co., Ltd.

Preparation of Plant Extracts The whole plant A. decumbens (200 g) was grown in China and a voucher specimen (HS050601) was placed in our laboratory. The dried A. decumbens was refluxed with 70% aqueous ethanol for 30 min. The alcoholic extract was then concentrated to 50 g. The concentrate was freeze-dried, finally producing 20 g (i.e., 10% yield) of a dark brown, A. decumbens extract (KE).

Formation of Osteoclast-Like Cells from Co-culture Bone marrow cells were isolated from the femoral bones of eight to ten-week-old female ddY mice. MC3T3-E1 cells (1.5×10⁴ cells/well) were co-cultured with bone marrow cells (5×10⁵ cells/well) in α-minimum essential medium Eagle (MEM) that contained 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 10⁻⁸ M 1α,25(OH)₂ vitamin D₃ and 10⁻⁷ M dexamethasone in 24 celled micro-plate. KE was supplied simultaneously and the medium was changed 3 d after cells plating. The cells were incubated for 7 d.

TRAP Activity of Co-culture Cells The co-culture cells were rinsed twice and fixed with fixing solution (4.5 mm citric acid, 2.25 mm tri-sodium citrate, 3 mm hydrochloric acid, 3% formic acid, and 3% acetic acid) for 30 min. Subsequently they were rinsed and stained with tartrate-resistant acid phosphatase (TRAP) staining solution (Muto Pure Chemical Co., Ltd., Japan) for 5 h at 30 degrees. Then they were rinsed and stained with Hematoxyline for 2 min as a

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contrast staining. The numbers of red stained cell were then counted microscopically. **Mineralization** The MC3T3-E1 cells (1 × 10^4 cells/well) were maintained in complete medium (α-MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin). When the cells reached confluence, the medium was changed to a differentiation medium (complete medium contained 10 mM glyceral 2-phosphate, 50 μg/ml ascorbic acid), which was changed every 3 d. KE was supplied simultaneously. After an additional 21 d, the cells were stained using Alizarin red S staining to evaluate the level of mineralization. **Alizarin Red S Staining** The cells were rinsed 3 times with PBS and fixed with 95% ethanol for 10 min, washed 5 times with distilled water, and stained with alizarin red S (2%, w/v) for 5 min. After washing with distilled water 3 times, the stain was eluted by cetylpyridinium chloride (10%, w/v). The absorbance at 562 nm was measured with a plate reader (MULTISKAN SPECTRUM; Thermo, Japan). **Measurement of NO Production** NO production was determined by measuring as nitrite (NO_2^-) concentration. RAW264.7 macrophages were plated at a density of 2 × 10^5 cells/well in 96 celled micro-plate and incubated for 24 h. KE (0.01—1 mg/ml) was supplemented and the cells were also stimulated by the addition of interferon-γ (50 U/ml) and lipopolysaccharide (1 μg/ml) at the same time. The amount of NO production in the media was detected with the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) 24 h after the stimulation with interferon-γ and lipopolysaccharide. Each supernatant was mixed with the same volume of Griess reagent. The absorbance at 570 nm was measured with a plate reader (MULTISKAN SPECTRUM; Thermo, Japan), and nitrite concentration was determined using a dilution of sodium nitrite as a standard. **Expression of iNOS** RAW264.7 macrophages were plated at a density of 4 × 10^5 cells/well in DMEM in 6 celled micro-plates and incubated for 24 h. KE, lipopolysaccharide, and interferon-γ were supplemented as described above. Intracellular protein extracts were analyzed on a 7.5% (iNOS) or 10% (β-actin) polyacrylamide mini-gel electrophoresis at 100 V for 1 h at room temperature using a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Japan). After transfer onto nitrocellulose membranes using iBlotTM (Invitrogen, Japan), the membranes were blocked overnight at 4 degrees with blocking solution Tris-buffered saline (TBS) containing 0.5% Tween20 and 0.3% skim milk. After blocking, the membranes were incubated for 2 h at room temperature with a 1:200 dilution of mouse NOS2 (iNOS) monoclonal IgG (Santa Cruz Biotechnology, CA, U.S.A.) or a 1:1000 dilution of Goat anti-Actin polyclonal IgG (Santa Cruz Biotechnology, CA, U.S.A.) in blocking solution. After 3 times washing with TBS containing 0.5% Tween20, the membranes were incubated for 1 h at room temperature with a 1:5000 dilution of horseradish peroxidase-labeled anti-mouse IgG1 (iNOS; Santa Cruz Biotechnology, CA, U.S.A.) and horseradish peroxidase-labeled anti-goat IgG (Actin; ZYMED, CA, U.S.A.) in blocking solution. After incubation, the membranes were washed extensively with TBS containing 0.5% Tween20. The immunoreactive bands were detected with ECL plus detecting reagent (GE healthcare Co., Ltd., Japan) and visualized using Light-capture II (ATTO Co., Ltd., Japan). **Ovariectomized Mice** Female ddY mice were sham operated or ovariectomized. Some of the ovariectomized mice were divided into three groups. Two groups were treated with KE (60—120 mg/kg, p.o.). Treatment started immediately after surgery and was administrated orally for three months. The mice were then dissected, and body, uterine, and femoral bone weights were measured. The left femoral bones were removed to measure the calcium content and the right femoral bones were used for histological analysis. **Measurement of the Calcium Content** The left femoral bones were dried at 100 degrees for 3 d and were then incinerated at 600 degrees. The ashes were dissolved in 1 M hydrochloric acid (1 ml), the solution was diluted with distilled water (1:100 or 1:200). Fifty micrometers of each sample was mixed with 5 ml of OCPC solution (82.5 μM o cresolphthalein complexone, 740 nm 2-aminoethanol, 36 nm boric acid, 1 μg/ml 8-quinoilinol) and incubated at room temperature for 5 min. The absorbance at 575 nm was measured with a plate reader (MULTISKAN SPECTRUM; Thermo, Japan). Calcium concentration was determined using a dilution of calcium chloride dihydrate as a standard. **Measurement of the Collagen Content** Lumbar were desiccated at 60 degrees and put into an ampoule with 1 ml of 4 M sodium hydroxide. They were dissolved at 120 degrees for 20 min in a closing condition. The sample solution was neutralized with 1.89 M citric acid (1 ml) and diluted with diluted hydrochloric acid. Chloramine-T solution (300 μl) was added and incubated at room temperature for 20 min. Perchloric acid (300 μl, 3.15 M) was added and incubated for an additional 5 min. Then, p-dimethyl benzaldehyde solution (300 μl) was added and incubated at 60 degrees for 20 min. After cooling, the absorbance at 555 nm was measured with a plate reader (MULTISKAN SPECTRUM; Thermo, Japan). Calcium concentration was determined using a dilution of hydroxyproline as a standard. **The Number of TRAP Positive Cells** The right femoral bones were fixed in a 15% formalin phosphate buffer for 2 d. After decalcification with 20% EDTA solution (pH 7.0—7.4) for 2 weeks, the samples were dehydrated in an ascending ethanol series (70, 80, 90, 95, 100%), embedded in paraffin, and cut into 7 μm thick frontal sections. The sections were stained with tartrate-resistant acid phosphatase (TRAP) staining solution (Muto Pure Chemical Co., Ltd., Japan) for 5 h at 30 degrees. Then they were rinsed and stained with Hematoxyline for 2 min as a contrast staining. The red stained cell numbers were counted microscopically using Image-pro Discovery (Media Cybernetics, Inc., MD, U.S.A.). **Adjuvant Induced Arthritic Rat** Freund’s complete adjuvant was prepared by suspending heat-killed Mycobacterium butyricum (Difco Laboratories, Detroit, MI, U.S.A.) in Freund’s incomplete adjuvant (Wako Pure Chemical Industries, Ltd., Japan) (12 mg/ml). Arthritis was induced by a single injection of 100 μl of Freund’s complete adjuvant/phosphate buffer emulsion intradermally in the right hind metatarsal footpad of the rats. Three groups were treated with KE (30, 150, 300 mg/kg, p.o.). Treatment started immediately after injection and the rats were administrated orally for 16 d. The severity of arthritis was monitored and the left hind paw volume was measured with a water replace-
ment method 5 d a week after the injection of Freund’s complete adjuvant. The grade of the ankle swelling was shown as the growth rate of the ankle volume. The severity of arthritis was evaluated with a clinical score: 0, paws with no swelling and focal redness; 1, paws with swelling of toe joints; 2, paws with mild swelling of ankle or wrist joints; 3, paws with severe swelling of ankle or wrist joints; 4, paws with deformities or ankylosis. Every paw was graded and the scores were totaled. The maximum possible score per rat was 16.

**Statistical Analysis** Results are expressed as means ± standard error (S.E.) for each experiment. Statistical analysis was performed by Dunnett test and Tukey–Kramer test. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**Effect of KE on Differentiation into Osteoclast-Like Cell** We studied the effect of KE on the level of differentiation into osteoclast-like cells using murine bone marrow cells and MC3T3-E1 cells co-culture incubation. Osteoclast-like cells differentiated from bone marrow cells were detected by using the TRAP stain method. As a result, KE inhibited differentiation into osteoclast-like cell in a concentration-dependent manner (Fig. 1).

**Effect of KE on Mineralization of Osteoblast** Figure 2 shows the level of mineralization of MC3T3-E1 cells cultured with 10 mM glycerol 2-phosphate and 50 μg/ml ascorbic acid. The level of mineralization was evaluated using Alizarin red S staining and the area stained red indicates the sites where calcium was deposited. Furthermore, the stain was eluted by cetylpyridinium chloride (10%, w/v) and determining the level of calcium incorporation. KE promoted osteoblast mineralization.

**Effect of KE on NO Production and Expression of iNOS** RAW264.7 macrophages were stimulated with lipopolysaccharide and interferon-γ for 24 h in order to induce NO synthesis. The production of NO was estimated from the accumulation of nitrite, which is a stable product of the NO metabolism in the medium using the Griess reagent. The lipopolysaccharide-stimulated cells produced 46 μM of nitrite over a 24 h period (Fig. 3). When the cells were incubated with KE, the production of nitrite was significantly inhibited in a concentration-dependent manner (Fig. 3). The effect of KE on the interferon-γ and lipopolysaccharide-induced expression of iNOS in RAW264.7 macrophages was involved in prevention from inflammatory mechanism. The western blot experiments showed the induction of the iNOS in the cells 24 h after the lipopolysaccharide and interferon-γ treatment (Fig. 4). KE significantly suppressed the expression of the iNOS in a concentration-dependent manner (Fig. 4).

**Anti-osteoporotic Effect of KE** A marked amount of bone loss occurred in the femoral bone in ovariectomized mice, and the loss was prevented by treatment with KE in a dose-dependent manner (Fig. 5). The femoral calcium and the lumbar collagen concentration in the ovariectomized group were significantly lower than in the sham-operated group (Fig. 6). In the 120 mg/kg of KE-fed group, the femoral calcium and the lumbar collagen concentration were higher than in the ovariectomized group (Fig. 6). The estrogen deficiency caused by ovariectomy stimulated markedly osteoclast differentiation, resulting in an increase in the num-

**Fig. 1. Effect of KE on Differentiation into Osteoclast-Like Cells**

The bone marrow cells and MC3T3-E1 cells were co-cultured with 1α, 25(OH2) vitamin D3, and dexamethasone for 7 d. Multi-nucleated osteoclast-like cells were detected by TRAP staining method. The numbers of red stained cells were counted microscopically. Each column represents the mean ± S.E. ∗∗p<0.01, ∗p<0.05 vs. Control (0 concentration of KE) (Dunnett test).

**Fig. 2. Effect of KE on Mineralization of MC3T3-E1 Cells**

MC3T3-E1 cells were incubated with glycerol 2-phosphate and ascorbic acid for 21 d. The level of mineralization was evaluated by Alizarin red S staining (A). The stain was eluted by cetylpyridinium chloride and quantified by colorimetry (B). Each column represents the mean ± S.E. ∗p<0.05 vs. Control (0 concentration of KE) (Dunnett test).

**Fig. 3. Effect of KE on the NO Production from RAW264.7 Cells**

RAW264.7 macrophages were stimulated with lipopolysaccharide and interferon-γ for 24 h. The accumulation of NO in the medium was detected by the Griess method. Each column represents the mean ± S.E. ∗∗p<0.01 vs. Control (0 concentration of KE) (Dunnett test).
ber of TRAP-positive multi-nucleated osteoclasts (Fig. 7). Administration of 120 mg/kg of KE restored it to the level in sham-operated mice (Fig. 7).

**Anti-arthritic Effect of KE** As shown in Fig. 8, inflammatory polyarthritis was induced in immunized rats. The swelling of non-injected hind ankle occurred on day 13 after immunization. Treatment with KE diminished the left hind ankle swelling and polyarthritis index of adjuvant induced-arthritis rats in a dose-dependent manner.

**DISCUSSION**

At the present time, we live in an aging society and aged people often suffer from illnesses such as osteoporosis and osteoarthritis. It is very important to prevent the deterioration of bone mass and joint cartilage, to maintain their quality of life. Therefore, we have undertaken this research.

Firstly, our results support the hypothesis that KE normally regulates the balance of bone resorption and bone formation. KE helps to prevent fracturing in the lumbar and the femur bones. The osteoclast is a tissue-specific polykaryocyte created by the differentiation of monocyte/macrophage precursor cells at or near the bone’s surface and it possesses characteristics which enable the resorption of mineralized bone. KE suppressed the up-regulation of bone resorption. It was caused by the inhibition of osteoclastogenesis and activation of osteoclast. The osteoblast is known to play a crucial role in the formation phase of bone remodeling by laying down the structural component of bone and secreting various cytokines and growth factors that influence both bone formation and resorption. KE activated and promoted mineralization of MC3T3-E1 osteoblast-like cells. Therefore, it is suggested that KE normalizes the condition of bone metabolism and helps to keep bone mineral density.

Secondly, the results of this study indicate that KE has an anti-inflammatory effect. KE inhibited synthesis of NO derived from RAW264.7 macrophages. The inhibition was caused by a down-regulation of expression of iNOS. NOS inhibitors of the treatment of NO mediated inflammatory reactions require specific links for iNOS. Accordingly, overproduction of NO by iNOS is considered to produce pro-inflammatory properties. Enhanced NO production by
macrophages upon exposure to substances of microbial origins such as lipopolysaccharide, facilitated a characteristic increase in iNOS activity. It was also demonstrated that iNOS was evident and critical during the developmental stages of osteoporosis consequent to systematic inflammation. It was proposed that the reduced bone formation mediated by iNOS is due to the dramatic increase in osteoblast apoptosis.\(^5\) As a result of the experiment using iNOS knockout mice, Armour \textit{et al.} identified that iNOS is a key signaling molecule that puts bone metabolism out of balance due to estrogen depletion.\(^5\) Cuzzocrea \textit{et al.} suggest that iNOS is a potential target for therapy of postmenopausal osteoporosis in women.\(^5\) We hypothesize that KE will be a useful agent in the treatment of osteoporosis and osteoarthritis because it has significant effects on the NO production and expression of iNOS in macrophages. We will investigate whether the anti-osteoporotic and anti-arthritic effect of KE are depressed by using the iNOS inhibitor.

Thirdly, to evaluate the effect of KE on osteoporosis, we used ovariectomized mice. KE significantly suppressed the bone loss and decrease of calcium and collagen concentration in the lumbar and femur. Collagen plays an important role in binding calcium in bone. In this study we found that KE prevents the loss of bone mineral density caused by upregulation of collagen synthesis from osteoblast. KE also decreased osteoclasts in femoral bones. These findings support the experimental results in co-culture experiment. These results suggest that KE up-regulates osteoblast activity and inhibits osteoclast differentiation. Therefore, KE improves the imbalance between osteoclast bone resorption and osteoblast bone formation. Ovariectomized models, characterized by estrogen depletion are used frequently in osteoporosis experiments. In this model, mice exhibited a loss of bone weight and a decrease in uterine weight.\(^2\) KE (60—120 mg/kg) did not increase uterine weight (data not shown). It is suggested that the side effects of KE on the uterus are minimal. There are many reports about the inhibitory effects of natural products on ovariectomized mice or rats bone loss, for example, \textit{Sambucus williamsii},\(^2\) soy isoflavone,\(^2\) black cohosh (\textit{Cimicifuga racemosa}),\(^3\) hesperidin,\(^3\) citrus flavonoids, etc. Soy isoflavone is a popular agent for the treatment or prevention of osteoporosis. We proved that KE up-regulated the synthesis of collagen in the lumbar in ovariectomized mice, whereas no activity was detected with soy isoflavone (data not shown). Therefore, we also studied the interaction between KE and soy isoflavone. KE and soy isoflavone potentiated each effect in ovariectomized mice (data not shown).

Fourthly, KE suppressed secondary immune response. KE suppressed the swelling of the left hind non-injected ankle of adjuvant induced arthritic rats causing an anti-inflammatory effect. It was reported that NOS inhibitor suppressed the swelling in adjuvant-injected paws of rats.\(^2\) It suggests that iNOS and NO have important roles to play in the initiation and development of arthritis in these specimens. Therefore, we can relate this suppressive effect in adjuvant induced arthritic rats to inhibition of NO products and iNOS expression. Adjuvant induced arthritis is an established laboratory model used in the study of rheumatoid arthritis.\(^3\) Accordingly, it suggests that KE has an anti-arthritic effect. Glucosamine is an aminosaccharide, acting as a preferred substrate for biosynthesis of glycosaminoglycan chains and, subsequently, for the production of aggrecan and other proteoglycans of cartilage. Glucosamine is also a useful preventative agent against adjuvant-induced arthritis.\(^4\) Therefore, we evaluated interaction between KE and glucosamine. As a result, KE and glucosamine potentiated each effect against adjuvant-induced arthritis (data not shown).

In conclusion, KE regulates the balance between bone resorption and bone formation, inhibits NO synthesis by RAW264.7 macrophages, and down-regulates expression of iNOS in macrophages. Accordingly, we think that KE is a beneficial medicine against osteoporosis and arthritis including osteoarthritis and rheumatoid arthritis.

**REFERENCES**