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Inhibitory effect of Kampo medicines on bone resorption *in vitro* and preventive effect on bone loss *in vivo*

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The inhibitory activities of 19 Chinese formulae on osteoclast formation and bone resorption *in vitro* were investigated. The inhibitory activity on osteoclast formation was evaluated using a mouse bone marrow cell culture in which osteoclast-like multinucleated cells were formed in the presence of parathyroid hormone (PTH). The inhibitory activity on bone resoption was evaluated using PTH-induced resorption of ^{45}Ca from ^{45}Ca-prelabeled neonatal mouse parietal bone. Among the 19 Chinese formulae, nine showed a strong inhibitory activity on osteoclast formation at a concentration of 50 μg/ml, and three of the nine also showed a strong inhibitory activity on bone resorption. Among these three formulae, Bu-Shen-Jian-Gu-Tang (補腎健骨湯) showed the most promising activity in a dose-dependent manner on the inhibition of bone resorption. A further *in vivo* experiment in ovariectomized (OVX) model rats using peripheral quantitative computed tomography (pQCT) revealed its preventive effect on bone loss.

Key words antiosteoporotic activity, Bu-Shen-Jian-Gu-Tang (補腎健骨湯), peripheral quantitative computed tomography (pQCT), Chinese formula, bone resorption, ovariectomy (OVX).

Introduction

Osteoporosis is a disease characterized by reduced bone density or bone mass, resulting in increased bone fragility and fracture risk. It is caused when the rate of bone resorption by osteoclasts exceeds that of bone formation by osteoblasts.[1] Thus, drugs inhibiting the formation or activity of osteoclasts or stimulating the proliferation of osteoblasts are valuable for treating osteoporosis. In order to develop the antiosteoporotic drug from natural sources, we examined the effect of eighteen medicinal plants on bone resorption,[2] and the active constituents of *Cimicifugae heracleifolia*,[2] *Boerhaavia repens*,[3] and *Sambucus sieboldiana*,[4] and their inhibitory activities on bone resorption were also investigated. On the other hand, we also screened Chinese medicinal plants for their activities to stimulate osteoblast proliferation and to inhibit the formation of osteoclast-like multinucleated cells,[5] and the active constituents of *Dioscorea spongiosa*, the most potent plant in both assay, were examined.[6-8] Further, *in vivo* experiment using ovariectomized rats as a model of postmenopausal bone loss was conducted.[5] On the other hand, according to the theory of traditional Chinese medicine, formulae, rather than one medicinal plant, are used for treatment of osteoporosis in clinic. Therefore, we also examined the effect of a Kampo formula, Tsu-Kan-Gan (通関丸), on bone resorption *in vitro* and *in vivo*.[9,10] In this study, we focused on nineteen Chinese formulae (Table 1)[11-24] used to cure bone diseases in clinics in China, and investigated their inhibitory activities on the formation of osteoclast-like multinucleated cells and on bone resorption. Furthermore, Bu-Shen-Jian-Gu-Tang (補腎健骨湯), which showed the most promising activity in both assays, was subjected to an *in vivo* experiment with ovariectomy-induced osteoporosis model rats.

Materials and Methods

General. Aged female rats (Wistar) and mice (ddY) were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Kansas, USA), and alpha modified Eagle's medium (α-MEM) and horse serum were obtained from ICN Biomedicals, Inc. (Ohio, USA). Ham's F-12 medium was purchased from Nissui Seiyaku Co. (Tokyo, Japan). Parathyroid hormone (human, 1-34, PTH) was obtained from Peptide Institute, Inc. (Osaka, Japan), and elcatonin from Asahi Kasei Corporation (Osaka, Japan). 17β-Estradiol was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and ^{45}Ca (772.34 MBq/mg) was from PerkinElmer Life and Analytical Sciences, Inc. (Boston, USA). All other reagents were from Wako Pure Chemicals Industries, Ltd. (Kyoto, Japan).

Nineteen Chinese formulae. Each formula used in the *in vitro* experiments (Table 1) was purchased from a pharmaceutical company in Liaoning Province, Shenyang, China, in March, 2004, as a mixture of the component plants. Each component plant of Bu-Shen-Jian-Gu-Tang (補腎健骨湯) used in the *in vivo* experiment was purchased from Chengdafangyuan Drug Store, Dalian, China, in January, 2005, and mixed as indicated (Table 1). Each formula was extracted with a threefold volume of water

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(reflux, 1 h, ×3), and the extract was concentrated under reduced pressure and lyophilized. The prepared Bu-Shen-Jian-Gu-Tang (補腎健骨湯) was analyzed by LC-MS (Fig. 3). The residual powder was dissolved in distilled water before use.

Bone marrow cell culture. Inhibitory activity on osteoclast-like cell formation was assessed using the method of Takahashi *et al.*[25] Osteoclast-like multinucleated cells were originated from a culture of mouse bone marrow cells. The marrow cavities of the tibiae of male ddY mice (7 weeks old) were flushed with α-MEM, and the bone marrow cells were cultured in α-MEM containing 10% FBS at 1.5×10^6 cells/ml in 24-well plates (0.5 ml/well) in the presence of PTH (24×10^{-9} M) and test samples at various concentrations. Elcatonin (2 U/ml) was used as a positive control. The medium was replaced with new medium containing test samples and PTH every 3 days. All cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. After culture for 8 days, cells adherent to the well surface were fixed with 10% formalin in phosphate-buffered saline (PBS, pH 7.4) for 10 min and then treated with ethanol-acetone (1:1) for 1 min. The cells were stained with tartrate-resistant acid phosphatase (TRAP) staining solution. TRAP-positive multinucleated cells containing three or more nuclei were recognized as osteoclasts and counted.

Assay of bone-resorbing activity *in vitro*. Bone-resorbing activity was assessed using the method of Shigeno *et al.*[26] ^{45}Ca (0.074 MBq) was injected subcutaneously into ddY mice (2 days old). Two days later, half parietal bones were taken out and cultured in sterile plastic 24-well culture plates on stainless steel grids on the top of glass rings that supported the bones near the gas-liquid interface. Bones were cultured in Ham's F-12 medium (1 ml/well) supplemented with 0.2% $NaHCO_3$, 1.0 mM $CaCl_2$, 25 μg/ml streptomycin, 15 μg/ml penicillin, and 5% heat-inactivated horse serum in an incubator at 37℃ under 5% CO_2 in air. Bones were randomly assigned to a control or treatment group and each group included 4 bones. Bones were incubated for a total of 168 h; the first 24 h allowed for efflux into the medium of readily exchangeable ^{45}Ca in bone. After being cultured for 24 h and 96 h, bones were transferred to the new medium in the presence of PTH (2×10^{-9} M) and test samples at various concentrations. Elcatonin (4 U/ml) was used as a positive control. After being cultured for 168 h, bones were extracted for 3 days with 0.01 M EDTA-acetate buffer solution (pH 5.5). ^{45}Ca released into the culture medium from parietal bones at 96 h and 168 h and into the EDTA solution was measured. The radioactivity of ^{45}Ca was measured by a liquid scintillation counter (Beckman LS 3801). Bone resorption was quantified on the basis of the percentage of bone ^{45}Ca released into the medium as shown below:

$$^{45}Ca\ released\ (\%)=\frac{^{45}Ca\ released\ into\ the\ medium}{^{45}Ca\ released\ into\ the\ medium\ +\ ^{45}Ca\ remaining\ in\ the\ bone}\times100$$

LC-MS analysis of Bu-Shen-Jian-Gu-Tang. Analysis of the chemical constituents of Bu-Shen-Jian-Gu-Tang was performed using a JEOL JMS-700T four-sector mass spectrometer (Tokyo, Japan), coupled to a Hewlett-Packard model HP1100 HPLC system (Waldbronn, Germany), operated in the negative atmospheric pressure chemical ionization (APCI) mode [capillary temperature, 520℃; ion injection time, 0.1 s; column, Supelco Discovery® C_{18} (250 × 4.6 mm i.d., 5μm) (Bellefonte, PA, USA); column temperature, 40 ℃; mobile phase, a gradient system of (A) MeOH-CH_3CN (1:1, v/v) and (B) 8 mM NH_4OAc: 0-10 min, 0% A, 100% B; 10-15 min, 0-15% A, 100-85% B; 15-30 min, 15-40% A, 85-60% B; 30-45 min, 40% A, 60% B; 45-50 min, 40-50% A, 60-50% B; 50-60 min, 50% A, 50% B; flow-rate, 1 ml/min]. The water extract of Bu-Shen-Jian-Gu-Tang was dissolved in distilled water and filtered with Cosmonice Filter W (Nacalai Tesque Inc., Kyoto, Japan), and the filtrate (20 μl) was used for analysis. Identification of each peak was carried out on the basis of the retention time and pseudomolecular ions $[M-H]^-$ and $[M+AcO]^-$.

Treatment of rats. Aged female Wistar rats (8 months, 240—300 g) were maintained in a 12-h light/dark cycle in a temperature- and humidity-controlled room. The animals were allowed free access to laboratory pellet chew (LABO MR STOCK, Nosan Corporation, Yokohama, Japan) and water *ad libitum* during the experiment. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of University of Toyama. Rats were matched according to body weight and assigned to 5 experimental groups of 10 animals each, as follows: group 1, sham control; group 2, ovariectomy (OVX) control; group 3—5, OVX + 17β-estradiol, OVX + Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6; 100 mg/kg), and OVX + Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6; 500 mg/kg). After 7 days of acclimatization, under pentobarbital (40 mg/kg, i.p.) anesthesia, each rat was either sham operated or bilaterally ovariectomized.[27] Sham operation was performed by only exposing the ovaries. Ovariectomy was confirmed by observation of an atrophic uterus at the termination of the experiment. Two weeks after the operation, Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) dissolved in distilled water was administered orally (100 mg/kg or 500 mg/kg) 6 times a week. 17β-estradiol (E_2) was dissolved in a mixture of 5% benzyl alcohol and 95% corn oil to a concentration of 30 μg/ml and was given at a dose of 0.1 mg/kg to rats by intraperitoneal injection. Body weight was measured once a week in order to monitor drug intake and proper development. Six weeks after the start of intervention, all animals were anesthetized by an intraperitoneal injection of pentobarbital and were subjected to peripheral quantitative computed tomography (pQCT) measurement. At autopsy, blood was collected from the abdominal aorta and serum samples were obtained by coagulation for 1 h at 4℃, followed by centrifugation (3600 rpm for 10 min at 4℃),[28] and stored at -80℃. Uteruses, tibiae, and femurs were removed, the wet weight of each was determined, and the lengths of tibiae and femurs were measured by a caliper (mm).

Peripheral quantitative computed tomography (pQCT) analysis. The left tibiae of rats were scanned with a pQCT system, the Stratec XCT Research M (Stratec Medizintechnik GmbH, Pforzheim, Germany). The voxel size was 0.12 mm, the slice thickness was 0.5 mm, and the

cortical threshold was 464 mg/cm^3. We used the measuring mode for dividing total bone into cortical bone or cancellous bone as peel mode 20. This mode can detect the inner threshold automatically. After a scout scan was obtained and the growth plate was identified, transverse image sets of four cross-sectional slices were scanned in a 1—5 mm region of the left proximal tibia below the proximal separation point between the femora and tibia. From each transverse image, the bone mineral content (BMC), bone mineral density (BMD), and area in the total region were measured. The cortical region was extracted from the total bone region using specific algorithms (separation mode 3 and contour mode 2), and the cortical and cancellous BMC and BMD, cortical thickness, periosteal and endosteal circumferences, X-axis strength stain index (XSSI), Y-axis strength stain index (YSSI), and polar-axis strength stain index (PSSI) were measured. The data were processed using XCT Research Series Manual Software Version 5.4 and the data from transverse images from 1 mm of the proximal tibiae.

Analysis of biochemical markers in serum. Bone formation was assessed by measuring the amount of the *N*-terminal middle portion of osteocalcin (N-MID) in serum with an immunoassay kit (Serum Rat-MID™ Osteocalcin ELISA, Nordic Bioscience Diagnostics, Denmark). Bone resorption was evaluated by measuring the serum concentration of *C*-terminal telopeptide (Glu-Lys-Ala-His-βAsp-Gly-Gly-Arg) of the collagen type I α1 chain (CTX) with an immunoassay kit (Serum RatLap™ ELISA, Nordic Bioscience Diagnostics, Denmark).

Statistical analysis. A paired two-tailed Student's *t*-test was used to compare data between two groups, and a p value of less than 0.05 was regarded as significant.

Results and Discussion

Osteoclasts are well recognized as primary cells responsible for bone resorption.[29,30] We determined the inhibitory activity of 19 Chinese formulae (Table 1) on the formation of osteoclast-like cells. When mouse bone marrow cells were cultured in the presence of PTH, osteoclast-like cells were formed. There were numerous TRAP-positive giant cells in the PTH group, which were recognized as osteoclast-like cells (Fig. 1). In presence of elcatonin, the number of osteoclast-like cells was less than in the PTH group. Adding each Chinese formula at a concentration of 50 μg/ml, Pi-Shen-Sheng-Hua-Tang (脾腎生化湯, F-1), Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6), Yi-Shen-Zhuang-Gu-Tang (益腎壯骨湯, F-7), Bu-Shen-Zhuang-Gu-Tang (補腎壯骨湯, F-8), Zuo-Gui-Wan (左帰丸, F-9), Xian-Ling-Gu-Bao-Jiao-Nang (仙霊骨葆膠嚢, F-12), Gu-Wei-Tang (骨痿湯, F-14), Gu-Bi-Tang (骨痹湯, F-15), and You-Gui-Wan-Jia-Jian (右帰丸加減, F-18) showed potent inhibitory activities (Table 2) compared with the positive control, and no giant cells were observed.

The inhibitory activity of these 19 formulae on bone resorption was indicated as the rate (%) of ^{45}Ca released from neonatal mouse parietal bone (Table 2). Among the 19 formulae, Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6), Yi-Shen-Zhuang-Gu-Tang (益腎壯骨湯, F-7), and Xian-Ling-Gu-Bao-Jiao-Nang (仙霊骨葆膠嚢, F-12) showed potent inhibitory activities on PTH-induced bone resorption, which were stronger than that of elcatonin (4 U/ml). The inhibitory activity of Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) was concentration-dependent, but that of Yi-Shen-Zhuang-Gu-Tang (益腎壯骨湯, F-7) was not (Fig. 2). Yi-Shen-Zhuang-Gu-Tang (益腎壯骨湯, F-7) inhibited bone resorption at a low concentration, but not at a high concentration. Thus, inhibition by Yi-Shen-Zhuang-Gu-Tang (益腎壯骨湯, F-7) seemed to be due to cytotoxity.

Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) effectively decreased the number and activity of osteoclasts, indicating its potent inhibitory activity against both osteoclast formation and bone resorption. Therefore, it was considered to be the most effective antiosteoporotic agent among the 19 Chinese formulae tested. Thus, its antiosteoporotic activity was then examined in an *in vivo* experiment using OVX rats as a postmenopausal osteoporosis model.

LC-MS analyses of both Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) used in both *in vitro* and *in vivo* revealed that they were almost the same extracts (Fig. 3).

The success of the ovariectomy procedure was confirmed by examinations of body and uterus weights. At the end of the experiment, the body weight of OVX rats significantly increased by 10.8% (Fig. 4) and the uterine weight significantly decreased by 68.2% (Table 3), compared with those of sham-operated rats. This proved that OVX rats became estrogen deficient. A six-week treatment with E_2, a common agent in the treatment of osteoporosis in postmenopausal women, as a positive control lowered the body weight (by 11.7% compared with that of the OVX control group) and restored the uterine weight to the level of sham-operated rats (83.0% of that in the sham group). The results of the present study were consistent with the previous observation that estrogen deficiency significantly increased weight gain in ovariectomized rats and this effect was attenuated by estrogen treatment.[31,32] Treatment with Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) slightly lowered the body weight (100 mg/kg, 1.2%; 500 mg/kg, 5.4% decrease) and slightly restored the uterine weight (100 mg/kg, 49.4%; 500 mg/kg, 59.5% of that in the sham group). Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) mildly prevented ovariectomy-induced uterine atrophy and body weight increase, suggesting that it had a slight estrogen-like effect. Measurements of the length and weight of the tibia and femur showed no significant differences among the groups (Table 3). Thus, changes in the weight and length of bone could not be inferred 8 weeks after OVX surgery.

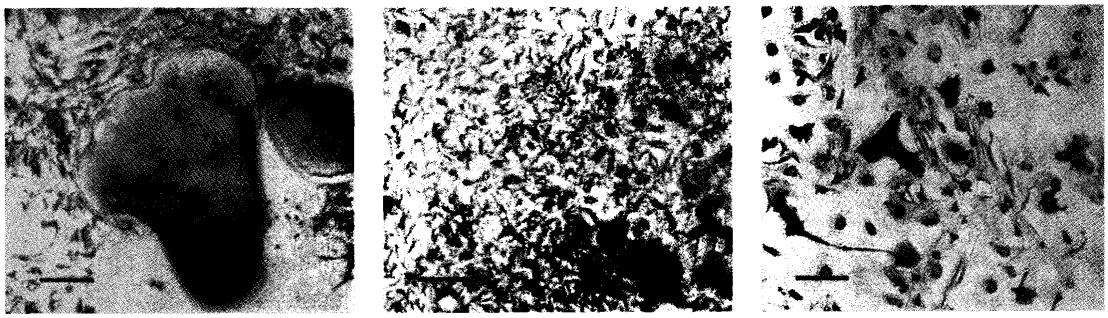
Densitometric and histomorphometric parameters in proximal tibiae, measured by pQCT, are shown in Table 4. At the end of the experiment, BMC and BMD of the total bone of OVX rats significantly decreased and the decrease in the BMD of cancellous bone was more than that in cortical bone. This is consistent with the findings of others, in which cancellous bone was found to be at a greater risk of fracture than cortical bone as a result of estrogen deficiency in women and female rats.[33,34] E_2 treatment significantly

Table 1. Chinese formulae used in this study

No.	Name	Composition
1	Pi-Shen-Sheng-Hua-Tang (脾腎生化湯) [9]	Radix Codonopsis (15 g), Rhizoma Atractylodis Macrocephalae (15 g), Radix Astragali (15 g), Poria (15 g), Rhizoma Dioscoreae (20 g), Pericarpium Citri Reticulatae (10 g), Radix Platycodi (6 g), Herba Dendrobii (10 g), Cortex Cinnamomi (6 g), Radix Polygoni Multiflori (15 g), Semen Arecae (6 g) Herba Epimedii (20 g), Fructus Psoraleae (10 g), Radix Glycyrrhizae (6 g), Fructus Jujubae (3 pieces), Fructus Hordei Germinatus (15 g)
2	Bu-Shen-Qiang-Gu-Tang (補腎強骨湯) [10]	Fossilia Dentis Mastodi (30 g), Concha Ostreae (30 g), Radix Dipsaci (12 g), Rhizoma Drynariae (12 g), Semen Cuscutae (9 g), Fructus Psoraleae (9 g), Colla Carapacis et Plastri Testudinis (9 g), Fructus corni (9 g), Fructus Lycii (9 g), Fructus Ligustri Lucidi (9 g), Rhizoma Dioscoreae (9 g), Poria (9 g)
3	Er-Xian-Jian-Gu-Tang (二僊堅骨湯) [11]	Pyritum (24 g), Fossilia Ossis Mastodi (24 g), Concha Ostreae (24 g), Radix Rehmanniae Preparata (24 g), Rhizoma Curculiginis (12 g), Herba Epimedii (12 g), Radix Angelicae Sinensis (15 g), Rhizoma Anemarrhenae (9 g), Radix Morindae Officinalis (9 g), Endothelium Corneum Gigeriae Galli (9 g), Cortex Phellodendri (6 g), Radix Astragali (30 g)
4	Bu-Shen-Jian-Gu -Tang (補腎堅骨湯) [12]	Radix Rehmanniae (10 g), Rhizoma Alismatis (10 g), Radix Morindae Officinalis (10 g), Herba Epimedii (10 g), Rhizoma Dioscoreae (15 g), Fructus Corni (15 g), Rhizoma Drynariae (15 g), Cortex Cinnamomi (3 g), Colla Cornus Cervi (6 g), Colla Carapacis et Plastri Testudinis (6 g)
5	Kang-Gu-Song-Pian (抗骨鬆片) [13]	Radix Rehmanniae Preparata (15 g), Rhizoma Dioscoreae (15 g), Poria (15 g), Fructus Corni (15 g), Cortex Moutan (10 g), Rhizoma Cibotii (10 g), Cortex Eucommiae (10 g), Radix Achyranthis Bidentatae (10 g), Fructus Psoraleae (10 g), Rhizoma Alismatis (6 g), Cortex Cinnamomi (6 g), Radix Glycyrrhizae (6 g), Membrana Follicularis Ovi (30 g)
6	Bu-Shen-Jian-Gu-Tang (補腎健骨湯) [12]	Radix Rehmanniae Preparata (20 g), Fructus Corni (10 g), Semen Cuscutae (10 g), Radix Achyranthis Bidentatae (10 g), Herba Epimedii (10 g), Herba Cistanches (10 g), Rhizoma Dioscoreae (15 g), Radix Salviae Miltiorrhizae (15 g), Fructus Lycii (8 g), Radix Notoginseng (3 g), Colla Cornus Cervi (10 g), Colla Carapacis et Plastri Testudinis (10 g)
7	Yi-Shen-Zhuang-Gu-Tang (益腎壮骨湯) [12]	Radix Rehmanniae Preparata (25 g), Caulis Spatholobi (25 g), Fructus Lycii (20 g), Rhizoma Cibotii (15 g), Cortex Eucommiae (25 g), Radix Dipsaci (20 g), Cortex Acanthopanacis (15 g), Radix Codonopsis (15 g), Rhizoma Drynariae (15 g), Radix Glycyrrhizae (10 g), Colla Cornus Cervi (10 g), Colla Carapacis et Plastri Testudinis (10 g)
8	Bu-Shen-Zhuang-Gu-Tang (補腎壮骨湯) [12]	Cortex Eucommiae (15 g), Radix Rehmanniae Preparata (15 g), Rhizoma Drynariae (12 g), Fructus Lycii (12 g), Herba Epimedii (12 g), Radix Codonopsis (12 g), Radix Glycyrrhizae (6 g), Fructus Corni (10 g), Radix Notoginseng (3 g)
9	Zuo-Gui-Wan (左帰丸) [14]	Radix Rehmanniae Preparata (24 g), Rhizoma Dioscoreae (12 g), Fructus Lycii (12 g), Fructus Corni (12 g), Radix Cyathulae (9 g), Semen Cuscutae (12 g), Colla Cornus Cervi (12 g), Colla Carapacis et Plastri Testudinis (12 g)
10	Kang-Gu-Song-Cong-Ji (抗骨鬆冲剤) [15]	Radix Rehmanniae Preparata (16 g), Herba Epimedii (16 g), Rhizoma Dioscoreae (8 g), Fructus Corni (8 g), Semen Cuscutae (8 g), Poria (6 g)
11	Lu-Jiao-Wan (鹿角丸) [16]	Cornu Cervi Pantotrichum (10 g), Radix Rehmanniae Preparata (20 g), Semen Cuscutae (10 g), Radix Polygalae (10 g), Herba Cistanches (10 g), Radix Aconiti Lateralis Preparata (10 g), Fructus Schisandrae (10 g), Cortex Eucommiae (10 g), Rhizoma Corydalis (10 g), Placenta Hominis (10 g), Radix Angelicae Sinensis (20 g), Radix Achyranthis Bidentatae (20 g)
12	Xian-Ling-Gu-Bao-Jiao-Nang (仙霊骨葆膠嚢) [17]	Herba Epimedii (14 g), Radix Dipsaci (14 g), Radix Salviae Miltiorrhizae (1 g), Rhizoma Anemarrhenae (1 g), Fructus Psoraleae (1 g), Radix Rehmanniae (1 g)
13	Bu-Shen-Zhuang-Gu-Wan (補腎壮骨丸) [18]	Fructus Ligustri Lucidi (15 g), Radix Rehmanniae Preparata (20 g), Semen Cuscutae (15 g), Cortex Eucommiae (15 g), Rhizoma Drynariae (12 g), Herba Epimedii (12 g), Rhizoma Polygonati (10 g)
14	Gu-Wei-Tang (骨痿湯) [12]	Radix Rehmanniae Preparata (25 g), Rhizoma Dioscoreae (20 g), Herba Epimedii (15 g), Radix Angelicae Sinensis (12 g), Rhizoma Chuanxiong (10 g), Pyritum (12 g), Fructus Corni (15 g), Lumbricus (8 g), Semen Cuscutae (12 g), Herba Pyrolae (20 g), Rhizoma Atractylodis Macrocephalae (12 g), Poria (10 g), Radix Codonopsis (12 g), Radix Glycyrrhizae (6 g), Cortex Cinnamomi (6 g), Cortex Eucommiae (12 g)
15	Gu-Bi-Tang (骨痺湯) [19]	Rhizoma Drynariae (15 g), Herba Pyrolae (15 g), Cornu Cervi Degelatinatum (15 g), Rhizoma Homalomenae (15 g), Fructus Psoraleae (15 g), Rhizoma Cibotii (30 g), Herba Cistanches (30 g), Radix Rehmanniae Preparata (30 g), Caulis Spatholobi (30 g), Radix Achyranthis Bidentatae (10 g)
16	Qing-E-Wan-Jia-Wei-Fang (青娥丸加味方) [20]	Cortex Eucommiae (12 g), Semen Juglandis (12 g), Fructus Psoraleae (12 g), Herba Epimedii (12 g), Radix Rehmanniae (12 g), Radix Achyranthis Bidentatae (12 g)

Table 1. (continued)

No.	Name	Composition
17	Sheng-Gu-Ling (生骨霊) [21]	Radix Astragali (30 g), Radix Dipsaci (15 g), Fructus Lycii (15 g), Rhizoma Drynariae (15 g), Radix Morindae Officinalis (15 g), Carapax Trionycis (9 g), Fructus Amomi (6 g), Fossilia Ossis Mastodi (30 g), Concha Ostreae (30 g), Radix Angelicae Sinensis (15 g), Radix Achyranthis Bidentatae (15 g), Semen Cuscutae (15 g), Plastrum Testudinis (12 g), Radix Glycyrrhizae (1 g)
18	You-Gui-Wan-Jia-Jian (右帰丸加減) [14]	Radix Rehmanniae Preparata (30 g), Fructus Lycii (15 g), Radix Dipsaci (9 g), Radix Aconiti Lateralis Preparata (3 g), Rhizoma Dioscoreae (15 g), Cortex Eucommiae (12 g), Cortex Cinnamomi (3 g), Fructus Corni (12 g), Fructus Psoraleae (15 g), Herba Cistanches (10 g), Radix Angelicae Sinensis(12 g), Herba Epimedii(12 g), Herba Taxilli (12 g), Eupolyphaga seu Steleophaga (6 g), Radix Glycyrrhizae (6 g), Carapax Trionycis (12 g)
19	Gui-Zhi-Fu-Zi-Jia-Zhu-Tang (桂枝附子加朮湯) [22]	Cortex Cinnamomi (20 g), Radix Aconiti Lateralis Preparata (3 pieces), Rhizoma Atractylodis Macrocephalae (20 g), Rhizoma Zingiberis Recens (15 g), Radix Glycyrrhizae (10 g), Fructus Jujubae (12 pieces)



a b c

Fig. 1. Bone marrow-derived multinucleated cells, stained for tartrate-resistant acid phosphatase (TRAP). PTH (24×10^{-9} M) group (a), elcatonin (2 U/ml) group (b), and Bu-Shen-Jian-Gu-Tang (F-6, 50 μg/ml) group (c). Bars = 100 μm.

Table 2. Inhibitory activity on osteoclast-like cell formation and bone resorption

No.	Name	Osteoclasts/well		^{45}Ca release (%)	
		50 μg/ml	500 μg/ml	50 μg/ml	500 μg/ml
F-1	Pi-Shen-Sheng-Hua-Tang (脾腎生化湯)	9.2 ± 2.7 **	1.0 ± 0.4 **	76.2 ± 11.4	69.4 ± 7.9
F-2	Bu-Shen-Qiang-Gu-Tang (補腎強骨湯)	23.6 ± 6.5 *	1.2 ± 0.4 **	77.8 ± 6.5	72.4 ± 2.9
F-3	Er-Xian-Jian-Gu-Tang (二僊堅骨湯)	18.2 ± 4.3 *	0.8 ± 0.4 **	77.1 ± 9.5	69.7 ± 5.5
F-4	Bu-Shen-Jian-Gu-Tang (補腎堅骨湯)	20.0 ± 2.6 *	0.6 ± 0.4 **	63.6 ± 7.5	75.8 ± 5.1
F-5	Kang-Gu-Song-Pian (抗骨鬆片)	24.0 ± 7.2 *	0.2 ± 0.2 **	71.8 ± 7.6	58.9 ± 4.7 *
F-6	Bu-Shen-Jian-Gu-Tang (補腎健骨湯)	12.2 ± 3.3 *	0.6 ± 0.2 **	77.5 ± 7.8	44.5 ± 9.4 **
F-7	Yi-Shen-Zhuang-Gu-Tang (益腎壮骨湯)	11.2 ± 3.5 **	0.4 ± 0.4 **	39.1 ± 10.3 **	76.4 ± 8.1
F-8	Bu-Shen-Zhuang-Gu-Tang (補腎壮骨湯)	9.4 ± 3.0 **	0.8 ± 0.6 **	64.7 ± 3.0 *	73.9 ± 9.1
F-9	Zuo-Gui-Wan (左帰丸)	12.6 ± 5.1 *	2.4 ± 1.9 **	66.0 ± 10.9	65.2 ± 16.0
F-10	Kang-Gu-Song-Cong-Ji (抗骨鬆冲剤)	20.8 ± 8.2 *	0.4 ± 0.2 **	75.2 ± 10.7	86.6 ± 7.6
F-11	Lu-Jiao-Wan (鹿角丸)	22.4 ± 8.5 *	1.0 ± 0.8 **	85.7 ± 6.7	61.7 ± 2.2 *
F-12	Xian-Ling-Gu-Bao-Jiao-Nang (仙霊骨葆膠嚢)	9.8 ± 2.7 **	0.0 ± 0.0 **	74.4 ± 9.3	54.1 ± 11.1 *
F-13	Bu-Shen-Zhuang-Gu-Wan (補腎壮骨丸)	38.6 ± 8.3	3.2 ± 1.0 **	74.1 ± 11.3	88.8 ± 4.9
F-14	Gu-Wei-Tang (骨痿湯)	14.6 ± 3.4 **	1.0 ± 0.4 **	86.6 ± 6.3	89.4 ± 7.6
F-15	Gu-Bi-Tang (骨痺湯)	5.0 ± 1.4 *	1.4 ± 0.7 **	75.3 ± 7.0	67.0 ± 5.6
F-16	Qing-E-Wan-Jia-Wei-Fang (青娥丸加味方)	19.6 ± 2.3 *	0.0 ± 0.0 **	74.7 ± 7.7	60.5 ± 5.8 *
F-17	Sheng-Gu-Ling (生骨霊)	38.6 ± 7.0	15.4 ± 4.1 **	82.3 ± 5.9	76.8 ± 8.6
F-18	You-Gui-Wan-Jia-Jian (右帰丸加減)	13.0 ± 1.4 *	0.2 ± 0.2 **	78.9 ± 6.6	76.4 ± 6.2
F-19	Gui-Zhi-Fu-Zi-Jia-Zhu-Tang (桂枝附子加朮湯)	22.8 ± 4.1 *	5.6 ± 1.6 **	76.6 ± 6.7	79.5 ± 9.1
	Elcatonin	18.2 ± 1.8 * (2 U/ml)		55.4 ± 12.0 * (4 U/ml)	
	PTH	64.6 ± 15.4 (24×10^{-9} M)		76.3 ± 8.8 (2×10^{-9} M)	

Inhibition on osteoclast-like cell formation: bone marrow cells were cultured with PTH (24×10^{-9} M); each value represents the mean ± S.E.M. (n = 5). Inhibition on bone resorption: bone was cultured with PTH (2×10^{-9} M); each value represents the mean ± S.D. (n = 4). Significant decrease compared to the PTH group, * $p < 0.05$, ** $p < 0.01$.

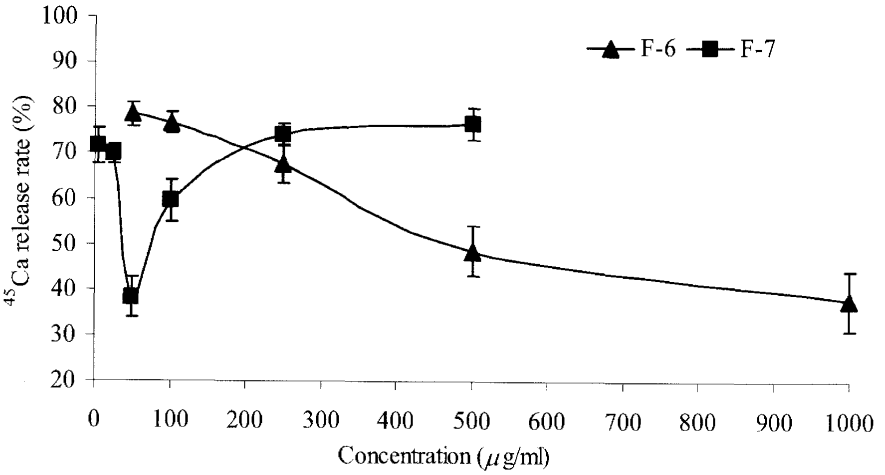


Fig. 2. Effects of Bu-Shen-Jian-Gu-Tang (F-6) and Yi-Shen-Zhuang-Gu-Tang (F-7) on ^{45}Ca release (%) from neonatal mouse parietal bone cultured for 6 days. Values are the mean±S.D. (n = 4).

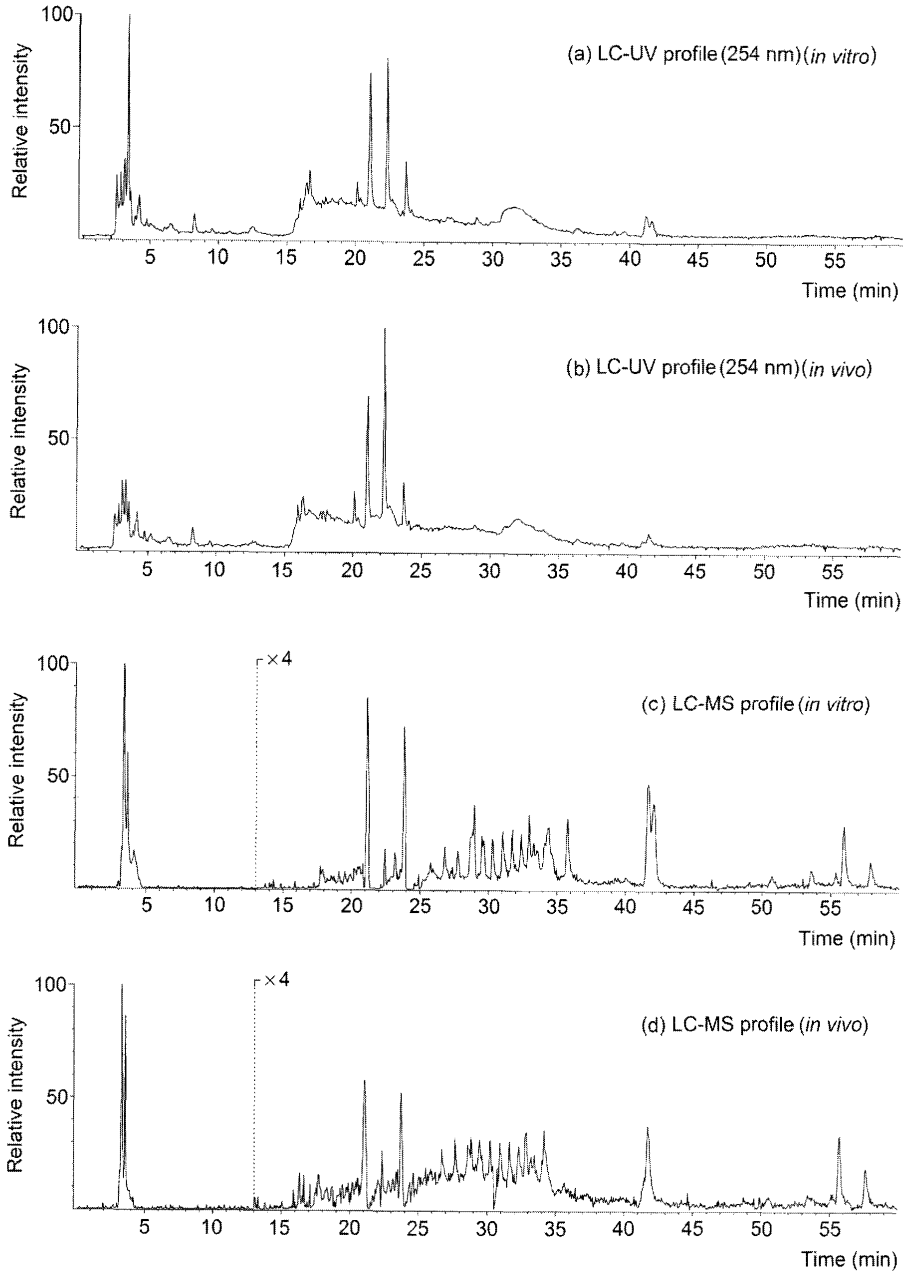


Fig. 3. LC-UV chromatogram at 254 nm of Bu-Shen-Jian-Gu-Tang used in the *in vitro* experiment (a) and *in vivo* experiment (b) and LC-MS chromatogram (4 multiples after 13 min) of Bu-Shen-Jian-Gu-Tang used in the *in vitro* experiment (c) and *in vivo* experiment (d).

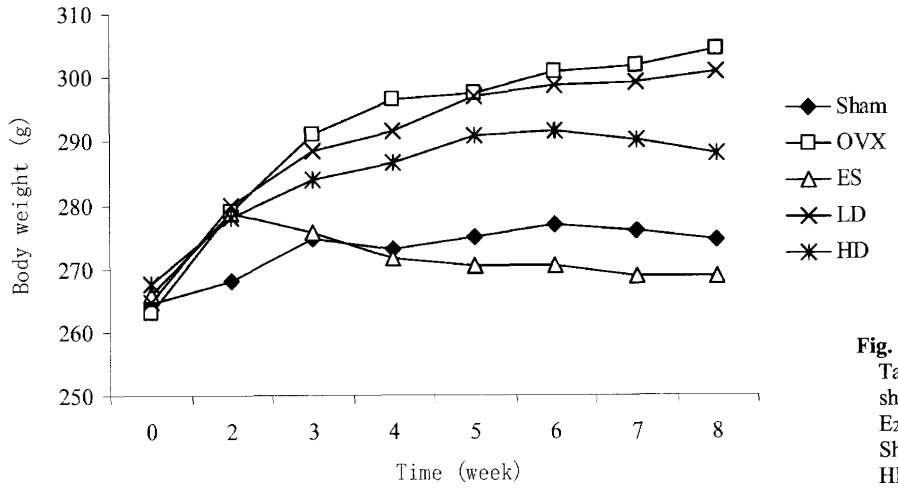


Fig. 4. Effects of ovariectomy, E_2, or Bu-Shen-Jian-Gu-Tang treatment on body weight gain of OVX rats. Sham: sham-operated group; OVX: ovariectomy control group; E_2: 17β-estradiol (0.1 mg/kg/d) treated group; LD: Bu-Shen-Jian-Gu-Tang (F-6, 100 mg/kg/d) treated group; HD: Bu-Shen-Jian-Gu-Tang (F-6, 500 mg/kg/d) treated group.

Table 3. Effects of Bu-Shen-Jian-Gu-Tang (F-6) and E_2 treatment on uterine weight, bone length and weight, and serum biochemical markers

Treatment	Uterine weight (g)	Bone length (cm)		Bone weight (g)		Serum biochemical markers	
		Tibia	Femur	Tibia	Femur	N-MID (ng/ml)	CTX (ng/ml)
Sham operation	0.504 ± 0.150	3.956 ± 0.056	3.617 ± 0.071	0.659 ± 0.053	0.831 ± 0.056	94.5 ± 19.1	27.9 ± 9.5
OVX	0.160 ± 0.055 ##	4.005 ± 0.177	3.659 ± 0.048	0.621 ± 0.068	0.777 ± 0.045	137.2 ± 21.3 #	44.1 ± 8.0 #
E_2 (0.1 mg/kg)	0.419 ± 0.155 *	3.923 ± 0.063	3.620 ± 0.064	0.632 ± 0.048	0.804 ± 0.027	78.5 ± 25.6 **	15.1 ± 10.5 **
F-6 (100 mg/kg)	0.249 ± 0.081 *	3.925 ± 0.092	3.665 ± 0.050	0.626 ± 0.044	0.779 ± 0.074	114.5 ± 26.3	21.2 ± 10.1 **
F-6 (500 mg/kg)	0.300 ± 0.137 *	3.937 ± 0.058	3.636 ± 0.031	0.629 ± 0.029	0.792 ± 0.026	113.8 ± 27.5	21.8 ± 7.7 **

Data represent the mean ± S.D. (n=7—10). # $p < 0.01$, ## $p < 0.001$ compared with the sham group. * $p < 0.01$, ** $p < 0.001$ compared with the OVX group.

Table 4. Effects of Bu-Shen-Jian-Gu-Tang (F-6) and E_2 on densitometric and geometric parameters of proximal tibiae

Parameters	Sham	OVX	E_2 (0.1 mg/kg)	LD (100 mg/kg)	HD (500 mg/kg)
Total bone					
BMC (mg/mm)	9.07 ± 0.56	6.96 ± 0.42 ###	8.36 ± 0.59 ***	7.50 ± 0.42 *	7.81 ± 0.54 **
BMD (mg/cm3)	780 ± 38	654 ± 46 ###	727 ± 30 **	653 ± 31	694 ± 44
Cancellous bone					
BMC (mg/mm)	0.52 ± 0. 08	0.48 ± 0.18	0.52 ± 0.11	0.59 ± 0.16	0.69 ± 0.11 *
BMD (mg/cm3)	177 ± 43	101 ± 45 #	130 ± 22	118 ± 43	152 ± 32 *
Cortical bone					
BMC (mg/mm)	7.34 ± 0.38	6.12 ± 0.49 ###	7.02 ± 0.31 **	6.41 ± 0.30	6.58 ± 0.32
BMD (mg/cm3)	1181 ± 15	1178 ± 16	1197 ± 14 **	1178 ± 14	1176 ± 10
Bone thickness (mm)	0.61 ± 0.04	0.53 ± 0.04##	0.58 ± 0.03 *	0.53 ± 0.02	0.55 ± 0.03
Endosteal circumference (mm)	8.23 ± 0.37	8.28 ± 0.48	8.39 ± 0.40	8.70 ± 0.30	8.42 ± 0.27
Periosteal circumference (mm)	12.08 ± 0.28	11.57 ± 0.41 #	12.01 ± 0.34 *	12.01 ± 0.29 *	11.89 ± 0.81
Bone strength index					
X-axis strength strain index (XSSI)	3.65 ± 0.16	2.84 ± 0.28 ###	3.42 ± 0.32 **	3.15 ± 0.28 *	3.14 ± 0.23
Y-axis strength strain index (YSSI)	3.93 ± 0.43	3.51 ± 0.45	4.18 ± 0.22 **	4.01 ± 0.15 *	4.00 ± 0.26 *
Polar-axis strength strain index (PSSI)	7.17 ± 0.58	5.87 ± 0.71 ##	6.84 ± 0.49 *	6.37 ± 0.50	6.64 ± 0.53 *

Data represent the mean ± S.D. of 7 to10 animals. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the sham group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the OVX group.

prevented the decrease in the BMC and BMD of total bone, BMC and BMD of cortical bone, and cortical bone thickness. Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) treatment prevented the decrease in the BMC of total bone (100 mg/kg, 7.8%; 500 mg/kg, 12.2% improvement, respectively, compared with that of the OVX control), and treatment with 500 mg/kg prevented the decrease in the BMC and BMD of cancellous bone (43.8% and 50.5% improvement, respectively, compared with those of the OVX control). The bone strength of rats in the OVX group was significantly reduced in XSSI and PSSI (22.2% and 18.1% decrease, respectively, compared with those of the sham group). Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) treatment improved bone strength in XSSI, YSSI, and PSSI (100 mg/kg, 10.9%, 14.2%, 8.5%; 500 mg/kg, 10.6%, 14.0%, 13.1% improvement, respectively, compared with those of the OVX group). These effects reflect its potent activity against bone fracture, the most serious symptom in postmenopausal osteoporosis.

Biochemical markers reflect the process involved in bone remodelling and are therefore useful in the assessment of osteoporotic risk and in monitoring the efficacy of treatment. Osteocalcin, a peptide of 49 amino acids, is produced by osteoblasts and has a high affinity for calcium.[35] Sixty—ninety % synthesized osteocalcin is incorporated into the bone matrix to form new bone, while the remainder is released into circulation and cleaved into two parts, a 45—49 *C*-terminus and a 1—43/44 *N*-terminus.[36] The latter peptide (N-MID fragment) is stable in serum and is a more sensitive marker of bone resorption than whole osteocalcin itself.[36] While Glu-Lys-Ala-His-βAsp-Gly-Gly-Arg (CTX fragment) is a specific epitope of eight amino acids located in the *C*-terminal telopeptide of CTX chain, is generated by normal osteoclastic degradation of bone, and is a biomarker of bone resorption.[37,38] The serum concentrations of N-MID and CTX fragments in OVX rats were increased by 45.2% and 58.1%, respectively, of those in the sham group (Table 3), indicating an increase in the bone turnover rate. The serum concentrations of both biomarkers in rats of the E_2 treatment group were decreased to below the sham control level, indicating that bone formation and bone resorption might have been inhibited. Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) slightly decreased the serum concentration of the N-MID fragment to 114.5±26.3 and 113.8±27.5 ng/ml at 100 and 500 mg/kg (OVX, 137.2±21.3 ng/ml), respectively, and significantly decreased the serum concentration of CTX to 21.2±10.1 and 21.8±7.7 ng/ml at 100 and 500 mg/kg (OVX, 44.1±8.0 ng/ml), respectively. Thus, the preventive effect of Bu-Shen-Jian-Gu-Tang (補腎健骨湯, F-6) on bone loss would be due to its inhibition of bone resorption, and not due to the stimulation of bone formation.

The formula Bu-Shen-Jian-Gu-Tang (補腎健骨湯) consisted of Radix Rehmanniae Preparata (Shu-Di-Huang, 熟地黄, 20 g), Fructus Corni (Shan-Yu-Rou, 山萸肉, 10 g), Semen Cuscutae (Tu-Si-Zi, 菟糸子, 10 g), Radix Achyranthis Bidentatae (Niu-Xi, 牛膝, 10 g), Herba Epimedii (Yin-Yang-Huo, 淫羊藿, 10 g), Herb Cistanchis (Rou-Cong-Rong, 肉蓯蓉, 10 g), Rhizoma Dioscoreae (Shan-Yao, 山薬, 15 g), Radix Salviae Miltiorrhizae (Dan-Shen, 丹参, 15 g), Fructus Lycii (Gou-Qi-Zi, 枸杞子, 8 g), Radix Notoginseng (Tian-Qi, 田七, 3 g), Deer Horn Glue (Lu-Jiao-Jiao, 鹿角膠, 10 g), and Tortoise Shell Glue (Gui-Ban-Jiao, 亀板膠, 10 g).[14] Ecdysterone, which is contained in Radix Achyranthis Bidentatae (Niu-Xi, 牛膝), was reported to promote the proliferation of osteoblast-like cells,[39] and tanshinone IIA isolated from Radix Salviae Miltiorrhizae (Dan-Shen, 丹参) was reported to inhibit osteoclast differentiation and bone resorption.[40] Rhizoma Dioscoreae (Shan-Yao, 山薬) prevented bone loss in a clinical study.[41] On the other hand, Herba Epimedii (Yin-Yang-Huo, 淫羊藿) had antiosteoporotic activity[42] and one constituent of Herba Epimedii, icariin, also identified in the water extract of Bu-Shen-Jian-Gu-Tang (補腎健骨湯), was reported to significantly promote osteoblast-like cell proliferation[43]. These features may be responsible for the antiosteoporotic activity of Bu-Shen-Jian-Gu-Tang (補腎健骨湯).

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Japanese abstract

本研究では19種の漢方方剤による *in vitro* での破骨細胞形成及び骨吸収の抑制活性を検討した。破骨細胞形成抑制活性はマウス骨髄細胞培養系を用い，副甲状腺ホルモン（PTH）存在下での破骨細胞様多核細胞の形成により評価した。骨吸収抑制活性は ^{45}Ca 標識した新生児マウスの頭頂骨での，PTH 誘導による ^{45}Ca 吸収を評価した。19種の漢方方剤のうち，9処方が50 μg/ml の濃度で強い破骨細胞形成抑制活性を示し，この中で，3処方は骨吸収を強く抑制した。中でも，「補腎健骨湯」は用量依存的に骨吸収を抑制し，最も有望な活性を示した。さらに，卵巣摘出（OVX）ラットを用いた *in vivo* での実験では，pQCT（末梢骨定量的コンピュータ一断層撮影法）での評価の結果，骨減少を予防する効果が認められた。

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