

# The Anti-osteoporotic Effect of Aqueous Extracts of *Gastrodiae Rhizoma* *In Vitro* and *In Vivo*

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The anti-osteoporotic effects of aqueous extracts of *Gastrodiae Rhizoma* (GR) were observed *in vitro* and *in vivo*. The effects on proliferation and alkaline phosphatase activity of primary osteoblasts, bone nodule formation, pit formation of osteoclasts and osteoclastogenesis were observed *in vitro*, and to observe the *in vivo* efficacy GR were orally administered once a day for 28 days to bilateral ovariectomy-induced osteoporosis mice at 125, 250 and 500 mg/kg. GR extracts enhanced the proliferation, differentiation, and bone nodule formation of primary cultured osteoblasts, but they inhibited the pit formation and the number of multinucleated osteoclast-like cells, osteoclastogenesis *in vitro*. As results of ovariectomy-induced osteoporotic process, dramatical decreases of bone weights and thickness of bone at epiphyseal regions, bone Ca and P contents, bone mineral density and failure load, serum Ca and P levels with increase of serum osteocalcin level. At histopathology-histomorphometry, dramatical decreases of trabecular and cortical bone masses were detected with classic histomorphometrical changes of bones including decrease of trabecular bone volume, thickness, length and number and cortical bone thickness and increase of osteoclast/bone perimeter. However, these estrogen-deficient osteoporotic changes were also dramatically and dose-dependently inhibited by treatment of all three different dosages of GR extracts. It was concluded that GR extracts has relatively good favorable effect to prevention and/or treatment of ovariectomy (OVX)-induced osteoporosis through osteoblast activation and inhibition of osteoclastogenesis and osteoclast activity.

**Key words** — *Gastrodiae Rhizoma*, osteoporosis, bone mineral density, ovariectomy, mice

## INTRODUCTION

Postmenopausal osteoporosis is the most frequent metabolic bone disease, it is characterized by a rapid loss of mineralized bone tissue, disruption of trabecular architecture of the bone and changes in the crystalline properties of mineral deposits, which result in the structural failure (fracture) of sites rich in cancellous bone, such as the vertebrae, hip and distal forearm.<sup>1,2)</sup>

It is widely recognized as a major public health problem, affecting both female and male. Most cases of osteoporosis occur in postmenopausal women due to the dramatic estrogen withdrawal as-

sociated with menopause. In America, it threatens an estimated 44 million people, or 55 percent of the people of 50 years of age and older.<sup>3)</sup>

Osteoblasts derived from mesenchymal cells synthesize and deposit bone matrix and increase bone mass.<sup>4)</sup> Therefore, the proliferation and activity of osteoclast cells have been regarded as a key on the bone formation. The efficacy of test articles on the osteoblast cells have been tested base on the cell proliferation and activity.<sup>5)</sup>

Osteoclasts originated from hematopoietic cells are the key participant in bone remodeling, because these cells are predominantly involved in bone resorption and lead to an imbalance in bone remodeling. The mature, multinucleated osteoclast-like cells (OCLs) maintain the proper actin cytoskeletal organization, ruffled boarder, and acidic condition during bone resorption.<sup>6)</sup> The efficacy of anti-osteoporotic agents therefore, has been continually

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evaluated on the inhibition of osteoclastogenesis and their activity like pit formation assay.<sup>5,6)</sup> In the rat, ovariectomy (OVX)-induced bone loss can be prevented by treatment with estradiol. For the similarities between rats and humans in their skeletal responses to estrogen deficiency, the mature OVX rat was considered as a good animal model for studying early postmenopausal bone loss.<sup>7)</sup>

The effects of a drug would be based on bone weight, bone mineral density (BMD), bone strength (failure load; FL), bone mineral contents and histomorphometrical changes of trabecular bone and thickness cortical bone in this model with serum biochemistry as osteocalcin, calcium (Ca) and inorganic phosphorus (P). In addition, the effects of various anti-osteoporotic agents have been evaluated using OVX mouse models.<sup>8,9)</sup>

The current anti-resorptive agents are extensively used, but there still remains a demand for a highly efficacious resorptive inhibitor with an excellent safety and efficacy profile. Anabolic agents that can stimulate bone formation are less known compared to anti-resorptive agents.<sup>10)</sup> Continuous trials to develop the anabolic agents have been accomplished with an advanced understanding of osteoblast differentiation and bone formation.

Many approaches have been tried searching for small natural extracts that exerted a therapeutic effect against bone loss. We primarily conducted random screening of a domestic natural product library and found *Gastrodia Rhizoma* (GR) a dried underground root-crust of *Gastrodia elata* (*G. elata*) Blume (Orchidaceae), with bioactivities in bone metabolism. GR is a traditional herbal agent that has been used as an anticonvulsant, analgesic, and sedative to treat general paralysis, epilepsy, vertigo, and tetanus in oriental countries for centuries.<sup>11,12)</sup>

Alendronate (brand name: Fosamax), a nitrogen-containing bisphosphonate, is a potent inhibitor of bone resorption used for the treatment and prevention of osteoporosis, and the anti-osteoporotic effect of alendronate in the OVX rodent models were already reported.<sup>13,14)</sup>

In the present study, we observed the *in vitro* [proliferation and alkaline phosphatase (ALP) activity of primary osteoblasts, bone nodule formation, pit formation of osteoclasts and osteoclastogenesis] and *in vivo* (OVX-induced osteoporosis mice) effects of GR extracts on osteoporosis as compared with alendronate, a well-known bisphosphonate anti-osteoporosis drug 10 mg/kg administered group.

## MATERIALS & METHODS

**Preparations and Administration of GR Extracts**—Aqueous GR extracts (yield: 41.32%) were prepared from the dried root-crust of *G. elata* that was commercially purchased in Seoul, Korea. Briefly, the dried roots (2 kg) were pulverized and extracted exhaustively with hot water (4000 ml) under reflux three times. The concentrated extract in aqueous suspension was lyophilized. Total 826.4 g of lyophilized GR water extracts were obtained, and used in this study.

### **In vitro assays**

*Reagents and Animals:* Minimal essential medium alpha modification ( $\alpha$ -MEM), fetal bovine serum (FBS), penicillin, streptomycin, and Trypsin-EDTA used for cell culture were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). For osteoblast study, ascorbic acid,  $\beta$ -glycerophosphate, and all reagents for ALP enzyme assay and Alizalin Red-S (AR-S) staining were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). For osteoclast study, 1,25(OH)<sub>2</sub>D<sub>3</sub>, dexamethasone, tartrate resistant acid phosphatase (TRAP) staining kit, and any other chemicals were purchased from Sigma-Aldrich. Newborn ICR mice were purchased from Biolinks Co. (Seoul, Korea) and 6 week-old ddY mice were purchased from SLC (Osaka, Japan).

*Primary Osteoblast Culture:* Primary osteoblastic cells were isolated from calvariae of newborn ICR mice by sequential digestion with 0.2% collagenase. Cells were grown to confluence in  $\alpha$ -MEM containing 10% FBS (growth medium). These cells were plated at a density of  $1 \times 10^5$  cell/ml in tissue culture dishes. After 24 hr, cells were changed with a differentiation medium containing ascorbic acid (50  $\mu$ g/ml) and  $\beta$ -glycerophosphate (10 mM) and the medium was changed every 3 days. At the desired days, cells were washed twice with phosphate buffered saline (PBS) and harvested.

*Cell Proliferation and ALP Activity in Primary Osteoblasts:* Cell proliferation was determined by counting the cell number at the end of each experiment. Cell counts were performed using a hemacytometer. For the measurement of ALP activity and total protein contents, cells were extracted into 0.05% Triton X-100 in PBS containing 1 mM MgCl<sub>2</sub> and centrifuged at  $1000 \times g$  for 5 min. Enzyme activity was determined in supernatants by colorimetric procedure using p-nitrophenyl phosphate (PNP) as a substrate at a wavelength of

**Table 1.** Changes on the Body Weight in the Present Study

Body weights	At OVX <sup>a)</sup>	1 wk after OVX	At Dosing <sup>a)</sup>	Wks after dosing			At Sacrifice <sup>a)</sup>
				1 wk	3 wks	4 wks	
Sham	25.50 ± 0.55	28.58 ± 1.11	25.18 ± 0.73	30.12 ± 1.13	31.54 ± 1.27	32.00 ± 0.95	29.64 ± 1.10
Control	25.50 ± 1.68	28.72 ± 1.67	25.90 ± 1.66	30.78 ± 1.78	32.56 ± 2.72	33.44 ± 3.00	31.18 ± 3.01
Alendronate	24.68 ± 1.33	27.76 ± 1.61	24.56 ± 1.23	30.22 ± 2.22	31.50 ± 1.74	32.10 ± 1.65	29.52 ± 1.37
GR extract							
125 mg/kg	25.28 ± 1.32	28.48 ± 0.83	25.78 ± 0.86	30.00 ± 2.35	31.10 ± 1.66	33.16 ± 2.23	30.58 ± 2.04
250 mg/kg	25.44 ± 0.68	28.20 ± 1.14	26.04 ± 1.35	30.54 ± 0.82	31.98 ± 0.86	33.36 ± 0.64	31.02 ± 1.11
500 mg/kg	25.24 ± 1.21	28.00 ± 2.20	25.70 ± 1.46	30.02 ± 1.98	31.96 ± 2.60	33.90 ± 2.52	31.66 ± 2.99

Values are expressed as mean ± S.D. of five mice, g; a) overnight fasted.

405 nm and the protein content was determined by a Bio-Rad protein assay kit. The ALP was expressed as nmol PNP/μg protein per min.

**Mineralized Nodule Formation:** The bone nodule formation was determined by measuring the amount of mineral deposited into bone matrix using AR-S staining. After the confluent cells were grown in the above culture conditions for 14 days with or without GR extract, cells were fixed with 10% formalin solution. For quantitative measurement, nodules stained with AR-S were destained with incubation in 10% cetylpyridinium chloride solution for 30 min. The quantity of eluted AR-S was determined by measuring the absorbance at 562 nm.

**Preparation of Osteoclast-like Cells and Osteoclast Formation Assay:** OCLs were developed on collagen gel plates by co-culture between bone marrow cells ( $2.5 \times 10^5$  cell/cm<sup>2</sup>) and calvaria-derived osteoblastic cells ( $4 \times 10^4$  cell/cm<sup>2</sup>) in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> ( $10^{-8}$  M) and dexamethasone ( $10^{-7}$  M) with α-MEM containing 10% FBS. Bone marrow cells were isolated from the femora of 6-week-old ddY mice and osteoblastic cells were from calvariae of newborn ICR mice. The medium was changed every two days with fresh osteoclastic medium. On day 6–8 of the co-culture, multinucleated OCLs were detached from plates by 0.2% collagenase treatment (crude OCLs) and then replated for desired studies using OCLs. An index of osteoclast formation in the culture was determined by counting TRAP-positive multinucleated osteoclasts [TRAP(+) multinucleated cells (MNCs)]. To investigate the effect of GR extract on osteoclast differentiation, the co-culture was conducted in 96-well plates with or without GR extract as described above. On day 6, the cells were fixed with 10% formaldehyde and stained for TRAP. The number of TRAP(+) MNCs containing more than 6–7 nuclei was counted under a microscope (Leica; Berlin,

Germany).

**Osteoclast Bone Resorption (Pit Formation) Assay:** Crude OCLs suspended in α-MEM containing 10% FBS were replaced on a dentine slice (4 mm diameter) in a 96-well culture plate. After 2 hr of preincubation, the dentine slice was further transferred to wells of 48-well plates containing GR extract and vehicle and incubated for 24 hr more. At the end of incubation, cells were removed by 1 M NH<sub>4</sub>OH treatment and resorption pits formed on the slices were stained with Mayer's hematoxylin. Resorption pits were enumerated with an image analyzer and compared to the control group.

#### OVX Induced Osteoporosis

**Animals and Husbandry:** Sixty female ddY mice (6-wk old upon receipt, SLC) were used after acclimatization for 12 days. Animals were allocated 5 per polycarbonate cage in a temperature (20–25°C) and humidity (40–45%) controlled room. Light: dark cycle was 12 hr: 12 hr and feed (Samyang; Seoul, Korea) and water were supplied free to access. Fifty mice were bilateral OVX and remainder ten mice were sham operated. For recovery of animals, treatment was initiated 1 week after OVX and then each sample was administered for 4 weeks. About half of healthy animals were selected at 1 week after OVX based on the body weight and gross inspection of their behavior according to the Guide for the Care and Use of Laboratory Animals by Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, U.S.A. on 1996, and Washington D.C.

**Administration of Test Articles:** GR extract was suspended in distilled water and dosed by oral gavage using a sonde attached to 3 ml syringes containing test article at dosage 125, 250 or 500 mg/kg (of body weight) from 7 days after OVX for 4 weeks. In addition, alendronate was orally dosed at 10 mg/kg. The administered dose and schedule of these drugs

were showed as Table 1.

**OVX:** Bilateral OVX was conducted under Ketamine hydrochloride (ICN Biochemicals Inc.; Irvine, CA, U.S.A.) and Xylazine hydrochloride (Wako; Osaka, Japan) anesthesia in all OVX groups. In Sham, bilateral ovaries were exposed and then closed by skin suture not removed.

**Body and Bone Weight Changes:** Changes of body weight and its gains were calculated at operation, 1 week after OVX, at dosing and once a week during experimental periods with at sacrifice. At dosing and sacrifice day, experimental animals were overnight fasted (water was not; about 18 hr) to reduce the erratum of feeding. In addition, Body weight gains during induction period (Gains I) and dosing (Gains II) were calculated as following equation. At sacrifice, the weight of bilateral side of femur and tibia was calculated at g levels and to reduce the erratum rose from individual body weight differences, the relative weight (%) was calculated using body weight at sacrifice and absolute weight as following equation.

**Bone Thickness/Length Measurement:** After weighing of bones, the thickness at epiphyseal regions and longitudinal length of right side of femur and tibia was calculated at mm levels using electronic digital caliper.

**Bone Mineral Content Measurement:** At sacrifice, the right side of tibia was harvest and dried at 120°C for 8 hr. Dried tibiae were carbonized at 800°C for 6 hr in furnace and dissolved in nitric acid. In diluted solution, Ca and P contents were calculated as mg/g bone using orthocresolphthalein complexon and enzyme methods, respectively. In addition, Ca/P ratio was calculated as following equation.

**Serum Biochemistry:** At sacrifice, 1 ml of venous blood was collected from vena cava. All blood samples were centrifuged at 3000 rpm  $\times$  g for 15 min under room temperature using clotting activated serum tube. Serum osteocalcin levels were detected with an automated gamma counter (HE model, ICN Biochemicals Inc) as ng/ml using RIA kit. Serum Ca levels were detected by o-cresolphthalein complexone (OCPC) methods using Ca strips (Wako) and automated blood analyzer (Toshiba 200FR, Toshiba; Tokyo, Japan) as mg/ml levels. Serum P levels were detected by Kinetic UV methods using P strips (Wako; Osaka, Japan) and automated blood analyzer as mg/ml levels.

**BMD and FL:** BMD of the epiphyseal regions of left side femur was detected by dual-energy x-ray

absorptionmetry (DEXA), Lunar (Madison, WI, U.S.A.) as mg/cm<sup>2</sup>. In addition, bone strength was detected as FL. FL of mid-shaft regions of left side tibia was detected by a three-point bending test to failure using computerized testing machine (Instron 6022, Instron; Norwood, MA, U.S.A., speed 20 mm/min) as N (Newton).

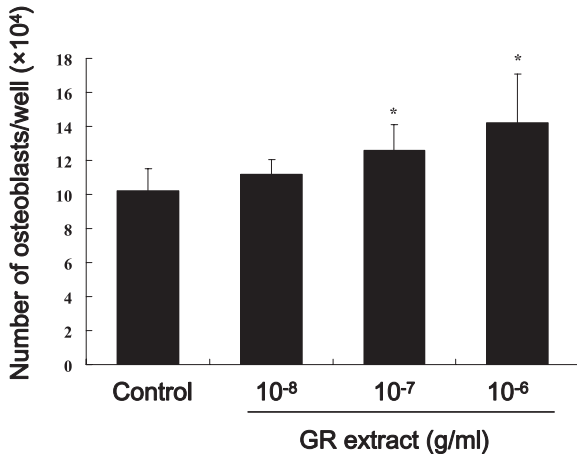
**Histological Procedures:** The right side of femur of each rat were separated and fixed in 10% neutral buffered formalin (NBF), then decalcified in decalcifying solution (24.4% formic acid, and 0.5 N sodium hydroxide) for 5 days (mixed decalcifying solution was exchanges once a day for 5 days). After that, embedded in paraffin, sectioned (3–4  $\mu$ m) and stained with Hematoxylin & Eosin (H&E) stain.

**Histomorphometry:** In each prepared histological samples of right side of femur, trabecular bone volume (TBV), thickness of trabecular bone (Tbt), trabecular bone number (Tbn) and length of trabecular bone (Tbl), cortical bone thickness (Cbt) and osteoclast cell number (Ocn) were measured using automated image analysis (analySIS Image Processing, SIS; Berlin, Germany) under magnification 100 of microscopy in the uniform area of right femur (growth plate regions were excluded). TBV was calculated as percentage (%) levels. Tbt and Tbl were calculated as  $\mu$ m levels, and Tbn as number/whole cross sectional part of epiphyseal regions. Tbt and Tbl were calculated in the most well developed trabecular in uniform area of trochlea epiphyseal regions. Cbt was detected in trochlea neck regions of femur as  $\mu$ m levels. Ocn/bone parameter was calculated as seen in 200  $\mu$ m<sup>2</sup> of trochlea cross sectioned epiphyseal regions as number/cross (of 200  $\mu$ m<sup>2</sup> of epiphyseal regions levels).

**Statistical Analyses:** All data was calculated as mean  $\pm$  S.D. Statistical analyses was conducted using Mann-Whitney U-Wilcoxon Rank Sum W test (MW test) with SPSS for Windows (Release 6.1.3., SPSS Inc.; Chicago, IL, U.S.A.). Values of  $p < 0.05$  were regarded as significant.

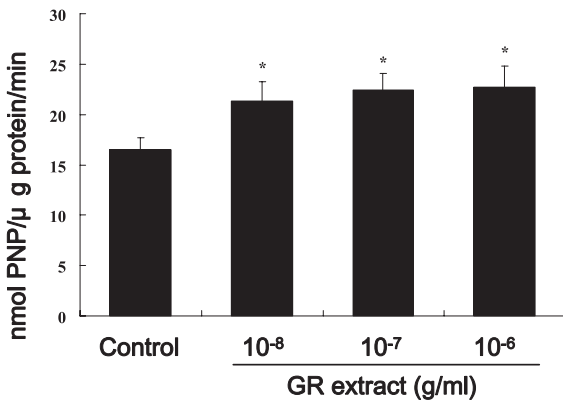
## RESULTS

The effects on the osteoblast were monitored using proliferation and alkaline phosphatase activity of primary osteoblasts *in vitro*. Significant ( $p < 0.05$ ) increases of number of primary cultured osteoblasts were dose-dependently observed in all GR extracts treated groups compared to that of vehicle control except for 10<sup>-8</sup> g/ml treated group



**Fig. 1.** GR Extracts Increases the Proliferation of Primary Osteoblast Cells

Primary osteoblast cells were cultured in growth medium containing the vehicle (water) and different concentrations of GR extracts for 3 days. All data are represented as the mean  $\pm$  S.D. of five independent experiments. \* $p < 0.05$  compared to that of control by MW test.

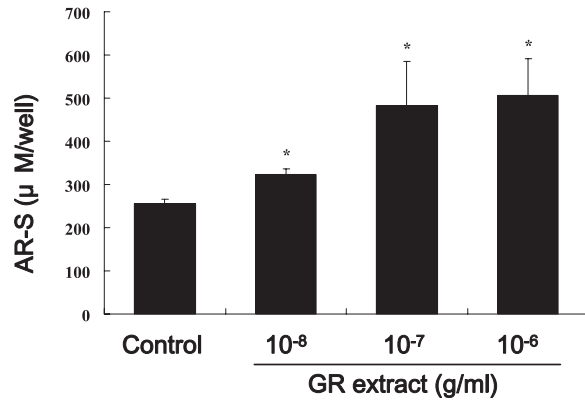


**Fig. 2.** GR Extracts Increased the ALP Activity of Primary Osteoblast Cells

Dose-response effect on ALP activity. The primary osteoblast cells were treated for 3 days with graded doses of GR extracts in differentiation medium. All data are represented as the mean  $\pm$  S.D. of five independent experiments. \* $p < 0.01$  compared to that of control by MW test.

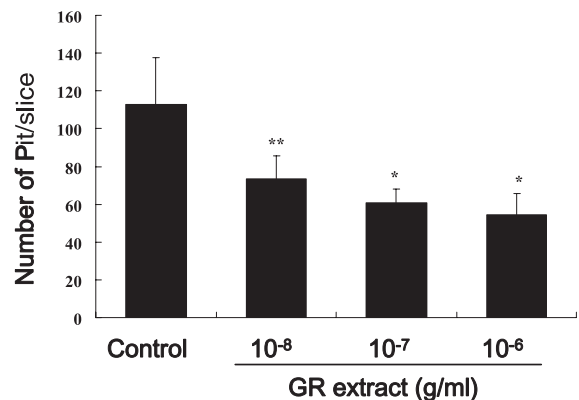
in which non-significantly increases were also detected (Fig. 1). In addition, ALP activity of osteoblasts were dose-dependently increased by treatment of GR extracts as compared with control (Fig. 2).

The effects on the osteoclast and osteoclastogenesis were observed as bone nodule formation and pit formation assays *in vitro*. Significant ( $p < 0.01$ ) increases of bone nodule formation by osteoblasts were dose-dependently observed in all GR extracts treated groups compared to that of vehicle control (Fig. 3), and pit formation on the dentin slices by



**Fig. 3.** GR Extracts Stimulated the Formation of Mineralized Bone Nodules

Primary osteoblast cells were plated in 12-well plates at a density of  $1 \times 10^5$  cell/well, cultured until confluence, and then treated with GR extracts for 14 days in differentiation medium. The bound AR-S was eluted from the matrix with 10% cetylpyridinium chloride solution and quantified by measuring the absorbance at 562 nm. All data are represented as the mean  $\pm$  S.D. of five independent experiments. \* $p < 0.01$  compared to that of control by MW test.



**Fig. 4.** GR Extracts Inhibits Osteoclastic Bone Resorption

OCLs obtained from co-cultured cells were plated on dentine slices and cultured in the presence of the indicated concentration of GR extracts for 24 hr. After removing the cells by 1 M  $\text{NH}_4\text{OH}$  treatment, resorption pits on dentine slices were visualized by staining with hematoxylin. Number of resorption pit was measured under microscope. All data are represented as the mean  $\pm$  S.D. of five independent experiments. \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of control by MW test.

osteoclasts were dose-dependently and significantly ( $p < 0.01$  or  $p < 0.05$ ) decreases by treatment of GR extracts as compared with (Fig. 4). In addition, the number of OCLs were dose-dependently and significantly ( $p < 0.05$ ) decreased in the all treated of GR extracts compared to that of vehicle control except for  $10^{-8}$  g/ml treated group in which non-significantly decreases were also detected (Fig. 5).

*In vivo* efficacy GR extracts were observed on the OVX mice. The body weight change, bone weight, bone length/thickness, bone Ca and P con-

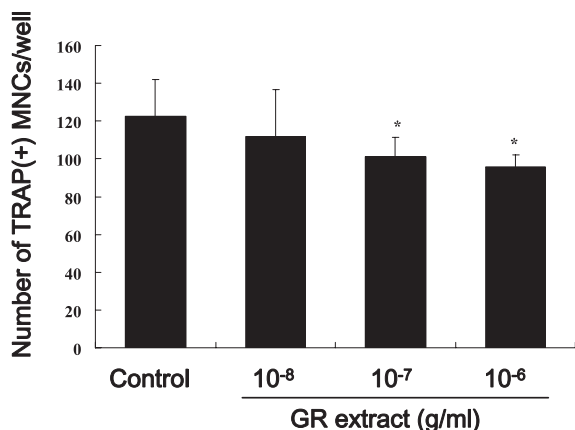
tents, BMD, bone strength, histological profiles and histomorphometrical analyses at sacrifice were conducted with serum osteocalcin, Ca and P levels.

In vehicle control, slight but non-significant increases of body weight and gains were detected as compared with non-OVX Sham. No meaningful changes on the body weight and gains in all dosing groups tested in the present study were detected as compared with vehicle control (Tables 1 and 2). Significantly ( $p < 0.05$ ) decrease of absolute and relative bone weight on the femur and tibia were demonstrated in vehicle control compared to that of Sham. Significant ( $p < 0.05$ ) increases of femur relative weight and absolute weight of tibia were observed in alendronate-dosing group. Although clear dose-dependency was not observed, significantly ( $p < 0.01$  or  $p < 0.05$ ) increases of bone weights were also detected in GR extracts-dosing

groups compared to that of vehicle control, respectively (Table 3).

Significantly ( $p < 0.05$ ) decrease of thickness of the epiphyseal regions of the femur and tibia were demonstrated in vehicle control compared to that of Sham. Significant ( $p < 0.01$  or  $p < 0.05$ ) increases of thickness in both femur and tibia were observed in all dosing group tested except for GR extracts 125 mg/kg-dosing group in which non-significantly increases of tibia thickness were also detected and the femur thickness was significantly ( $p < 0.05$ ) increased (Table 4). However, no meaningful changes on the longitudinal length of the femur and tibia were demonstrated including vehicle control (Table 4).

Significantly ( $p < 0.01$ ) decreases of tibia Ca and P contents were detected in vehicle control compared to that of Sham. However, significant ( $p < 0.01$  or  $p < 0.05$ ) increases of bone mineral contents were observed in all dosing group tested (Table 5). Bu no meaningful changes on the Ca/P ratio were demonstrated including vehicle control



**Fig. 5.** GR Extracts Inhibits Osteoclastogenesis

Mouse bone marrow cells and primary osteoblasts were cocultured in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> and dexamethasone with GR extracts for 6 days. Cells were fixed and stained for TRAP. The number of TRAP(+) MNCs containing six or more nuclei were counted. All data are represented as the mean ± S.D. of five independent experiments. \* $p < 0.05$  compared to that of control by MW test.

**Table 2.** Changes on the Body Weight Gains in the Present Study

Group	Body weight gains during	
	Recovery periods	Dosing periods
Sham	3.08 ± 0.78	4.46 ± 0.70
Control	3.33 ± 0.58	5.28 ± 1.62
Alendronate	3.08 ± 1.03	4.96 ± 0.79
GR extract		
125 mg/kg	3.20 ± 1.38	4.80 ± 1.91
250 mg/kg	2.76 ± 0.91	4.98 ± 2.13
500 mg/kg	2.76 ± 1.28	5.96 ± 2.70

Values are expressed as mean ± S.D. of five mice, g; Gains during recovery periods = (Body weight at 1 week after operation - Body weight at operation); Gains during dosing periods = (Body weight at sacrifice - Body weight at dosing).

**Table 3.** Changes of Bone Weights after OVX and GR Extract Administration

Bone weight	Absolute bone weights (g)		Relative bone weights (% vs. body weight)	
	Femur	Tibia	Femur	Tibia
Sham	0.079 ± 0.005	0.058 ± 0.004	0.266 ± 0.019	0.196 ± 0.013
Control	0.069 ± 0.006*	0.050 ± 0.004*	0.221 ± 0.024*	0.161 ± 0.025*
Alendronate	0.071 ± 0.004*	0.054 ± 0.002* <sup>##</sup>	0.241 ± 0.015* <sup>##</sup>	0.183 ± 0.009**
GR extract				
125 mg/kg	0.075 ± 0.006 <sup>##</sup>	0.056 ± 0.003 <sup>#</sup>	0.248 ± 0.028 <sup>##</sup>	0.185 ± 0.015 <sup>##</sup>
250 mg/kg	0.076 ± 0.006 <sup>##</sup>	0.058 ± 0.004 <sup>#</sup>	0.245 ± 0.017* <sup>##</sup>	0.186 ± 0.015 <sup>##</sup>
500 mg/kg	0.078 ± 0.009 <sup>##</sup>	0.059 ± 0.004 <sup>#</sup>	0.247 ± 0.019* <sup>##</sup>	0.187 ± 0.019 <sup>##</sup>

Values are expressed as mean ± S.D. of five mice; \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of Sham by MW test; <sup>#</sup> $p < 0.01$  and <sup>##</sup> $p < 0.05$  compared to that of vehicle control by MW test.

**Table 4.** Changes of Bone Thickness/Lengths after OVX and GR Extract Administration

Bone measurement	Bone thickness (mm)		Bone lengths (mm)	
	Femur	Tibia	Femur	Tibia
Sham	1.85 ± 0.12	2.00 ± 0.04	16.50 ± 0.51	19.06 ± 0.30
Control	1.49 ± 0.07*	1.89 ± 0.06**	16.38 ± 0.49	18.96 ± 0.32
Alendronate	1.74 ± 0.16 <sup>##</sup>	1.99 ± 0.06 <sup>##</sup>	16.66 ± 0.42	19.07 ± 0.44
GR extract				
125 mg/kg	1.65 ± 0.05 <sup>*,#</sup>	1.97 ± 0.04	16.61 ± 0.43	19.17 ± 0.35
250 mg/kg	1.67 ± 0.09 <sup>*,#</sup>	2.03 ± 0.09 <sup>##</sup>	16.55 ± 0.55	19.02 ± 0.43
500 mg/kg	1.73 ± 0.09 <sup>#</sup>	2.04 ± 0.08 <sup>##</sup>	16.64 ± 0.85	19.32 ± 0.38

Values are expressed as mean ± S.D. of five mice; \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of Sham by MW test; # $p < 0.01$  and <sup>##</sup> $p < 0.05$  compared to that of vehicle control by MW test.

**Table 5.** Changes on Bone Mineral Contents after OVX and GR Extract Administration

Bone contents	Ca levels (mg/g bone)	P levels (mg/g bone)	Ca/P ratio <sup>a)</sup>
Sham	59.64 ± 2.49	40.89 ± 1.90	146.18 ± 10.64
Control	37.41 ± 5.53*	22.36 ± 1.80*	167.81 ± 25.46
Alendronate	49.76 ± 6.57 <sup>*,#</sup>	31.86 ± 6.74 <sup>*,#</sup>	159.32 ± 22.55
GR extract			
125 mg/kg	47.32 ± 5.24 <sup>*,##</sup>	27.06 ± 4.13 <sup>*,##</sup>	179.71 ± 42.30
250 mg/kg	49.40 ± 2.45 <sup>*,#</sup>	28.74 ± 2.53 <sup>*,#</sup>	172.86 ± 16.43
500 mg/kg	49.96 ± 6.94 <sup>*,##</sup>	32.36 ± 3.58 <sup>*,#</sup>	155.65 ± 26.30

Values are expressed as mean ± S.D. of five mice; a) Ca/P ratio (%) = (Tibia Ca contents/Tibia P contents) × 100; \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of Sham by MW test; # $p < 0.05$  compared to that of vehicle control by MW test.

**Table 6.** Changes on Serum Biochemistry after OVX and GR Extract Administration

Serum contents	Osteocalcin levels (ng/ml)	Ca levels (mg/dl)	P levels (mg/dl)
Sham	20.68 ± 1.33	11.20 ± 0.65	11.82 ± 1.81
Control	33.07 ± 3.31*	8.66 ± 0.64*	7.34 ± 0.98*
Alendronate	25.41 ± 2.86 <sup>*,#</sup>	9.60 ± 0.28 <sup>*,##</sup>	9.08 ± 0.23 <sup>*,#</sup>
GR extract			
125 mg/kg	29.15 ± 0.93 <sup>*,##</sup>	9.58 ± 0.44 <sup>*,##</sup>	8.96 ± 0.72 <sup>*,##</sup>
250 mg/kg	27.28 ± 4.44 <sup>##</sup>	9.98 ± 0.23 <sup>*,#</sup>	9.32 ± 0.88 <sup>*,##</sup>
500 mg/kg	27.36 ± 1.63 <sup>*,#</sup>	10.16 ± 0.80 <sup>*,#</sup>	9.56 ± 0.93 <sup>*,#</sup>

Values are expressed as mean ± S.D. of five mice; \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of Sham by MW test; # $p < 0.01$  and <sup>##</sup> $p < 0.05$  compared to that of vehicle control by MW test.

(Table 5). Significantly ( $p < 0.01$ ) increase of serum osteocalcin level with was detected in vehicle control compared to that of Sham with significantly ( $p < 0.01$ ) decreases of serum Ca and P levels, respectively. However, significantly ( $p < 0.01$  or  $p < 0.05$ ) increases of serum Ca and P levels, and decreased in serum osteocalcin levels were detected in all dosing groups (Table 6).

Significantly ( $p < 0.01$ ) decrease of the femur BMD and FL were detected in vehicle control compared to that of Sham. However, significantly ( $p < 0.05$ ) increases of the femur BMD and FL were observed in all dosing group tested compared to that of vehicle control (Table 7).

**Table 7.** Changes on the BMD of Femur and FL of Tibia after OVX and GR Extract Administration

BMD/FL	BMD (mg/cm <sup>2</sup> )	FL (N)
Sham	0.68 ± 0.03	1.71 ± 0.16
Control	0.41 ± 0.03*	0.97 ± 0.10*
Alendronate	0.52 ± 0.07 <sup>*,##</sup>	1.29 ± 0.15 <sup>*,##</sup>
GR extract		
125 mg/kg	0.45 ± 0.03 <sup>*,##</sup>	1.15 ± 0.12 <sup>*,##</sup>
250 mg/kg	0.48 ± 0.03 <sup>*,##</sup>	1.20 ± 0.12 <sup>*,#</sup>
500 mg/kg	0.50 ± 0.03 <sup>*,##</sup>	1.22 ± 0.07 <sup>*,#</sup>

Values are expressed as mean ± S.D. of five mice; \* $p < 0.01$  compared to that of Sham by MW test; # $p < 0.01$  and <sup>##</sup> $p < 0.05$  compared to that of vehicle control by MW test.

**Table 8.** Histomorphometrical Changes of Trabecular and Cortical Bone at Trochlea Epiphyseal Regions of Right Femur with Osteoclast Cell Numbers after OVX and GR Extract Administration

Femur	Sham		Control		Alendronate		GR extract					
							125 mg/kg		250 mg/kg		500 mg/kg	
TBV <sup>a)</sup>	42.06 ± 6.91	21.03 ± 2.78*	48.20 ± 7.84 <sup>#</sup>	26.40 ± 2.87 <sup>*,##</sup>	29.88 ± 3.04 <sup>*,#</sup>	31.74 ± 3.97 <sup>*,#</sup>						
Tbt <sup>b)</sup>	93.22 ± 13.42	42.23 ± 8.98*	44.45 ± 13.27*	53.24 ± 16.67*	53.16 ± 12.61*	48.29 ± 8.47*						
Tbn <sup>c)</sup>	11.80 ± 0.84	7.20 ± 1.48*	16.40 ± 2.97 <sup>*,#</sup>	9.60 ± 1.52 <sup>*,##</sup>	10.60 ± 2.30 <sup>##</sup>	11.40 ± 1.14 <sup>#</sup>						
Tbl <sup>d)</sup>	826.64 ± 106.33	422.67 ± 49.63*	636.95 ± 143.28 <sup>*,#</sup>	571.33 ± 104.00 <sup>*,##</sup>	577.95 ± 118.43 <sup>*,##</sup>	594.81 ± 105.50 <sup>*,##</sup>						
Cbt <sup>e)</sup>	196.39 ± 10.52	77.51 ± 17.37*	82.52 ± 14.62*	99.33 ± 14.55 <sup>*,##</sup>	106.89 ± 7.42 <sup>*,##</sup>	111.97 ± 13.92 <sup>*,#</sup>						
Ocn <sup>f)</sup>	4.40 ± 1.82	13.80 ± 2.49*	21.60 ± 3.05 <sup>*,##</sup>	13.20 ± 2.68*	15.00 ± 1.58*	13.20 ± 2.86*						

Values are expressed as mean ± S.D. of five mice; a) TBV, trabecular bone volume (Unit, %); b) Tbt, trabecular bone thickness (Unit, μm); c) Tbn, trabecular bone number (Unit, number/epiphyseal); d) Tbl, trabecular bone length (Unit, μm); e) Cbt, cortical bone thickness (Unit, μm); f) Ocn, osteoclast cell number (Unit, number/epiphyseal); All histomorphometrical indices were calculated using automated image analyzer; \* $p < 0.01$  and \*\* $p < 0.05$  compared to that of Sham by MW test; # $p < 0.01$  and ## $p < 0.05$  compared to that of vehicle control by MW test.

In vehicle control, dramatically decreases of trabecular bone mass and the bone mass cortical bone area results from the increases of connective tissues, a classic histopathological change of osteoporotic processes were detected. In addition, the TBV, Tbt, Tbl, Tbn and Cbt were histomorphometrically significantly ( $p < 0.01$ ) decreased with significantly ( $p < 0.01$ ) increase of Ocn in the epiphyseal regions (Table 8). In alendronate-dosing group, the decreases on the trabecular bone mass were dramatically inhibited but the changes on the cortical bone mass were not changed, and significantly ( $p < 0.01$ ) increases of TBV, Tbn and Tbl were detected compared to that of vehicle control but they also significantly increased the Ocn not inhibited. In GR extracts-dosing groups, the decreases of trabecular bone mass were dramatically and dose-dependently inhibited and the changes on the cortical bone mass were also inhibited, and dramatically and dose-dependent increases of the TBV, Tbn, Tbl, Tbt and Cbt were detected compared to that of vehicle control. However, the Ocn in all GR extracts-dosing groups was quite similar to that of vehicle control (Table 8).

## DISCUSSION

Bone remodeling by bone cells plays a very important role in determining and increasing bone mass under the pathological condition such as bone disorder.<sup>15)</sup> In the search for the development of anti-osteoporosis agents, we found that GR extracts, a dried root-crude water extracts of *G. elata* enhance the osteoblast proliferation, differentiation and activity, and they inhibit the osteoclastogenesis and activity of osteoclasts. In addition, GR ex-

tracts showed favorable effects on prevention and/or treatment of OVX-induced osteoporotic mice in the present study.

We used the culture system of primary osteoblasts isolated from the calvariae of newborn mice due to the usefulness for analysis of the differentiation process of osteoblasts.<sup>16)</sup> GR extracts enhanced the ALP activity and bone-forming activity of primary osteoblasts with their proliferation. These results showed that GR extracts stimulated early osteoblast differentiation, which may consequently influence the bone-forming activity by accelerating the differentiation process because ALP activity was increased as osteoblast differentiation and their activity.<sup>17,18)</sup> Considering that matrix mineralization is the final fate of the osteoblast differentiation process to complete this bone-forming function and to increase the bone density, GR extracts might be a positive agent boosting the bone-forming activity of osteoblasts. In addition, the current studies on osteoclasts revealed that GR extracts significantly inhibited the main function/activation of osteoclasts, bone resorption. The regulation of osteoclastic bone resorption is believed to largely associate with the formation of osteoclasts.<sup>19)</sup> In osteoclastogenesis study test, this study showed that GR extracts had an effective activity on inhibiting the number of TRAP(+) osteoclasts in co-culture system, suggesting that GR extracts affected the differentiation into osteoclast lineage from osteoclast progenitors.

As OVX-induced osteoporotic processes, dramatically decrease bone weights and thickness of bone at epiphyseal regions were detected. In addition, the bone Ca and P contents, BMD and FL of bones and serum Ca and P levels were significantly ( $p < 0.01$  or  $p < 0.05$ ) decreased in vehicle con-



trol group compared to that of Sham with increase of serum osteocalcin level. In histopathology-histomorphometry, dramatical decreases of trabecular and cortical bone mass were detected in vehicle control compared to that of sham control with classic histomorphometrical changes of bones, including decrease of TBV, Tbt, Tbl, Tbn, Cbt, and an increase of Ocn. These estrogen-deficient osteoporotic changes were dramatically decreased in all GR extracts-dosing groups, respectively. Especially middle dosage of GR extracts effectively and significantly ( $p < 0.01$  or  $p < 0.05$ ) inhibited the loss bone strength and bone quality.

OVX in the rat is associated with an increase in body weight.<sup>20)</sup> Existing data on the effect of OVX on the body weight in mice are inconsistent: one study has shown that ovariectomy results in an increased body weight,<sup>21)</sup> whereas other reports have failed to do so.<sup>22)</sup> Slight increase of body weight and gains were detected in OVX groups compared to that of non-OVX sham but significant effect on body weight was not seen in the present study. In the present study we used young (but sexually mature) mice, whereas older mice were used in previous studies. This difference may, at least partly, explain the difference with respect to body weight gain after OVX.

Although, it is generally accepted that the changes of bone weight was not critical index to detect the efficacy of anti-osteoporotic agents except for ASH bone weight,<sup>23)</sup> dramatical increased trends of bone weights were detected in all dosed levels tested in this study. Therefore, it is considered that these increase trends of bone weight directly related to the potency of anti-osteoporotic potentials of GR extracts.

In generally, the thickness of bones was significantly decreased in OVX-induced rodents but no significantly or meaningful changes were detected on the bone length.<sup>24, 25)</sup> However, they were showed dose-dependent increase patterns in GR extracts-dosing groups in both femur and tibia. The increase or inhibit the reduction of bone thickness was considered as the direct evidence of GR extracts preserved the bone mass or increased bone formation. Similar effects on the bone thickness were also observed in parathyroid hormones, a bone anabolic agent.<sup>26)</sup>

Ca is a ubiquitous intracellular second messenger that plays a key role in variety of cellular function including growth, intracellular signal transduction, survival, and death.<sup>27)</sup> Clinically, reduced

Ca levels in blood in elderly population results in secondary hyperparathyroidism, which in turn increases bone turnover, accelerates bone loss, and increases the risk of osteoporosis.<sup>28)</sup>

Generally, bone mineral contents are significantly decreased in osteoporotic status. Among bone mineral contents, Ca and P contents are the most dramatically decreased mineral contents in the bone but their ratio, Ca/P, was not generally changed.<sup>29, 30)</sup> The increase or inhibit the reduction of bone mineral contents was considered as the direct evidence of GR extracts preserved the bone mass or increased bone formation, and it therefore promising the increase of bone strengths.

Osteocalcin is vitamin K-dependent alpha-carboxyglutamic acid released by osteoblasts and serum osteocalcin levels are an indication of bone formation. Although, the variable changes according to the time of blood collection, types of study and used animals were reported, and some augments were existed in interpret base on bone turnover and bone formation, serum osteocalcin levels were generally accepted as a marker of bone turnover.<sup>31, 32)</sup> GR extracts showed dramatical and dose-dependent inhibition on the increase of serum osteocalcin induced by OVX osteoporosis, thus it is considered as indirect evidence that GR extracts has an anti-resorption of bone. Although, somewhat another reason of increase of serum Ca and P levels were exist, it is generally accepted that the increase of serum Ca and P levels were marker of bone formations in osteoporotic animal models.<sup>33, 34)</sup> GR extracts showed dramatical and dose-dependent inhibition on the decreases of serum Ca and P levels induced by OVX osteoporosis, it therefore, is considered as indirect evidence that GR extracts has an action to improve bone formation with dose dependent trends.

BMD has been regarded as a valuable index to test the changes of bone quality in clinics especially to human and they were generally and significantly decreased in osteoporotic animals regardless of the cause. BMD of bone provided good predictable information about efficacy of anti-osteoporotic agents.<sup>35)</sup> It also provides diagnostic profiles of bone qualities in human clinical researches.<sup>36)</sup> The BMD of epiphyseal regions of Femur of GR extracts-dosing groups were dose-dependently increased compared to that of vehicle control group. Therefore it is considered that GR extracts inhibited the decrease of the BMD on femur induced by OVX, and it is expected that GR

extracts has favorable effect to the prevention and/or treatment of fractures frequently encountered on the osteoporotic patients.

Because the FL directly indicates cortical bone strength<sup>37)</sup> and one of the most important indicators to predict the efficacy of anti-osteoporotic agents.<sup>38,39)</sup> The FL of mid-shaft regions of left tibia of GR extracts-dosing groups were increased compared to that of vehicle control group. These increases of FL was considered that GR extracts has direct effect to inhibit the decrease of bone strength induced by OVX, and as direct evidence that GR extracts has a favorable effects to improve the bone strength.

Although this method is generally used, a microscopic observation of bone provided good information about bone morphology.<sup>37)</sup> In the osteoporosis, some histomorphometrical indices are clearly decreased including TBV, Cbt, Tbt, Tbl and osteoblasts/bone perimeter or increased as osteoclasts/bone perimeter (Ocn) and they provided trustworthy information to predict the efficacy of anti-osteoporotic agents.<sup>40,41)</sup> As results of OVX-induced osteoporotic process, dramatical decreases of trabecular and cortical bone masses were detected in vehicle control compared to non OVX Sham with classic histomorphometrical changes of bones including decrease of TBV, Tbt, Tbl, Tbn, Cbt and increase of Ocn. In GR extracts-dosing groups, these histopathology-histomorphometry changes were dramatically reduced with dose-dependent patterns especially on the cortical bone mass. The effects on the trabecular bone mass of GR extracts were much lower than that of alendronate. However, more favorable effects on the cortical bones were detected in the GR extracts compared to that of alendronate. These inhibitions of decreases of bone mass by GR extracts will be providing relatively good bone strength and quality.

Our results that GR extracts effectively inhibited the estrogen-deficient osteoporotic changes were considered as the results of positively regulates osteoblast differentiation and bone formation and negatively regulates bone resorption and osteoclast activity detected *in vitro* assay in the present study. Although somewhat slight effects were detected, 125 mg/kg of GR extracts constantly and significantly ( $p < 0.01$  or  $p < 0.05$ ) inhibited the loss bone strength and bone quality. Roughly calculated based on the body surface (1/12 of human in mouse), the effect dose of GR extracts for osteoporotic patients is 625 mg/day (in case of 60 kg body weight).

Based on the present results, it was concluded that GR extracts (125, 250 or 500 mg/kg; orally dosing) has an efficacy to prevention and/or treatment of OVX-induced osteoporosis. In addition, GR extracts enhance the osteoblasts proliferation, differentiation and activity, and they inhibit the osteoclastogenesis and activity of osteoclasts. Although the efficacy was slighter than that of alendronate on the inhibition of trabecular bone loss, it is expected that GR extracts will be promising as a new anti-osteoporotic agents for prevent the fracture induced in osteoporotic patients because natural herbal medicine origin GR extracts will be dose not show serious side effects especially the problem in upper alimentary irritation of bisphosphonate,<sup>42)</sup> and hypocalcaemia and hormone disturbance of parathyroid hormone analogs.

Main active compounds of GR extracts generally known as phenolic compounds — gastrodin, vanillin, 4-hydroxybezymethyl ether, 4-hydroxybezy alcohol, bis(4-hydroxyphenyl)methane, 4-hydroxybezyaldehyde with fatty acid such as palmitic acid and some steroids —  $\beta$ -sitosterol.<sup>43,44)</sup> In the present study, we only focused on the anti-osteoporotic effects of crud extract itself not on the active compounds. Thus, these active compound searches should be proceeding in future.

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