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Osteoprotective Effect of *Monascus*-fermented Dioscorea in Ovariectomized Rat Model of Postmenopausal Osteoporosis

Shen-Shih Chiang,[†,‡] Shang-Ping Chang,[†] and Tzu-Ming Pan[*,†]

[†]Department of Biochemical Science and Technology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan

[‡]Department of Food Science and Biotechnology, National Chung Hsing University, No. 250, Kuokuang Road, Taichung 40227, Taiwan

ABSTRACT: This experiment established the ovariectomized (OVX) rat model of postmenopausal osteoporosis and examined the effect of the oral administration of different dosages of dioscorea, red mold dioscorea (RMD), and soy isoflavones on bone mineral density (BMD). Three months after osteoporosis had been induced and 4 weeks after feeding had begun, the tibia and femur BMD of OVX rats administered RMD showed significant increases compared with that of all other groups of OVX rats. Closer examination using microcomputed tomography also revealed that the RMD-administered rats had denser trabecular bone volume and a higher trabecular number compared to all other rat groups. Reconstructed 3D imaging indicated increases in cancellous bone mineral content, cancellous bone mineral density, and cortical bone mineral content of the proximal tibia in OVX rats. These findings indicate that administration of monacolin K and phytoestrogen diosgenin could prevent bone loss induced by estrogen deficiency.

KEYWORDS: ovariectomized rat, osteoporosis, phytoestrogen, red mold dioscorea, microcomputed tomography

INTRODUCTION

Osteoporosis, a major skeletal disease associated with aging, has a number of subtypes, including senile osteoporosis and postmenopausal or menopause-related osteoporosis.[1] Loss of estrogen seems to be the most important mechanism in the development of osteoporosis. The use of ovariectomized (OVX) rat or mice models, a well-established and reproducible method of simulating the postmenopausal condition, has been found effective in mimicking postmenopausal cancellous bone loss over relatively short periods.[2] Following ovariectomy, these models show a biphasic loss of bone that is characterized by an initial rapid phase of bone loss for up to 100 days, followed by an intermediate period of relative stabilization of cancellous bone volume at an osteopenic level.[3]

Current therapies for the treatment of osteoporosis, including administration of estrogen and related compounds, are primarily based on the goal of blunting the resorption component of bone homeostasis, a tightly coupled process of bone formation and bone resorption.[4] The three main classes of phytoestrogens, plant-derived estrogens that can bind to the estrogen receptor and have agonist or antagonist effects on the estrogen receptor, are isoflavones, lignans, and coumestans.[5] Daidzein and genistein, the main isoflavones in soybeans, have been found to prevent OVX-induced decreases in bone mineral density (BMD) and bone mechanical strength and exert a moderately protective effect on the microarchitecture of trabecular bone in rats.[6,7]

Diosgenin, an aglycone of the steroid saponin in yam (*Dioscorea* spp.) obtained from the hydrolysis of the yam saponins,[8] is the principal raw material in the industrial production of steroid drugs. Recent studies have indicated that diosgenin may have actively contributed to several biological activities that have been observed both in vitro and in vivo, including antioxidative and hypolipidemic,[8] antithrombic,[9] immunomodulatory,[10] and estrogenic (as phytoestrogenic in antiosteoporotic) activities.[11−14]

In our previous research into the secondary metabolites of *Monascus* species such as monacolins and monascin, we examined the activities of *Monascus*-fermented dioscorea, also referred to as red mold dioscorea (RMD). When fermented by *Monascus purpureus* NTU 568,[15] the new *Monascus* product was found to have several beneficial effects, including reducing blood serum cholesterol,[16,17] decreasing hypertension,[18] protecting against DMBA-induced oral cancer in hamsters via anti-inflammatory and antioxidative potentials,[19,20] inducing G2/M arrest and apoptosis in human oral cancer cells,[21] and preventing diabetes development.[22,23] In this study, we investigated the phytoestrogen content in dioscorea and RMD and evaluated the differences in skeletal morphology in response to different dosages of dioscorea, RMD, and soy isoflavones (as a positive control) in OVX rats, using an especially adapted microcomputed tomography (μCT) machine to determine baseline bone morphology.

MATERIALS AND METHODS

Preparation of RMD. The *M. purpureus* NTU 568 culture strain was maintained on potato dextrose agar (PDA; Difco Co., Detroit, MI, USA) slanted at 10 °C and transferred monthly. The dioscorea (*Dioscorea batatas* Dence) root was used as the solid substrate to produce RMD.[16] The substrate was prepared by soaking 500 g of dioscorea in distilled water for 8 h, draining the excess water with a sieve, then autoclaving the substrate for 20 min at 121 °C in a "koji dish," a dish made of wood with dimensions of 30 cm × 20 cm × 5 cm. After cooling, the substrate was inoculated with a 5% (v/w) spore suspension (10^7 spores/mL) and 0.3% (v/w) ethanol and then cultivated at 30 °C for 10 days. After this period, the crushed and dried product with mold was further freeze-dried into powder and stored at −20 °C until used in the experiments.

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Determination of Monacolin K, Monascin, and Ankaflavin Concentrations in Dioscorea and RMD. Dioscorea or RMD (0.5 g) was extracted with 5 mL of ethanol at 70 °C for 30 min and filtered with a 0.45 μm syringe filter before analysis. The HPLC system consisted of model PU2089 plus (Jasco Co., Tokyo, Japan) and a Luna C_{18} column (Phenomenex, 150 mm × 4.6 mm i.d.; 5 μm particle size) equipped with a 2-cm LC-18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase gradient consisted of A eluent (0.05% triflouracetate in acetonitrile solution) and B eluent (0.05% trifluoroacetate in water), at a gradient elution of A/B (51/49) for 0−29 min, A/B (80/20) for 29.1−53 min, and A/B (51/49) for 53.1−60 min at a flow rate of 1.0 mL/min. Monacolin K, monascin, and ankaflavin were detected using a UV-2075 plus detector (Jasco Co.) set at 238 nm.

Determination of Diosgenin Concentration in Dioscorea and RMD. The extraction of diosgenin in dioscorea and RMD was modified from the method described by Taylor et al.[24] Dioscorea or RMD (10 g) was transferred to thimble filter paper (28 mm × 100 mm) equipped with a Soxhlet extractor. After the addition of 150 mL of methanol, the mixture was heated at 85 °C for 10 h with refluxing and extraction. After the extract had evaporated at 43 °C, the pellet that remained in the test tube was dissolved by vortex mixing in 2 mL of 70% 2-propanol containing 1 M of sulfuric acid. Each extract was refluxed by heating at 80 °C for 30 min to evaporate the propanol, after which 20 mL of *n*-hexane was added to re-extract and combine the fraction. The mixture was washed with 50 mL of water three times and evaporated to remove the *n*-hexane. Finally, the residue was dissolved in 5 mL of methanol and filtered with a 0.45 μm syringe filter before HPLC analysis.

The HPLC system consisted of model PU2089 plus (Jasco Co.) and Luna C_{18} column (Phenomenex, 150 mm × 4.6 mm i.d.; 5 μm particle size) equipped with a 2 cm LC-18 guard column (Phenomenex, Torrance, CA, USA). The mobile eluent of acetonitrile/water = 95/5 (v/v) was applied for 25 min at a flow rate of 1.0 mL/min. Diosgenin was detected using the UV-2075 plus detector PU2089 (Jasco Co.) set at 203 nm.

Ovariectomized Rat Model and Experimental Protocol. Seventy-two 2-month-old female Sprague−Dawley rats were purchased from BioLASCO Taiwan Co. (Taipei, Taiwan). The rats were housed in polycarbonate cages and maintained under a 12 h light/dark cycle and given access to food (lab rodent diet 5001; Purina Mills LLC, St. Louis, MO, USA) and water ad libitum during a 4-week adaptation period before the study. At 12 weeks of age, 64 rats were anesthetized with sodium pentobarbital (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and ovariectomized (referred to as the OVX rats), while 8 sham-operated rats (referred to as the sham rats, subsequently the sham group) were fed a normal diet. Twelve weeks after surgery according to the method of Bone et al.,[25] the OVX rats were divided into eight groups. The feed compositions used in parts I and II of the experiment were based on the findings of Chen et al. [26] regarding the effective dosage of dioscorea (750 mg·kg^{-1}·day^{-1}) and the findings of Aradhana et al. [27] regarding the effective dosage of diosgenin. The components of the feed administered to each group were as follows: group 1 (Sham group), lab rodent diet 5001; group 2 (OVX group), lab rodent diet 5001; group 3 (ISO group), isoflavones (80 mg·kg^{-1}·day^{-1}; VSC Leader Source Products, Vancouver, Canada) mixed with dextrinized corn starch (ICN Biochemicals Co., Costa Mesa, CA, USA) and lab rodent diet 5001; group 4 (I D1X group), dioscorea powder (750 mg·kg^{-1}·day^{-1}, containing 0.15 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001; group 5 (I RMD1X group), RMD (375 mg·kg^{-1}·day^{-1}, containing 0.15 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001; group 6 (I RMD2X group), RMD (750 mg·kg^{-1}·day^{-1}, containing 0.3 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001; group 7 (II D1X group), dioscorea powder (29.4 g·kg^{-1}·day^{-1}, containing 20 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001; group 8 (II RMD1X group), RMD (14.7 g·kg^{-1}·day^{-1}, containing 20 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001; group 9 (II RMD2X group), RMD (29.4 g·kg^{-1}·day^{-1}, containing 40 mg of diosgenin) mixed with dextrinized corn starch and lab rodent diet 5001.

Each group (n = 8) was subjected to daily gavage for 4 weeks. All experimental procedures were reviewed and approved by the Animal Care and Research Ethics Committee of the National Taiwan University. From the commencement of the experiment until feeding began, all rats were anesthetized with 5% isoflurane and maintained in an anesthetized state with 3% isoflurane to measure BMD and obtain 2D images of the distal femur and proximal tibia of the right leg. Measurements were taken using the small animal PET/CT scanner (eXplore Vista DR; GE Healthcare, Waukesha, WI, USA) operated at 40 kV, 300 μA, and scanned for 20 min at 60 μm/pixel scan resolution. At the end of treatment, the rats were euthanized by carbon dioxide inhalation and blood samples were collected via the retro-orbital sinus. After whole blood had clotted at room temperature for 2 h, the serum was separated by centrifugation at 700*g* for 10 min and stored at −80 °C until analysis. The content of the serum biomarker osteocalcin was determined using the MID osteocalcin ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark). The serum was also analyzed to determine the levels of alkaline phosphatase (ALP) activity.

Preparation of Bone Specimens. The right femur was dissected, and the adherent soft tissues were removed. The right femur bone specimens were placed in sealed plastic Eppendorf tubes containing 4% formalin for 48 h, which was subsequently changed to 75% ethanol to preserve the specimens. The specimens were analyzed using the SkyScan 1076 (SkyScan, Kontich, Belgium) microcomputed tomography (μCT) system.

Trabecular and Cortical Bone Assessment by μCT Imaging. The cortical and trabecular microstructure and a 3D image of the right femur were analyzed using the SkyScan1076 in vivo μCT system at 50 kV, 200 μA, at a rotation step of 0.4° using a 0.5 mm aluminum filter and a 9 μm/pixel scan resolution. The voxel size was 9 μm × 9 μm × 9 μm. Cross sections were reconstructed using NRecon cone-beam algorithm software (SkyScan), and the files were imported into CTAn software (SkyScan) for 3D analysis and image generation.

The region of interest (ROI) of trabecular bone was defined using 100 slices approximately 0.8 mm distant from the growth plate of the distal femur. The ROI of femoral cortical geometry was assessed for slices located at the diaphyses of the midfemur. The trabecular morphometric parameters were analyzed by imaging and measured using the CTAn software (SkyScan). The position of trabecular bone commencing approximately 0.8 mm from the growth-plate level was placed in the direction of the metaphysic and extended from this position for an additional 3.0 mm. Separation of the trabecular bone was performed using a freehand drawing tool for delineating complex ROIs, and 3D morphometric parameters were calculated for the trabecular selected ROIs. Trabecular bone volume (BV/TV) was calculated using measurement of bone volume (BV) and total tissue volume (TV). Mean trabecular thickness (Tb.Th) was determined using measurement of local thickness. Trabecular separation (Tb.Sp) and trabecular number (Tb.N) were estimated using the plate model, and Tb.N and Tb.Sp were measured according to the parallel-plate model.[28]

Statistical Analysis. All data are expressed as the mean ± standard deviation. The statistical significance of the behavioral and biochemical effects was determined by performing a one-way analysis of variance (ANOVA) using the general linear model procedure of Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL) software followed by performing an ANOVA with Duncan's test. Differences at a $p < 0.05$ level of significance were considered statistically significant.

RESULTS

Monacolin K, Monascin, Ankaflavin, and Diosgenin Concentrations in Dioscorea and RMD. Table 1 shows the quantities of monacolin K, monascin, ankaflavin, and diosgenin found

Table 1. Monacolin K, Monascin, Ankaflavin, and Diosgenin Contents in Dioscorea and Red Mold Dioscorea

compositions/sample	dioscorea	red mold dioscorea
monacolin K (μg/g)	ND[a]	104.8 ± 7.0
monascin (μg/g)	ND[a]	369.0 ± 32.5
ankaflavin (μg/g)	ND[a]	602.8 ± 46.4
diosgenin (μg/g)	680 ± 60.5	1360 ± 89.8

[a] ND, Not detected.

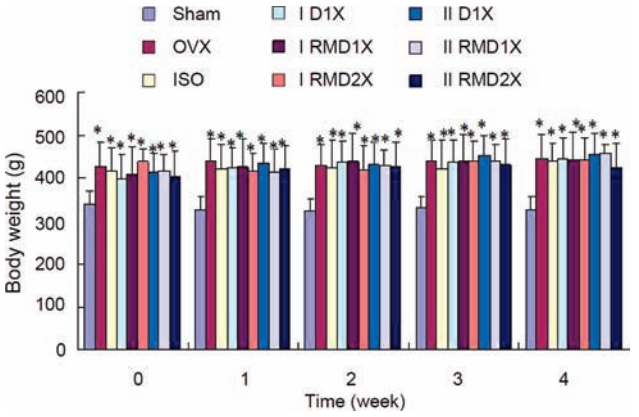

Figure 1. Average body weights of OVX rats within the experimental time frame. Data are expressed as the mean ± standard deviation ($n = 8$). Asterisks indicate comparison with the OVX group (∗, $p < 0.05$). The abbreviations that appear here are the following: Sham, sham-operated and normal diet group; OVX, ovariectomized and normal diet group; ISO, ovariectomized and isoflavone group; I D1X, ovariectomized and 750 (mg/kg)/day dioscorea powder group; I RMD1X, ovariectomized and 375 (mg/kg)/day RMD group; I RMD2X, ovariectomized and 750 (mg/kg)/day RMD group; II D1X, ovariectomized and 29.4 (g/kg)/day dioscorea powder group; II RMD1X, ovariectomized and 14.7 (g/kg)/day RMD group; II RMD2X, ovariectomized and 29.4 (g/kg)/day RMD group.

in dioscorea and RMD. As can be observed, whereas dioscorea was found to contain no monacolin K, monascin, or ankaflavin, RMD was found to contain 104.8, 369.0, and 602.8 μg/g of monacolin K, monascin, and ankaflavin, respectively. In contrast, both dioscorea and RMD were found to contain phytoestrogen diosgenin, with dioscorea found to contain 680 and RMD 1360 μg/g.

Body Weights and Serum Biochemical Parameters. The body weights of all the OVX groups were found to be significantly higher than those of the rats in the sham group within the experimental time frame (Figure 1). However, no significant differences were found among the weights of the different OVX groups ($p < 0.05$). As can be observed in Figure 2, serum ALP levels were elevated in all OVX surgery groups except for the IIRMD2X group. Administration of ISO increased serum ALP value and was statistically significant from that of the OVX group. The level of osteocalcin is secreted solely by osteoblasts and is related to pro-osteoblastic and bone-building processes. As shown in Figure 3, administration of isoflavone and RMD treatments significantly increased osteocalcin levels in the OVX rats from that of the sham group.

Effects of Dioscorea and RMD on Knee, Proximal Tibia, and Distal Femur BMD. Figure 4A presents a longitudinal section view of the rats' distal femur and proximal tibia before OVX surgery. Figure 4B presents the view 3 months after OVX

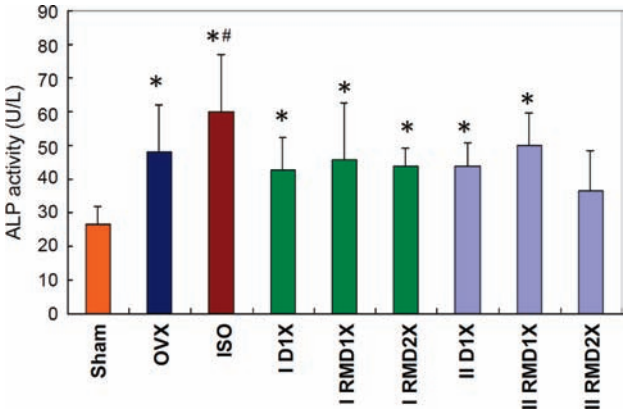

Figure 2. Serum bone ALP activity of OVX rats. Asterisks indicate comparison with the sham group (∗, $p < 0.05$) and pound signs comparison with the OVX group (#, $p < 0.05$). The abbreviations that appear here are the same as those used in Figure 1.

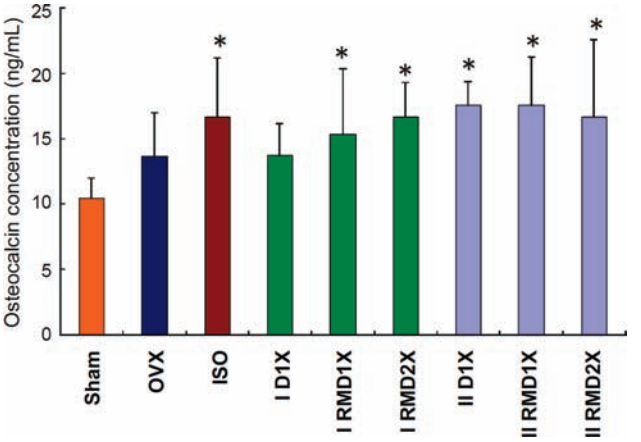

Figure 3. Serum osteocalcin concentrations of OVX rats. Asterisks indicate comparison with the OVX group (∗, $p < 0.05$). The abbreviations that appear here are the same as those used in Figure 1.

surgery, and Figure 4C presents the view 3 months after OVX surgery and then fed with the dioscorea and RMD products for 4 weeks. Table 2 shows the findings regarding the quantification of BMD by white fractions in the knee and distal femur before OVX surgery, 3 months after OVX surgery, and after feeding with the dioscorea and RMD products for 4 weeks. Regarding the first time interval, the knee BMD was found to be similar for all the groups (between 24.1% and 29.7%), and no significant differences were found among them. Regarding the second time interval, the knee BMD for all the OVX groups (48.6−56.0%) was found to be significantly lower than that of the sham group (70.9%). Regarding the third time interval, the knee BMD of the sham group (51.2−62.3%) was found to have increased to a greater extent than that of the OVX group (48.3%), indicating a dose-dependent effect of dioscorea or RMD feeding. The distal femur BMD of all the groups was found to be highest (60.3−68.3%) at the first time interval (before OVX surgery). At the second time interval, the white areas of the OVX groups were found to have decreased by 20% compared with the sham group. At the third time interval, the results were increased in high dosage group and the values of RMD in categories I and II

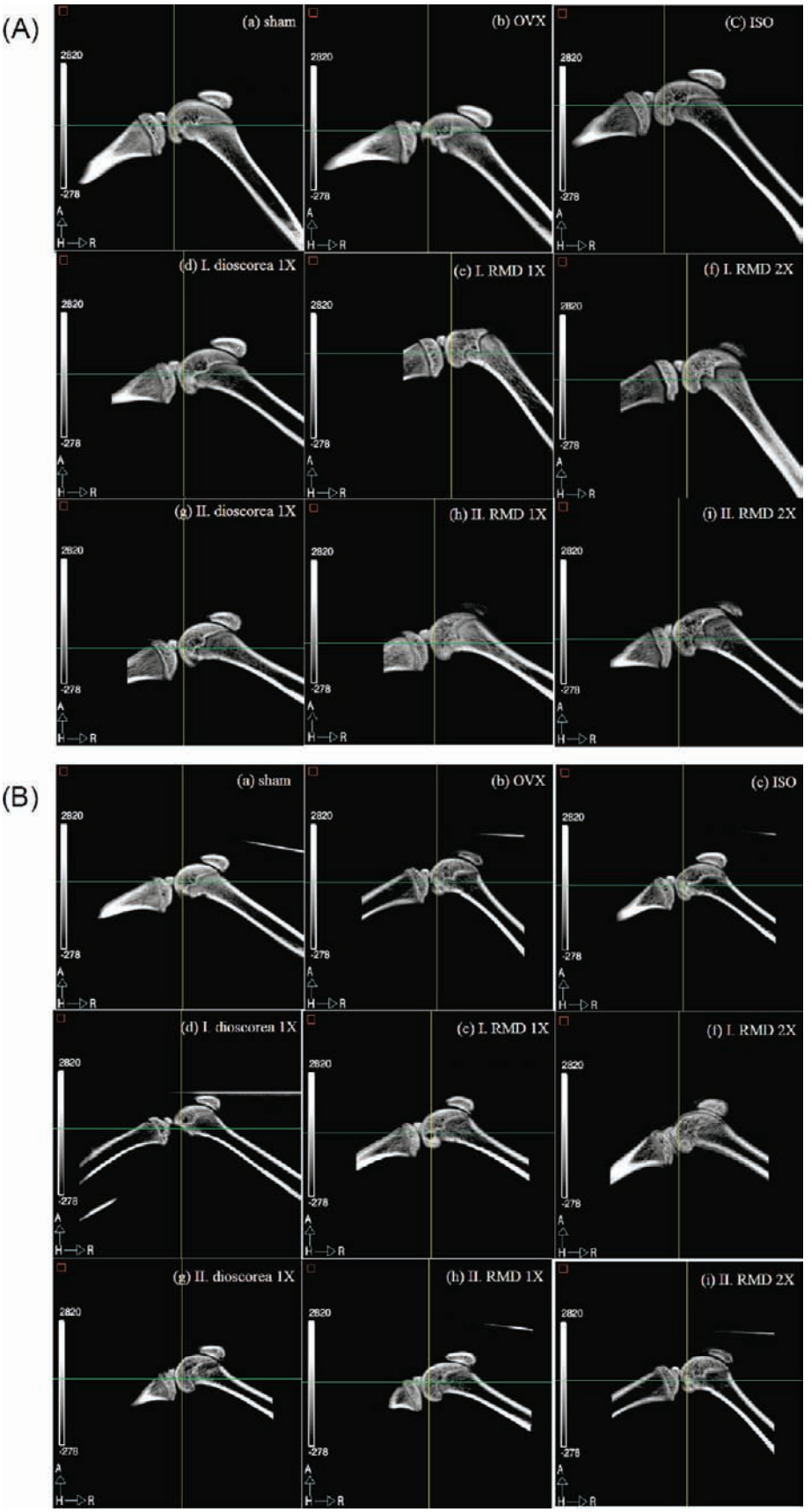

Figure 4. Continued

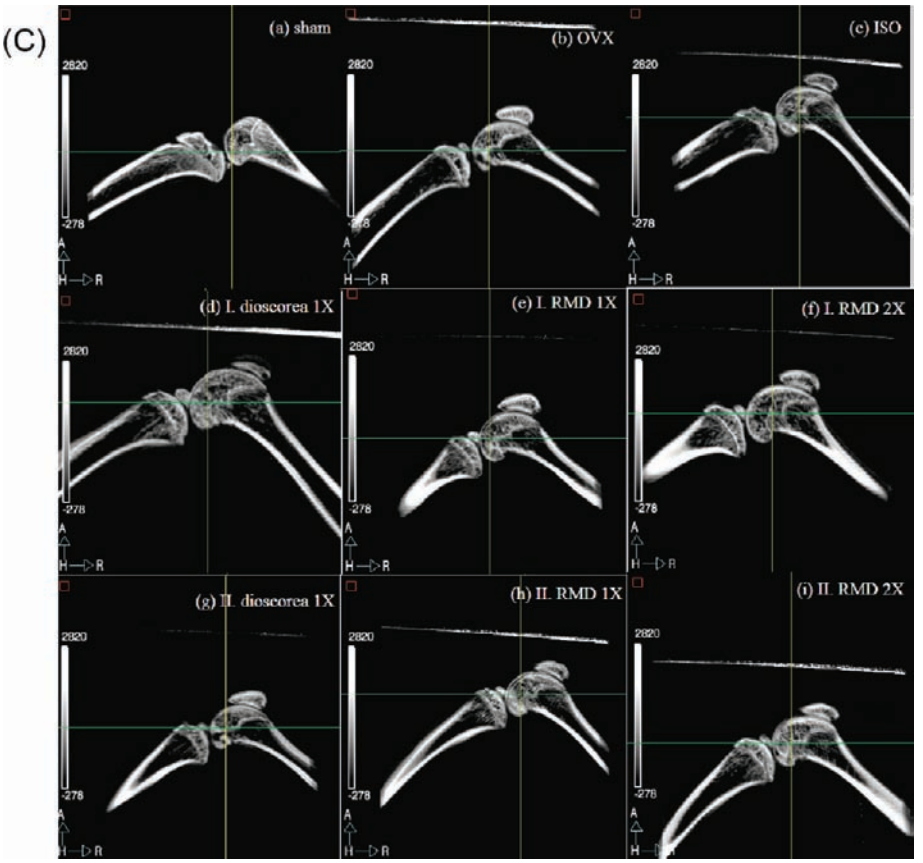

Figure 4. Longitudinal section views of rat distal femur and proximal tibia (A) before OVX surgery, (B) 3 months after OVX surgery, and (C) 3 months after OVX surgery and 4 weeks after beginning feeding. The abbreviations that appear here are the same as those used in Figure 1.

were higher (51.2−56.4%) than those in dioscorea (50.1%). These findings indicate that osteoporotic symptoms could be successfully induced 3 months after OVX surgery.

Three-Dimensional μCT Imaging of Trabecular Bone in the Femoral Diaphysis. Parameters of trabecular structure estimated by μCT imaging are shown in Table 3. Analysis of data from the trabecular bone of OVX mice given normal diet, isoflavone, dioscorea, and red mold dioscorea shows a decrease in BV/TV compared with the data from the sham group. The trabecular bone thickness of OVX rats fed isoflavones and high dosages of dioscorea and RMD was found to be significantly higher than that of the OVX control and the sham group. Although no significant differences were found among the eight OVX groups regarding trabecular separation and number, these values were found to be higher and lower than those of the sham group, respectively.

The 3D reconstruction of μCT imaging results from CTAn and CTvol software analysis shown in Figure 5 reveals the complexity of the rat femur bone structure. As can be observed in Figure 5c,f,i, the trabecular bone in the sham group was found be significantly thicker than that of the OVX groups, as well as that the trabecular bone thickness and volume of the ISO and RMD2X groups significantly increased.

DISCUSSION

Osteoporosis is a bone disease that affects individuals worldwide and is particularly prevalent among elderly postmenopausal women. Many drugs currently used to treat osteoporosis, including bisphosphonates, estrogen, selective estrogen receptor modulators, and statins, act as antiresorptive agents in the prevention of bone loss.[29,30] Many studies have found that statins, specific competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, increase bone formation and bone mass in rodents,[31] suggesting that they could be an important treatment for osteoporosis, particularly when significant amounts of trabecular bone have been lost.[32,33] The group of statins includes mevastatin, lovastatin, simvastatin, and pravastatin, which are all naturally occurring statins derived by fermentation.

In our previous study, we examined whether a *M. purpureus* NTU 568 fermented dioscorea product could serve as a hypolipidemic functional food due to its high content of the metabolites monacolin K (identified as lovastatin) and the yellow pigments monascin and ankaflavin.[17] In this study, we found that the greater antiosteoporosis effect of RMD compared with dioscorea may result from the rich content of these metabolites in RMD (Table 1). When we investigated the content of phytoestrogen diosgenin in dioscorea and RMD, we found that RMD contained twice the quantity of diosgenin compared with dioscorea (Table 1). Diosgenin, a steroid saponin extracted from the root of wild yam, is hypothesized to have osteogenic properties. In an in vitro experiment, Yin et al. [11,14] found that water-extracted *Dioscorea spongiosa* contained many glycosides that could stimulate osteoblast proliferation and inhibit activity against osteoclast formation. In an in vivo experiment, Yin et al.[11] found that the antiosteoporotic activity was determined by highly cancellous bone mineral content, cancellous bone mineral density, and cortical bone mineral content of the proximal tibia in ovariectomized rats. Alcantara et al.[34]

Table 2. Quantification by μCT Imaging of Knee and Distal Femur BMD in OVX Rats[a]

	white color fraction of knee (%)			white color fraction of distal femur (%)		
group	A[b]	B	C	A	B	C
sham[c]	29.6 ± 1.7	70.9 ± 3.9#	66.2 ± 3.6#	65.8 ± 3.6	65.6 ± 3.2#	67.4 ± 3.7#
OVX	26.3 ± 2.1	53.3 ± 2.1*	48.3 ± 2.3*	61.7 ± 3.7	48.6 ± 2.9*	47.5 ± 2.3*
ISO	29.1 ± 1.7	48.6 ± 3.2*	54.1 ± 3.2*	60.3 ± 2.7	46.6 ± 2.0*	52.8 ± 2.6*,#
I D1X	29.7 ± 2.4	54.5 ± 3.1*	55.5 ± 2.6*	60.7 ± 2.9	47.0 ± 2.8*	50.1 ± 3.1*
I RMD1X	29.6 ± 1.7	50.4 ± 3.0*	55.7 ± 3.8*	67.2 ± 3.8	48.9 ± 2.3*	51.2 ± 2.5*
I RMD2X	24.1 ± 2.0	52.4 ± 2.5*	61.3 ± 3.2#	62.5 ± 2.9	50.3 ± 3.0*	53.7 ± 2.9*,#
II D1X	26.9 ± 1.2	49.0 ± 2.8*	51.2 ± 2.9*	60.5 ± 3.5	49.7 ± 2.3*	50.1 ± 3.2*
II RMD1X	28.1 ± 1.5	50.6 ± 3.2*	55.1 ± 3.0*	68.3 ± 3.1	47.3 ± 2.8*	56.4 ± 2.9*#
II RMD2X	29.7 ± 1.9	56.0 ± 2.6*	62.3 ± 3.6#	60.6 ± 4.2	47.4 ± 2.6*	55.5 ± 3.1*,#

[a] Data are expressed as mean values ($n = 8$). Asterisks indicate comparison with the sham group (*, $p < 0.05$), and pound signs indicate comparison with the OVX group (#, $p < 0.05$). [b] BMD (A) before OVX surgery, (B) 3 months after OVX surgery, (C) 3 months after OVX surgery and 4 weeks after dioscorea or RMD administration. [c] The abbreviations that appear here are the same as those used in Figure 1.

Table 3. Three-Dimensional Microstructural Properties of the Distal Femoral Metaphyseal Trabecular Bone in OVX Rats after 4 Weeks of Dioscorea or RMD Administration[a]

group	BV/TV (%)[b]	trabecular thickness (mm)	trabecular separation (mm)	trabecular number (1/mm)
sham[c]	34.00 ± 4.71#	0.11 ± 0.01	0.22 ± 0.04#	3.22 ± 0.38#
OVX	17.43 ± 8.00*	0.11 ± 0.01	0.63 ± 0.22*	1.57 ± 0.76*
ISO	20.82 ± 14.30*	0.12 ± 0.01*	0.56 ± 0.19*	1.73 ± 1.03*
I D1X	16.67 ± 5.78*	0.12 ± 0.01	0.62 ± 0.15*	1.43 ± 0.37*
I RMD1X	14.53 ± 2.93*	0.11 ± 0.01	0.64 ± 0.15*	1.30 ± 0.23*
I RMD2X	19.71 ± 5.88*	0.11 ± 0.01	0.48 ± 0.16*	1.77 ± 0.60*
II D1X	16.90 ± 5.60*	0.12 ± 0.01*	0.57 ± 0.13*	1.43 ± 0.43*
II RMD1X	16.09 ± 7.85*	0.11 ± 0.01	0.65 ± 0.20*	1.46 ± 0.69*
II RMD2X	24.27 ± 18.05*,#	0.12 ± 0.02*	0.55 ± 0.29*	1.99 ± 1.24*,#

[a] Data are presented as the mean ± standard deviation ($n = 8$). Asterisks indicate comparison with the sham group (*, $p < 0.05$), and pound signs indicate comparison with the OVX group (#, $p < 0.05$). [b] BV/TV: the ratio of trabecular bone volume (BV) to total tissue volume (TV). [c] The abbreviations that appear here are the same as those used in Figure 1.

also suggested that diosgenin can enhance bone formation in osteoblastic MC3T3-E1 cells by stimulating the synthesis and secretion of type 1 collagen, ALP, the bone marker protein Runx2, and osteopontin expression. The data obtained in this study, including those indicating a positive relationship between the levels of these molecules and the prevention of bone loss, suggest that diosgenin in dioscorea and RMD may be effective alternative forms of treatment for osteoporosis.

In part I of this experiment, we examined the effects of administering a low dosage of dioscorea and RMD products according to the total effective amount of dioscorea ingestion. In part II, we divided the high dosage from the effective dosage by diosgenin. We found the diversity dosage in two categories was about 40-fold. In our clinical observation of all the experimental groups, we found that the body weights of the eight OVX groups were higher than those of the sham group. In images of dissections, we observed the formation of much fatty tissue in the abdominal cavity of the OVX groups. We found that the level of ALP activity, a major osteoblastic differentiation marker whose synthesis is largely dependent on the synthesis and processing of type 1 collagen, was highest in the ISO group and lowest in the sham group (Figure 2). An elevated serum alkaline phosphatase can be due to rapid growth of bone. However, previous surveys have indicated that treatment with raloxifene at 150 mg/day for 24 months or at 60 mg/day for 12 weeks reduced serum ALP levels in the postmenopausal women.[35,36] These findings appear to be consistent with the high dose of treatment and may account for the discrepancy in ALP concentrations after 4 weeks of treatment in our study.

We found that the number of bone formation sites as indicated by osteocalcin formation, a widely used marker of early and mature osteoblast function in bone formation, increased in postmenopausal osteoporosis animals and the OVX groups.[37] It has been routinely observed that higher serum osteocalcin levels are relatively well correlated with increases in BMD. We found that the serum levels of osteocalcin were significantly higher in the OVX groups (Figure 3), likely because the administration of isoflavones and RMD increased the osteocalcin levels to levels significantly higher than those of the sham group. We note that levels of osteocalcin can be increased by replacement of estrogen with phytoestrogen or by statin supplementation.

We also investigated femur and tibia bone from multiple skeletal sites to identify the microstructural changes of bone in the OVX groups. Physiological bone changes generally begin as a result of reduced estrogen levels after ovariectomy. Osteopenia increases with increases in bone turnover rate and when bone resorption exceeds formation.[38] In our model, the knee and distal femur BMD of the sham group remained at a higher level than that of the OVX groups throughout the experiment. We found

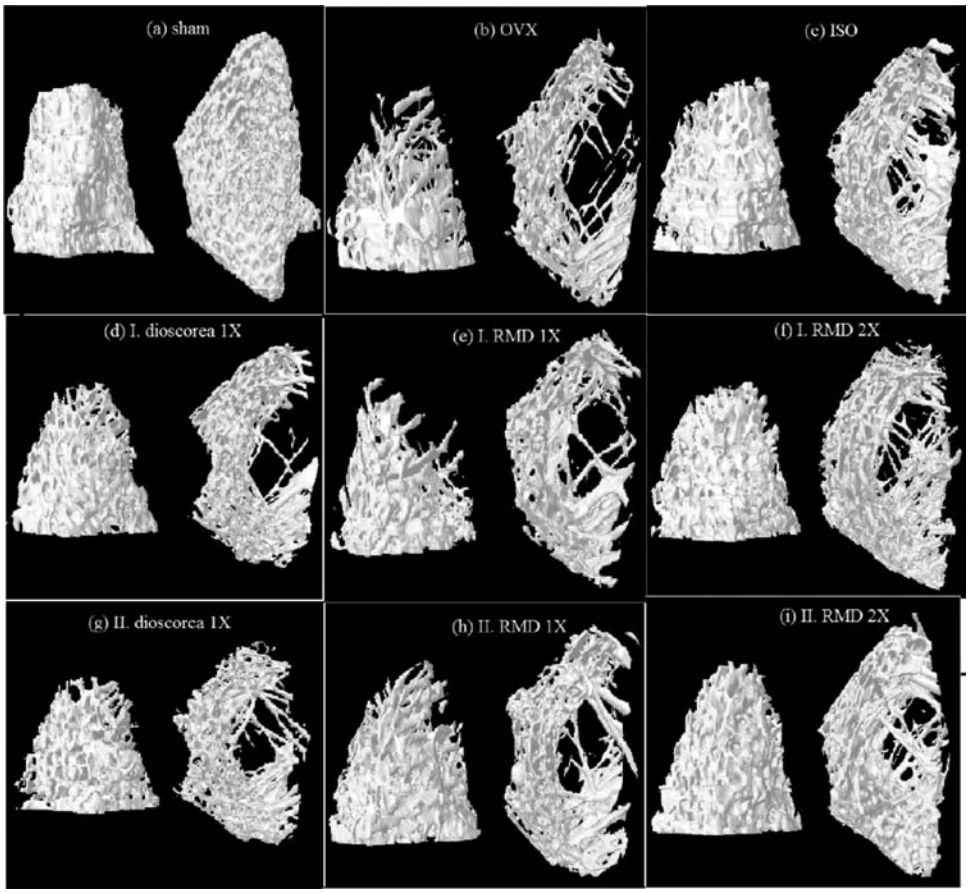

Figure 5. 3D tomographic rendering of a 3.0-mm-thick distal femoral metaphysis trabecular rat bone reveals the complexity of the bone structure. The image on the left shows the side view, and the image on the right shows the top view. The abbreviations that appear here are the same as those used in Figure 1.

that although the BMD of all the OVX groups had decreased 3 months after ovariectomy, the BMD of the ISO and high-dosage RMD groups subsequently significantly increased (Table 2).

We found that the sham group had the highest BV/TV and Tb.N values and the lowest Tb.Sp values in trabecular bone among all the groups (Table 3). Comparing the all OVX treatment groups, we also found that the BV/TV and Tb.N values of the II RMD2X group were the highest. As the II RMD2X group had been fed large quantities of RMD, which contains many active compounds including greater amount of diosgenin. This finding suggests that the osteoprotective effect was major from the diosgenin and is superior to that of isoflavones in the OVX rat model.

Figures 4 and 5 show the 2D and reconstructed 3D images, respectively, obtained from μCT imaging. The trabecular parameters were taken from 3.0-mm-thick sections of trabecular bone in the distal metaphysic femur. When compared with the reconstructed 3D image of the distal femur shown in Figure 5, the BV and trabecular bone network from the sham group can be observed to be more complete and denser than that of the other groups. The trabecular bone network of the ISO, I RMD2X, and II RMD2X groups can be observed to be obviously denser than that of the other OVX groups.

Our findings regarding the OVX specimens evaluated in this study indicate a specialized therapeutic application of RMD. We found that although the OVX process reduces trabecular bone volume to a significant degree, the administration of the aglycone type of isoflavones or other bioactive compounds, such as monacolin K and diosgenin, or high dosages of RMD can inhibit this event and enhance the inhibitory effect in OVX treatment. On the basis of our finding regarding the efficacy of RMD in the treatment of osteoporosis, we hypothesize that the combined effects of high levels of phytoestrogen diosgenin and monacolin K effectively prevent bone loss induced by estrogen deficiency and provide an osteoprotective effect in the OVX rat model.

AUTHOR INFORMATION

Corresponding Author

*Phone: +886-2-3366-4519, extension 10. Fax: +886-2-3366-3838. E-mail: tmpan@ntu.edu.tw.

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