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Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats

Bruce A. Watkins[a,b,*], Susan Reinwald[a], Yong Li[a], Mark F. Seifert[b]

[a]*Center for Enhancing Foods to Protect Health, Lipid Chemistry and Molecular Biology Laboratory, Purdue University, West Lafayette, IN 47907-2009, USA*
[b]*Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

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Abstract

Ovariectomy (OVX) in female rats precipitates a marked reduction in endogenous estrogen concentrations and induces bone remodeling abnormalities that augment bone loss and increase the risk of developing osteopenia. This research examined the combined effects of two levels of soy isoflavones (IFs), trace (−IF) and high (+IF) (0.03 and 3.43 mg/g protein, respectively), and two levels of n-3 polyunsaturated fatty acids (PUFAs) on bone conservation in 2-month-old sexually mature OVX Sprague–Dawley rats. All dietary treatments provided 110.4 g/kg of fat from either safflower oil (N6) or a blend of safflower oil and menhaden oil (N3). OVX rats were randomly assigned to the N6−IF, N6+IF, N3−IF and N3+IF groups. The OVX and sham rats were euthanized after 12 weeks of feeding. Data for sequential femoral and tibial in vivo bone mineral density and bone mineral content (BMC) measurements were determined every 4 weeks. The hindlimb mineral data indicated a trend toward a positive bone mineral-sparing effect related to +IF. Among the OVX rats, those fed the N3+IF diet had a significantly higher value for tibial BMC. The concentration of serum pyridinoline cross-links was significantly lower in the N3+IF group. These findings indicate a complementary action of soy IFs and n-3 PUFAs for attenuating bone mineral reduction in OVX rats.

Keywords: Soy isoflavones; n-3 PUFAs; Bone; Ovariectomy; Rat

1. Introduction

The pathogenesis of postmenopausal osteoporosis is manifest by increased bone turnover with a relative increase in bone resorption resulting in a sharp decline in bone mass with the loss of estrogen after menopause. In sexually mature female rats, ovariectomy (OVX) precipitates a marked reduction in endogenous estrogen concentrations, inducing a negative bone remodeling balance that augments bone loss and osteopenia [1,2]. Extensive comparison of OVX-induced bone loss in rats with postmenopausal bone loss in women reveals many similar characteristics [3,4] that justify the use of the OVX rat as an appropriate model to investigate agents that could help in the prevention of osteoporosis.

Sources of long-chain (LC) n-3 polyunsaturated fatty acids (PUFAs) have been shown to promote bone formation in growing animals undergoing bone modeling. Our laboratory reported that feeding LC n-3 PUFAs to growing male rats elevated eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) but reduced arachidonic acid (AA; 20:4n-6) in various bone tissue compartments, reduced ex vivo bone prostaglandin (PG) E_2 production and increased bone formation rates as determined by histomorphometry in the tibia and the femur [5,6]. These results indicate that higher intakes of n-6 PUFAs [linoleic acid (LA); 18:2n-6] lead to a greater capacity of ex vivo bone PGE_2 production associated with an elevated ratio of AA to EPA in bone and reduced bone formation rates. Lower rates of bone formation likely diminish bone modeling for supporting greater deposition of bone mineral

Abbreviations: AA, arachidonic acid; ALP, alkaline phosphatase; COX, cyclooxygenase; DHA, docosahexaenoic acid; Dpd, deoxypyridinoline cross-links; DXA, dual-energy X-ray absorptiometry; EPA, eicosapentaenoic acid; IF, isoflavones; IL, interleukin; LA, linoleic acid; LPS, lipopolysaccharide; OVX, ovariectomy; PG, prostaglandin; PUFA, polyunsaturated fatty acid; Pyd, pyridinoline cross-links; TNFα, tumor necrosis factor-α.

* Corresponding author. Center for Enhancing Foods to Protect Health, Lipid Chemistry and Molecular Biology Laboratory, Purdue University, West Lafayette, IN 47907-2009, USA. Tel.: +1 765 494 5802; fax: +1 765 494 7953.

E-mail address: baw@purdue.edu (B.A. Watkins).

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later in adult life, and since the greatest deterrent for osteoporosis is maximal bone density achieved in adolescence, bone density would be compromised. A high dietary ratio of n-6 to n-3 PUFAs is therefore believed to reduce bone formation capacity and cause greater bone resorption activity [7] through increased endogenous production of PGE_2. Regression analysis demonstrated a significant positive correlation between ex vivo production of PGE_2 and the ratio of AA to EPA in rat femoral cortical bone but a significant negative correlation between bone formation rate and the ratio of AA to EPA [5]. Hence, these findings on reducing the capacity of PGE_2 biosynthesis in bone and in osteoblast-like cell cultures [7,8] are evidence for the premise that dietary LC n-3 PUFAs support bone cell activities for sustaining bone formation.

Reports from other laboratories corroborate our findings on n-3 PUFAs. In rats fed an n-6 PUFA diet, the level of urinary pyridinium cross-links (markers of bone resorption) was significantly higher compared with that in rats fed an n-3 PUFA diet [9]. Different dietary ratios of n-6 to n-3 PUFAs were also tested in piglets for their effects on growth and bone metabolism, which revealed that higher n-3 PUFA levels in blood were associated with lower bone resorption [10]. Additional studies indicated that an increase in bone formation markers occurred when PGE_2 production was decreased or inhibited in osteoblasts [11–13]. Moreover, an EPA-enriched diet was effective in minimizing bone loss induced by estrogen deficiency, which prevented the loss of bone weight and strength in OVX rats [14]; feeding fish oil to OVX mice also attenuated bone loss [15].

The physiological effects of soy isoflavones (IFs; genistein and daidzein) were similar to native estrogen in maintaining bone mass in OVX rats [16,17] and mice [15]. In a 3-month feeding study with aged female rats, soy protein was shown to be effective in maintaining bone formation rates after OVX compared with casein-based diets [18]. Soy IFs were also shown to reduce bone turnover in adult OVX rats but failed to reverse established osteopenia [19]. In addition, genistein suppressed osteoclastic activity in vitro and in vivo [20], and the anabolic effect of genistein on bone components was inhibited by tamoxifen, an anti-estrogen agent, indicating a bone-sparing effect via the estrogen pathway [21].

In human subjects, IF-rich soy protein helped reduce bone loss in perimenopausal [22] and postmenopausal [23,24] women. However, in a double-blind randomized study that compared the effect of dietary soy protein supplement with milk protein in healthy postmenopausal subjects aged 60 years or older, it was concluded that supplements of soy protein containing IFs at this stage did not show an apparent sparing effect on bone mineral density (BMD) [25]. One could postulate that phytoestrogens may serve as an anti-resorptive agent to help decrease bone resorption postmenopause to maintain bone mass; however, the timing of supplementation appears to be an important factor to achieve these bone benefits.

Although the research on soy protein and bone health is promising, researchers have yet to establish a clear mechanism of action for IFs. Since both LC n-3 PUFAs and genistein have inhibitory effects on bone cell PGE_2 production and may suppress bone resorption, our proposed investigation characterized their effects on bone density measurements. In the current study, we hypothesized that, by a combination of these two dietary factors, estrogen-deficient induced bone loss could be minimized with LC n-3 PUFAs and soy IFs, which could exert a complementary action in OVX rats.

2. Materials and methods

2.1. Animals and diets

Two-month-old female virgin Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) initially weighing 206±1 g were either ovariectomized (OVX) or sham operated (Sham). Success of OVX was confirmed by determination of wet weights of uterine horns compared with Sham rats at the time of tissue collection [1]. One week postsurgery, OVX rats (n=40) were randomly assigned to one of four treatment groups following a 2×2 factorial design: low n-3 PUFAs+trace IFs (N6−IF), low n-3 PUFAs+IFs (N6+IF), high n-3 PUFAs+trace IFs (N3−IF) and high n-3 PUFAs+IFs (N3+IF) (with 10 animals per group). Sham-operated rats (n=10) served as the reference control animals. All rats were housed individually in wire hanging cages with food and water available ad libitum in an animal facility maintained at 22±1°C and on a 12-h light–dark cycle. Animal care and experimental protocols were in compliance with the guidelines of the Purdue University Policy on Animal Care and Use.

All dietary treatments (Table 1) were formulated using a basal semi-purified diet (based on AIN-93G) with added fat providing 25% of dietary energy, a level similar to that in typical human intakes. Sham rats were fed a casein-based diet with a low level of n-3 PUFAs after adding 100% safflower oil (Dyets, Bethlehem, PA, USA). OVX rats were given soy protein-based diets (Protein Technologies International, St. Louis, MO, USA), which substituted for casein and were composed of two levels of IF content, either trace (−IF) or high (+IF) [0.03 and 3.43 mg (all forms)/g soy protein, respectively]. The aglycone content of IF was 0.02 and 2.00 mg/g soy protein for trace and high IF soy protein products, respectively. The lipid component of the soy diets was either 100% safflower oil (N6) or 72.6% safflower oil blended with 27.4% menhaden oil (N3), the latter provided a higher level of LC n-3 PUFAs.

2.2. Sample collections

After 12 weeks of feeding, rats were anesthetized with pentobarbital (60 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL, USA) and exsanguinated by cardiac

Table 1
Fatty acid and ingredient composition of the diets given to rats*

Fatty acid (wt.%)	Dietary treatments**				
	Sham	N6-IF	N6+IF	N3-IF	N3+IF
14:0 (myristic)	0.1	n.d.	n.d.	1.9	1.8
16:0 (palmitic)	6.4	6.5	6.5	9.3	9.5
16:1n-7 (palmitoleic)	0.1	n.d.	n.d.	2.4	2.4
18:0 (stearic)	2.3	2.3	2.3	2.6	2.6
18:1n-9 (oleic)	15.9	15.9	15.8	14.1	13.9
18:2n-6 (linoleic)	73.0	73.1	73.2	55.9	55.4
18:3n-3 (α-linolenic)	0.2	0.2	0.2	0.5	0.6
18:4n-3 (stearidonic)	n.d.	n.d.	n.d.	0.8	0.8
20:4n-6 (arachidonic)	n.d.	n.d.	n.d.	0.1	0.2
20:5n-3 (eicosapentaenoic)	n.d.	n.d.	n.d.	3.3	3.3
22:0 (behenic)	0.3	0.3	0.3	0.3	0.3
22:1n-9 (erucic)	0.3	0.3	0.3	0.1	0.3
22:5n-3 (docosapentaenoic)	n.d.	n.d.	n.d.	0.7	0.7
22:6n-3 (docosahexaenoic)	n.d.	n.d.	n.d.	4.3	4.2
SAT	28.2	28.1	28.1	33.0	33.2
MONO	16.6	16.6	16.5	17.2	17.2
PUFA	73.2	73.4	73.4	66.0	65.5
n-6 PUFAs	73.0	73.1	73.2	56.0	55.6
n-3 PUFAs	0.2	0.2	0.2	10.0	10.0
n-6/n-3	453.0	344.0	337.0	5.6	5.6

n.d. indicates not detected (peak detection at 10 ng); SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids.

* The semi-purified basal diet contained the following (g/kg): casein, 200; corn starch, 367.076; DYETROSE, 122; sucrose, 100; cellulose, 50; L-lysine, 3; choline bitartrate, 2.5; salt mix, 35; and vitamin mix, 10. Salt mix for the casein basal diet provided the following (mg/kg diet): $CaCO_3$, 357; K_2HPO_4, 196; $C_6H_5O_7K_3 \cdot H_2O$, 70.78; NaCl, 74; K_2SO_4, 46.60; MgO, 24; $C_6H_5O_7Fe$, U.S.P., 6.06; $ZnCO_3$, 1.65; $MnCO_3$, 0.63; $CuCO_3$, 0.3; KIO_3, 0.01; Na_2SeO_4, 0.01025; $(NH_4)_2MoO_4 \cdot H_2O$, 0.00795; $Na_2O_3Si \cdot 9H_2O$, 1.45; $CrK(SO_4)_2 \cdot 12H_2O$, 0.275; LiCl, 0.0174; H_3BO_3, 0.0815; NaF, 0.0635; $NiCO_3$, 0.0318; and NH_4VO_3, 0.0066. The soy treatments were prepared by substituting casein with soy protein (Dupont Protein Technologies International, product code FXP H0140 for the high IF soy protein and IB1.2 UN 30 CA0 for trace level IF soy protein) at 200 g/kg and contained the following (g/kg): corn starch, 367.076; DYETROSE, 122; sucrose, 100; cellulose, 50; L-cystine, 2.54; L-methionine, 2.54; choline bitartrate, 2.5; salt mix, 35; and vitamin mix, 10. Salt mix for the soy protein-based dietary treatments provided the following (mg/kg diet): $CaCO_3$, 102.97; $C_6H_5O_7K_3 \cdot H_2O$, 83.44; KCl, 82.36; K_2SO_4, 46.60; MgO, 19.40; $C_6H_5O_7Fe$, U.S.P., 1.21; $ZnCO_3$, 1.65; $MnCO_3$, 0.63; $CuCO_3$, 0.3; KIO_3, 0.01; Na_2SeO_4, 0.01025; $(NH_4)_2MoO_4 \cdot H_2O$, 0.00795; $Na_2O_3Si \cdot 9H_2O$, 1.45; $CrK(SO_4)_2 \cdot 12H_2O$, 0.275; LiCl, 0.0174; H_3BO_3, 0.0815; NaF, 0.0635; $NiCO_3$, 0.0318; and NH_4VO_3, 0.0066. Vitamin mix provided the following (mg/kg diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 7; niacin, 30; calcium pantothenate, 16; folic acid, 2; biotin, 0.2; cyanocobalamin (B_{12}) (0.1%), 25; vitamin A palmitate (500,000 IU/g), 8; vitamin E acetate (500 IU/g), 150; vitamin D_3, 2.5; vitamin K_1, 0.75.

** Dietary fat treatments included 100% safflower oil (Dyets) (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (Dyets) (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein of diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.02) (N6−IF and N3−IF) as aglycones.

puncture. Blood samples were immediately placed on ice to clot before the serum was separated by centrifugation. Right hindlimbs and vertebrae were harvested for ex vivo BMD measurements, the left femora were used for fatty acid analysis and the left tibiae were removed for preparation of frontal sections (10% neutral buffered formalin) of the proximal tibial metaphyses for static histomorphometric analyses. All other samples were kept on ice at the time of collection and promptly frozen at −80°C.

2.3. Dual-energy X-ray absorptiometry analysis

Initially (0 week, 1 week after OVX) and at 4-week intervals (i.e., 4, 8 and 12 weeks), in vivo two-dimensional BMD and bone mineral content (BMC) were assessed using peripheral dual-energy X-ray absorptiometry (DXA; pDEXA Densitometer Sabre, Norland Medical Systems, White Plains, NY, USA). Under ketamine–xylazine anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine; prescription obtained from the Veterinary Teaching Hospital Pharmacy, Purdue University), rats were placed in the supine position with the right hindlimb held at a right angle in external rotation and routinely scanned. The whole right femur and tibia, the cortical rich mid-shaft of the diaphysis as well as trabecular-rich subregions within the proximal and distal 25% ends of the bones were measured by DXA according to the protocol used by Dempster et al. [2]. Excised femora and tibiae with flesh removed were also subjected to DXA analysis. Briefly, bones were uniformly positioned and immersed in a saline water bath at a depth of 1.5 cm to enhance scanning resolution [26]. Excised lumbar vertebrae were scanned with flesh intact, and the BMD and BMC of the L4 and L1–4 regions were measured.

2.4. Serum bone biomarkers

Serum bone turnover markers for both formation and resorption were assessed. Bone-specific alkaline phosphatase (ALP) isoenzyme activity, an indicator of osteoblast activity in bones, was measured as previously described [5,6]. De novo serum carboxylated and decarboxylated rat osteocalcins, the unbound specific protein products released into circulation by osteoblast activity, were measured directly using a sandwich ELISA Kit (Biomedical Technologies, Stoughton, MA, USA). Bone resorption markers quantified included the bone and collagen degradation product pyridinoline cross-links (Pyd; Metra Serum Pyd EIA Kit, Quidel, USA) and the bone-specific Type 1 collagen degradation product deoxypyridinoline cross-links (Dpd; Metra Total Dpd EIA Kit, Quidel).

2.5. Fatty acid analysis of bone tissues

Lipids in the diet and in various tissue compartments of rat femora (periosteum and marrow) were extracted with chloroform/methanol (2:1, vol/vol). All excised femur bone samples were disarticulated but not defleshed to preserve the periosteal tissue and bone lipids during the frozen storage. Defrosted bones were carefully freed of musculature before procurement of the periosteum by gentle scraping with a scalpel to avoid disturbing or collecting the underlying bone matrix. Bone marrow was flushed out and harvested with methanol. Lipids from the diets and rat bone compartments

were saponified, and fatty acid methyl esters were prepared using boron trifluoride in methanol (10% wt/vol, Supelco Bellefonte, PA, USA) prior to gas chromatographic analysis as previously described [5,6].

2.6. Histomorphometry

Bone histomorphometric analyses were performed on methylmethacrylate-embedded undecalcified frontal sections (5-μm thickness) of proximal tibial metaphyses cut using a Leica RM2165 microtome. Static measurements were obtained from von Kossa/tetrachrome stained sections using a semiautomatic image analysis system (Osteomeasure Histomorphometry System, Osteometrics, Atlanta, GA, USA) [27]. Parameters measured included trabecular bone volume (BV/TV, %), osteoid volume (OV/BV, %), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm) and number (Tb.N, /mm), osteoblast and osteoclast surface (Ob.S/ and Oc.S/BS, %) and osteoblast and osteoclast number per millimeter bone perimeter (N.Ob/ and N.Oc/B.Pm, /mm). These measurements were performed in trabecular bone tissue in a standardized (2×2 mm) area beginning 1 mm distal to the growth plate–metaphyseal junction. This area contained only secondary spongiosae and excluded trabeculae connected to the osseous cortex. These parameters and their formulae are based on the recommendations of the American Society for Bone and Mineral Research Nomenclature Committee [28].

2.7. Statistical analyses

Evaluation of all data was performed by one-way (comparing all five groups) and two-way (comparing within the four OVX groups for main factors of two levels of soy IFs and two levels of n-3 PUFAs) ANOVAs using an SAS Data Analytical System. Results for variations between treatment groups are expressed as means±S.E.M. where applicable. When significant differences ($P \leq .05$) were identified, Tukey's Studentized Range Test was performed.

3. Results

3.1. Body weight, feed intake and OVX

The initial mean body weight for all rats was 206±1 g and was not significantly different among the dietary groups. However, at the termination of the study, differences in body weight gains ($P < .0001$) were found between the Sham (82±20 g) and OVX (N6−IF, 131±16 g; N6+IF, 144±28 g; N3−IF, 142±18 g; and N3+IF, 143±15 g) rats. Food consumption varied significantly ($P < .01$) between the Sham (95.1±6.6) and OVX rats [N6−IF, 107.0±11.8 g/week; N6+IF, 101.8±9.3 g/week; N3−IF, 105.9±7.8 g/week; N3+IF, 107.8±10.8 g/week], and feed efficiencies (total gram weight gain/total gram food consumed) ranged from 0.07 to 0.11 but were not different ($P > .8$). The success of OVX was confirmed by the observation of a marked atrophy of the uterine horns compared with the Sham rats ($P < .0001$).

3.2. DXA measurements

The in vivo measurements of BMD and BMC were determined at the beginning of the study (1 week after OVX) and at 4, 8 and 12 weeks into the feeding period (Figs. 1 and 2). The initial DXA results did not show any statistical difference among all dietary groups, although both femoral BMD and BMC measurements were more variable in the OVX rats compared with the values for the Sham rats. The initial femoral BMD value ranged from 0.149±0.009 to 0.152±0.009 g/cm^2 for the OVX rats and was 0.156±0.004 g/cm^2 for the Sham rats (Fig. 1A), and the BMC value ranged from 0.237±0.022 to 0.241±0.031 g for the OVX rats and was 0.246±0.011 g for the Sham rats (Fig. 1B).

No significant differences were detected in femoral BMDs among the OVX treatment groups at 4 (0.180±0.008−0.184±0.008 g/cm^2), 8 (0.190±0.006−0.194±0.006 g/cm^2) and 12 (0.192±0.006-0.199±0.007 g/cm^2) weeks; however, all OVX groups had lower values compared with the Sham group for each time interval ($P < .0002$ by one-way ANOVA). In contrast, the femoral BMC values (Fig. 1B) in the OVX treatment groups were

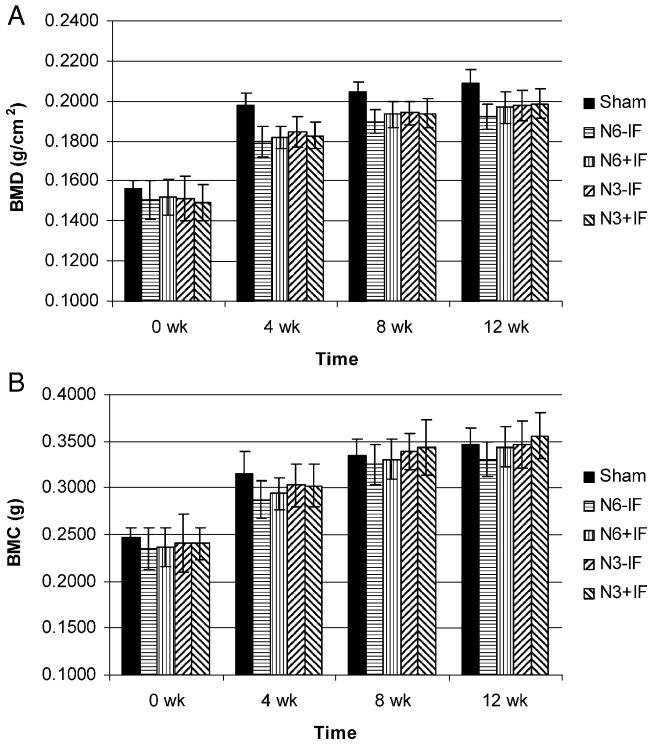

Fig. 1. Time course study on in vivo femoral BMD and BMC measurements in rats. Bars with different letters are statistically different (a and b for fat effect; x and y for soy IF effect) by two-way ANOVA. The Sham group was not included in the statistics. Dietary fat treatments included 100% safflower oil (Dyets) (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (Dyets) (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein in diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.04) (N6−IF and N3−IF) as aglycones.

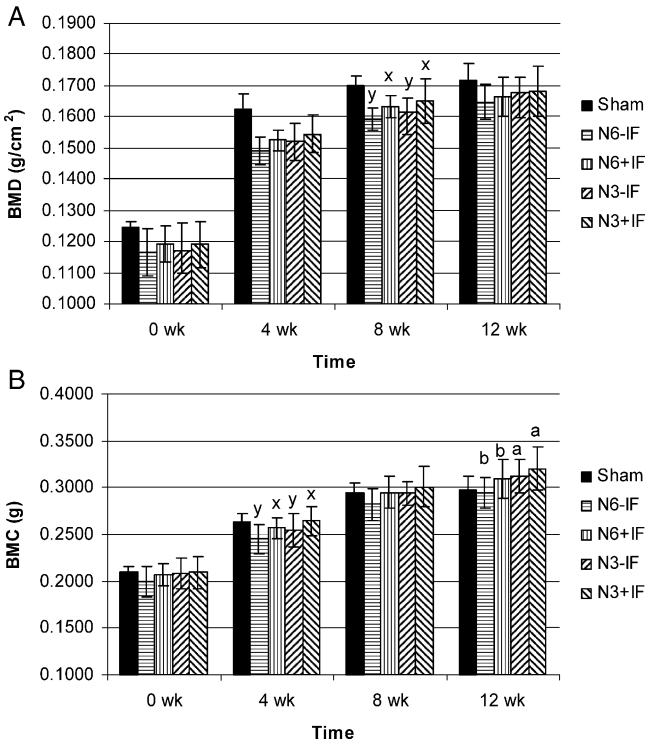

Fig. 2. Time course study on in vivo tibial BMD and BMC measurements in rats. Bars with different letters are statistically different (a and b for fat effect; x and y for soy IF effect) by two-way ANOVA. The Sham group was not included in the statistics. Dietary fat treatments included 100% safflower oil (Dyets) (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (Dyets) (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein in diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.04) (N6−IF and N3−IF) as aglycones.

similar to those in the Sham group for each time interval. At 4 weeks and beyond, the BMC values for the OVX rats given n-3 PUFAs (N3−IF or N3+IF) were not different from those for the Sham rats ($P>.06$ by one-way ANOVA).

The initial BMD and BMC values for tibiae in the OVX and Sham rats were very similar although the BMD values were highly variable in the OVX groups (Fig. 2A and B). The BMD measurements at 8 and 12 weeks in the OVX rats nearly approached the level for the Sham group; however, at 8 weeks, the +IF groups, independent of PUFA type, were higher compared with the −IF groups (Fig. 2A). At 12 weeks, the OVX rats fed the N3+IF diet were not different from the Sham rats. The BMC values for tibiae of OVX rats given the +IF diets (N6+IF and N3+IF) had higher values for BMC compared with rats fed the low −IF diets at 4 weeks (two-way ANOVA, $P=.03$; Fig. 2B). While at 12 weeks, a significant effect of dietary fat treatment was apparent in the rats fed the n-3 PUFA diets (N3±IF), which had higher tibial BMC values compared with those given the n-6 PUFA diets (N6±IF) independent of soy IFs (two-way ANOVA, $P=.03$; Fig. 2B).

An important finding is that the values for tibial BMC in OVX rats given LC n-3 PUFAs increased during the 12-week dietary study and were equal to or greater than the values for the Sham group. The results in tibiae were consistent with the values for femoral BMC in OVX rats at 8 and 12 weeks when compared with the Sham group.

At the termination of the 12-week feeding study, hindlimbs were collected from rats and the excised bones were subjected to DXA measurements to assess BMD and BMC and the data were analyzed by two-way ANOVA (Table 2). For tibial measurements, the BMC and bone area and length were all higher in rats given the n-3 PUFA diets independent of soy IFs (N3±IF) compared with the values for rats fed the high n-6 PUFA diets (N6±IF). Values for tibial BMC in the N3 group were greater than those in the N6 group and equal to those in the Sham group. The DXA measurements for excised bones are consistent with the higher in vivo BMC measurements for tibiae of OVX rats given the N3 diets

Table 2
BMD and BMC measurements on excised bones of rats after 12 weeks of feeding safflower and menhaden oils with different levels of soy protein IFs*

Measurement		Dietary treatments**					Pooled S.E.M.	ANOVA P		
		Sham	N6−IF	N6+IF	N3−IF	N3+IF		Soy	Fat	Soy × Fat
Femur	BMD (g/cm^2)	0.205	0.183	0.191	0.188	0.189	0.009	.2	.7	.2
	BMC (g)	0.378	0.352	0.369	0.375	0.367	0.028	.6	.2	.2
	Area (cm^2)	1.846	1.919	1.926	1.990	1.941	0.093	.5	.2	.4
	Length (mm)	36.0	36.6^b	36.9^b	37.5^a	37.7^a	0.9	.4	.009	.8
Tibia	BMD (g/cm^2)	0.179	0.166	0.171	0.170	0.170	0.006	.2	.4	.2
	BMC (g)	0.278	0.268^b	0.281^b	0.290^a	0.291^a	0.019	.2	.01	.3
	Area (cm^2)	1.549	1.614^b	1.645^b	1.702^a	1.710^a	0.069	.4	.001	.6
	Length (mm)	39.2	40.1^b	40.5^b	40.8^a	40.8^a	0.8	.5	.04	.4

* Mean values for the DXA measurements ($n=10$) within a row having different superscripts (x and y for soy effect; a and b for fat effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Tukey's range test ($P<.05$). The BMD for lumbar vertebrae was also measured and the Sham group had higher BMD values than all the treatment groups. The values for L1–4 BMD (g/cm^2) measurement, Sham group=0.185; the range for treatment groups, 0.160–0.167. The values for L4 BMD measurement, Sham group=0.185; the range for treatment groups, 0.156–0.166. Sham group values were not used in the statistical analysis.

** Dietary fat treatments (Dyets) included 100% safflower oil (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein in diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.02) (N6−IF and N3−IF) as aglycones.

presented in Fig. 2B. A longer femoral bone length in rats fed the N3 diets (N3±IF) was apparent and comparable with that found in rats fed the N6 diets (N6±IF); the femoral BMC values for the N3 group were similar to those for the Sham group.

Further analysis of the DXA data on specific regions of excised rat femoral and tibial bones indicated differences consistent with the whole bone measurements. In brief, although most of the BMD measurements were lower in OVX rats compared with Sham rats, the distal femur BMD values (rich in metabolically active trabecular bone) were higher in rats given the N6+IF treatment compared with those not given soy IFs (N6−IF). In addition, the BMC values for femoral diaphysis were higher in the rats given the N3+IF diet (0.173±0.003 g) compared with the Sham group (0.153±0.003 g) and the N6−IF group (0.156±0.003 g). These findings suggest that the dietary IF source protected against bone loss in OVX rats fed the n-6 PUFA diet (greater bone loss) and that LC n-3 PUFAs also conserved bone mineral in the estrogen-deficient state (OVX).

The whole bone in vivo DXA data were analyzed by one-way ANOVA that included the Sham group in the statistical model. The tibial BMC values were greater in rats given the N3+IF diet compared with those in the N6−IF group after 4 and 12 weeks of dietary treatment and soy (+IF) also improved the bone DXA measurements; however, the BMC values in the N6−IF group were lower in the femur and the tibia at 4 weeks when compared with those in the Sham group. The L4 BMC DXA values for excised lumbar vertebrae were lowest in rats given the n-6 PUFA diet (N6−IF treatment) compared with the Sham rats. Likewise, in vitro distal femur BMD at 12 weeks was lowest in rats fed the n-6 PUFA diets compared with Sham rats. Femur diaphysis BMC was lowest in the N6−IF rats compared with the N3+IF rats but not with the Sham rats. The DXA data revealed that feeding of the n-6 PUFA diet without soy IFs or a low intake of LC n-3 PUFAs led to greater bone loss during estrogen deficiency. The combination of EPA and DHA with soy IFs in this study was effective in maintaining a higher BMC in the rat model of estrogen-deficient bone loss.

3.3. Serum bone biomarkers

At the termination of the 12-week study, serum bone biomarkers for rats indicated a protective effect for n-3 PUFAs and soy IFs on reducing bone resorption during OVX (Table 3). Serum bone resorption marker Pyd was lowest in rats fed n-3 PUFAs (N3+IF diet) compared with rats given n-6 PUFAs (N6+IF diet). No significant changes were found for Dpd, another bone resorption marker, or for osteocalcin and bone-specific ALP activity, which are two commonly used bone formation biomarkers.

3.4. Tissue fatty acid analysis

Fatty acid compositional analysis of femoral bone compartments shows that the dietary source of LC n-3 PUFAs (N3 treatments, independent of soy IFs) led to significantly higher levels of EPA and DHA and a reduction in the amounts of n-6 PUFAs. The fatty acid composition of periosteum reflected the general influence of the dietary PUFA source on bone tissue fatty acid composition (Table 4). For example, periosteum isolated from rats fed the n-6 PUFA diets (N6±IF) demonstrated increased amounts of 18:2n-6, 20:4n-6, 22:4n-6, total n-6 PUFAs and the ratio of n-6 to n-3 PUFAs. Conversely, rats fed the n-3 PUFA diets (N3±IF) showed increased amounts of 18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3 and total n-3 PUFAs compared with the N6 rats (Table 4).

A similar pattern was also found in the marrow, which reflected the influence of the dietary PUFA treatments; however, marrow contained significantly lower amounts of n-3 PUFAs compared with the periosteum for the same dietary treatment groups. For example, for rats in the N3 groups, the total n-3 PUFA content in periosteum ranged from 9.6% to 10.0% of total fatty acids, while in the marrow it was only from 3.2% to 3.4% of total fatty acids, a level that was only one third of that found in the periosteum. The ranges (percentages) for major n-6 and n-3 PUFAs in the marrow were as follows: 18:2n-6, 44.5–44.8 for those in the N6 groups and 38.4–38.5 in the N3 groups; 20:4n-6, 3.0–3.3 in N6 and 2.0–2.4 in N3; 20:5n-3, not detected in

Table 3
Rat serum Pyd, osteocalcin, Dpd and bone-specific ALP activity (BALP) after 12 weeks of feeding safflower and menhaden oils with different levels of soy protein IFs*

Measurement	Dietary treatments**					Pooled S.D.	ANOVA *P*		
	Sham	N6−IF	N6+IF	N3−IF	N3+IF		Soy	Fat	Soy × Fat
Serum Pyd (nmol/L)	2.53	2.15^{AB}	2.75^{A}	2.26^{AB}	1.89^{B}	0.17	.03	.5	.006
Osteocalcin (nmol/L)	10.66	12.26	11.33	11.17	10.51	2.41	.4	.3	.9
Dpd (nmol/L)	6.68	8.87	9.38	8.34	10.11	3.29	.4	.95	.6
BALP (U/L)	10.30	10.10	10.11	11.00	8.90	2.67	.2	.9	.4

* Mean values for the DXA measurements (for osteocalcin and Dpd, $n=6$ for Sham group and $n=7$ for the rest; for BALP, $n=9$ for N6+IF and $n=10$ for the rest) within a row having different superscripts (x and y for soy effect; a and b for fat effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Tukey's range test ($P<.05$). Sham group values were not used in the statistical analysis.

** Dietary fat treatments (Dyets) included 100% safflower oil (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein in diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.02) (N6−IF and N3−IF) as aglycones.

Table 4
Fatty acid composition of femur periosteum from rats fed the different dietary treatments*

Fatty acid (area%)	Dietary treatments**					Pooled S.E.M.	ANOVA *P*		
	Sham	N6−IF	N6+IF	N3−IF	N3+IF		Soy	Fat	Soy × Fat
14:0	0.8	0.9^{b}	0.9^{b}	1.4^{a}	1.5^{a}	0.1	.4	<.0001	.2
16:0	18.2	17.8^{C}	17.6^{C}	19.2^{B}	20.8^{A}	0.4	.06	<.0001	.03
16:1n-7	1.7	2.0^{b}	2.1^{b}	2.8^{a}	3.0^{a}	0.3	.5	.0001	.7
17:0	0.2	0.1^{b}	0.2^{b}	0.3^{a}	0.2^{a}	0.1	.8	.0005	.3
18:0	8.6	7.6	7.7	7.8	7.9	0.4	.9	.8	.9
18:1n-9	13.0	14.0	13.8	14.0	13.6	0.5	.6	.9	.9
18:1n-7	2.1	1.8^{bx}	1.8^{by}	2.3^{ax}	2.2^{ay}	0.1	.01	<.0001	.9
18:2n-6	36.1	40.4^{a}	40.5^{a}	35.2^{b}	34.1^{b}	0.7	.7	<.0001	.6
18:3n-3	0.1	0.2^{b}	0.2^{b}	0.3^{a}	0.3^{a}	0.1	.8	.03	.4
18:4n-3	n.d.	$n.d.^{b}$	$n.d.^{b}$	0.1^{a}	0.1^{a}	0.1	.9	.0003	.9
20:1n-9	n.d.	0.1	0.03	0.9	0.04	0.1	.2	.7	.9
20:2n-6	0.3	0.3^{a}	0.3^{a}	0.2^{b}	0.1^{b}	0.1	.1	.002	.9
20:3n-6	0.3	0.2	0.3	0.3	0.3	0.1	.6	.8	.6
20:4n-6	9.6	7.8^{a}	7.9^{a}	4.2^{b}	4.1^{b}	0.5	.99	<.0001	.8
20:4n-3	n.d.	$n.d.^{b}$	$n.d.^{b}$	0.1^{a}	0.1^{a}	0.1	.5	.0008	.5
20:5n-3	n.d.	$n.d.^{b}$	$n.d.^{b}$	0.8^{a}	0.8^{a}	0.1	.06	<.0001	.06
22:0	0.03	0.1	0.1	0.04	0.1	0.1	.6	.7	.8
22:4n-6	1.2	0.8^{a}	1.0^{a}	0.2^{b}	0.1^{b}	0.2	.6	<.0001	.1
22:5n-3	0.2	0.1^{b}	0.1^{b}	1.1^{a}	1.1^{a}	0.1	.4	<.0001	.9
22:6n-3	2.8	1.6^{b}	1.9^{b}	7.2^{a}	7.7^{a}	0.4	.5	<.0001	.9
SAT	27.9	26.6^{b}	26.6^{b}	28.9^{a}	30.5^{a}	0.5	.2	<.0001	.2
MONO	16.8	17.9	17.6	19.1	18.8	0.5	.6	.1	.99
PUFA	50.5	51.4^{a}	52.1^{a}	49.5^{b}	48.6^{b}	0.5	.9	.0002	.2
n-6 PUFA	47.5	49.5^{a}	49.9^{a}	40.0^{b}	38.6^{b}	0.5	.5	<.0001	.3
n-3 PUFA	3.0	1.8^{b}	2.2^{b}	9.5^{a}	9.9^{a}	0.4	.4	<.0001	.9
$n\text{-}6_{LC}$	10.7	8.6^{a}	8.9^{a}	4.4^{b}	4.2^{b}	0.6	.9	<.0001	.7
$n\text{-}3_{LC}$	3.0	1.6^{b}	2.0^{b}	9.1^{a}	9.6^{a}	0.6	.4	<.0001	.9
$n\text{-}6_{LC}/n\text{-}3_{LC}$	18.5	26.6^{a}	24.5^{a}	0.5^{b}	0.4^{b}	0.3	.6	<.0001	.4
n-6/n-3	15.7	16.2^{a}	22.4^{a}	4.2^{b}	3.9^{b}	1.0	.8	<.0001	.7

$n\text{-}6_{LC}$=20:4n-6+22:4n-6; $n\text{-}3_{LC}$=20:5n-3+22:5n-3+22:6n-3.

* Mean values for the fatty acid measurements (n=8) within a row having different superscripts (x and y for soy effect; a and b for fat effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Tukey's range test (P<.05). Sham group values were not used in the statistical analysis.

** Dietary fat treatments (Dytes) included 100% safflower oil (Sham and N6±IF) and 72.6% safflower oil+27.4% menhaden oil (N3−IF and N3+IF). Total fat content in each diet was 110.4 g/kg of diet. IF containing compounds in the diet (mg/g protein in diet) provided genistein (1.04), daidzein (0.82), glycitein (0.14) or total IF (2.00) (N6+IF and N3+IF) and genistein (0.01) and daidzein (0.01) or total IF (0.02) (N6−IF and N3−IF) as aglycones.

N6 and 0.4–0.5 in N3; 22:4n-6, 0.6–0.7 in N6 and 0.2–0.2 in N3; 22:5n-3, not detected in N6 and 0.7–0.7 in N3; 22:6n-3, 0.01–0.06 in N6 and 1.5–1.8 in N3; total saturated fatty acids, 25.4–25.4 in N6 and 28.6–29.4 in N3; total monounsaturated fatty acids, 22.4–23.5 in N6 and 24.3–24.4 in N3; total PUFAs, 49.4–50.1 in N6 and 44.5–48.3 in N3; total n-6 PUFAs, 49.1–49.9 in N6 and 41.3–41.9 in N3. These results demonstrate that in OVX rats, dietary treatment with LC n-3 PUFAs significantly increased the amounts of these fatty acids in all bone tissue compartments and reduced those for n-6 PUFAs. These data in bone support the hypothesis that dietary LC n-3 PUFAs can reduce the concentrations of AA, a pro-inflammatory prostanoid precursor, while elevating the concentrations of EPA, which is much less inflammatory.

3.5. Histomorphometry

Static histomorphometric analyses were performed on left proximal tibiae, and, as expected, the data showed a clear difference between the Sham and the OVX rats inclusive of all the dietary treatments, indicating greater bone remodeling after OVX. The osteoblast and osteoclast surface (Ob.S/ and Oc.S/BS) values were higher (P<.02) in the OVX rats (7.7–9.33% and 13.3–17.9%, respectively) compared with the Sham rats (2.0–8.5%, respectively). Consistent with the osteoblast and osteoclast surface values, the N.Ob/ and N.Oc/B.Pm values were higher in the OVX groups compared with the Sham group (P<.01).

Although the differences for most of the parameters measured among the OVX treatment groups were not statistically different, a benefit of soy protein IF is apparent based on the higher values for trabecular number (Tb.N) in rats that received the +IF diets (Tb.N=1.03–1.61 for the +IF groups) compared with those in the low IF groups (Tb.N=0.58–0.63 for rats fed the −IF diets). Bone volume values for OVX rats were also marginally higher (P=.06) in the +IF treatment groups (3.98–5.03) compared with those in the trace IF (−IF) groups (2.27–2.56).

4. Discussion

In our current study, a positive effect on bone metabolism was observed when OVX rats were given soy protein IFs and LC n-3 PUFAs. In general, the mixture of soy IFs and LC n-3 PUFAs attenuated bone loss in OVX rats, which was reflected in higher femoral and tibial BMC values, compared with those given n-6 PUFAs. In the case of tibial bones, BMC values were higher in rats fed LC n-3 PUFAs compared with the n-6 PUFA treatment and Sham groups. The sparing effect of soy protein containing a higher amount of IFs on bone mineral (DXA measurements) was apparent in hindlimbs (femur and tibia) and lumbar vertebrae of OVX rats compared with those given diets containing trace amounts of soy IFs and/or n-6 PUFAs. The DXA data from OVX rats fed soy IF diets (+IF) indicated a protective action of reduced bone resorption following estrogen deficiency and were further supported by the bone histomorphometric data of a twofold to threefold greater trabecular number. Although in this study the comparison of OVX rats with Sham rats does not reflect a positive control such as estrogen treatment, it does provide a point of reference for OVX to the intact female rat.

These new findings are consistent with our previous investigations where LC n-3 PUFAs improved bone metabolism in growing animals. We showed in rats that supplementing a diet with LC n-3 PUFAs resulted in greater bone formation rates and reduced ex vivo PGE_2 production in bone organ cultures [5], and a protective effect was observed on minimizing bone mineral loss in OVX rats compared with Sham rats [7]. In osteoblast-like MC3T3-E1 cell cultures, we reported that LC n-3 PUFAs modulated cyclooxygenase (COX)-2 protein expression, reduced PGE_2 production and increased ALP activity [7].

Other studies support our view that n-3 PUFAs promote bone formation and exert a positive effect by inhibiting bone resorption. Iwami-Morimoto et al. [29] studied alveolar bone resorption in 4-week-old rats given diets supplemented with 10% of either fish oil or corn oil for 6 weeks. Dietary fish oil reduced osteoclastic activity (osteoclast number was only 60% of control) and alveolar bone resorption (80% of control). Claassen et al. [30] showed that feeding essential fatty acid-deficient growing male rats a mixture of γ-linolenic acid and EPA effectively inhibited bone resorption (lowered urinary excretion of pyridinium cross-links and hydroxyproline cross-links) compared with rats fed LA+α-linolenic acid.

The mechanism for the bone-sparing actions of n-3 PUFAs and IFs could be targeted on the regulation of prostanoid formation. It seems that COX-2-dependent PG synthesis is necessary for osteoclastogenesis and bone resorption induced by cytokines such as interleukin (IL)-1β, tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS) [31], which are elevated during and aggravate the symptoms of estrogen deficiency on bones [32]. Moreover, genistein stimulated the production of osteoprotegerin (a protective factor against bone resorption), suggesting that this phytoestrogen may help reduce bone resorption by decreasing osteoclastogenesis [33].

In contrast to LC n-3 PUFAs, the n-6 PUFAs have been shown to increase the production of pro-inflammatory cytokines (TNFα and IL-6) and reactive oxygen species such as the inducible nitric oxide (NO) that can potentially mediate some of the deleterious effects associated with cytokines on bone resorption in osteoarthritis [34]. In a human osteoblast-like cell line MG-63 culture, gene expression (mRNA level) of the pro-inflammatory cytokines IL-1α, IL-1β and TNFα was significantly increased by AA treatment while EPA caused a significant inhibition of expression [35]. Supplementation of n-3 PUFAs to cultures of human OA cartilage explants abolished the expression of mRNA for mediators of inflammation (COX-2, 5-lipoxygenase, TNFα and IL-1) [36]. An increase in the production of constitutive NO is associated with decreased number and diminished activity of osteoclastic cells and therefore decreased bone resorption [37]. Furthermore, activation of the inducible NO synthase pathway and an increase in the concentration of NO under inflammatory conditions have been linked to enhanced osteoclastic activity and bone resorption in inflammation-induced osteoporotic animals [38].

Genistein and daidzein are naturally occurring diphenolic IFs that exhibit structural similarities to natural and synthetic estrogens. Although a protein tyrosine kinase inhibitor, genistein selectively reduced COX-2 expression without modification of COX-1 after induction by PMA (phorbol ester activator of PKC) and IL-1 (bone resorption inducer) in human endothelial cells [39]. Genistein prevented IL-1 induced expression of COX-2 and production of PGE_2 in human islets [40] and inhibited PGE_2 synthesis in NRK cells (a fibroblastic clone) when induced by EGF [41]. Genistein also inhibited the induction of COX-2 protein and activity in rats with endotoxic shock [41,42], blocked the production of PGE_2 in macrophages stimulated by LPS [43] and inhibited COX activity in newborn pig endothelial cells [44]. These findings support the premise that genistein has a general negative effect on PGE_2 production, which could down-regulate osteoclastic activity to favor bone formation that would be additive to the recognized actions of LC n-3 PUFAs in moderating inflammation.

Relatively higher BMD and lower fracture incidence in postmenopausal women from China and Japan compared with women in western countries have promoted the supposition that a diet of n-3 PUFAs (specific fish and plant oils) and soy food (sources of IFs) protects against bone loss associated with estrogen decline. Investigations either in animal models or in human subjects have demonstrated that IFs from soy food products spare bone loss following estrogen deficiency. In contrast to these findings, soy protein administered for 7 months to monkeys did not prevent bone loss but actually increased bone turnover on the endosteal surface of the femoral mid-shaft when compared with those fed casein/lactabumin [45].

Register et al. [46] reported that feeding soy protein with or without phytoestrogens for 3 years to skeletally mature OVX macaques did not afford any protective effect on bone loss during estrogen deficiency. In a 3-month placebo-controlled double-blind randomized human study with 106 postmenopausal women, soy protein supplement decreased triglycerides and LDL cholesterol but did not affect the levels of urinary bone resorption markers Pyd and Dpd [47].

A recent human case–control (double-blind, randomized and placebo-controlled) study showed that administering postmenopausal subjects 25.6 g of soy protein (99 mg of total IFs, 52 mg genistein and 41 mg daidzein) for 1 year did not affect overall bone mineral measurements compared with control subjects who were given milk protein in place of the soy protein [25]. However, an interesting finding was that in women who became menopausal within the past 14 years, soy protein consumption did show a positive effect of increased BMD for the hip and the lumbar spine compared with those who became menopausal more than 22 years earlier [25]. An epidemiological study on Chinese postmenopausal women suggests that soy protein intake was not associated with BMD/BMC values within the first 4 years of menopause; but, with the progression of menopause, soy protein intake was significantly correlated with greater hip BMD and total body BMC by stepwise multiple linear regression analysis [48]. In a recent review that summarized several in vitro and in vivo (animal and human) studies on dietary phytoestrogens, the authors concluded that diets rich in phytoestrogens have a bone-sparing effect long term, although the mechanism of action and the extent of the benefits are not known [49]. From these studies, it seems that the timing of supplementation is a critical factor for achieving a desirable outcome with soy protein (IFs) intervention on estrogen-deficient bone loss.

Our study showed that soy IFs protected BMC in OVX rats. Although it is problematic to extrapolate findings in rats to humans, in our study, the IF intake when expressed as metabolic body size in rats was higher compared with humans (2.7 mg/kg BW, Asian subjects [25]). Yet, it is more difficult to compare dietary IF intake since bioavailability data are variable in humans [50] and limited data are available in rodents (estimated to be 15% in mice [51]).

In summary, this research examined the effects of dietary sources of soy IFs and LC n-3 PUFAs, which were hypothesized to augment bone preservation in 2-month-old sexually mature OVX rats. Data for sequential tibial BMD and BMC measurements revealed bone conservation in OVX rats fed IFs and LC n-3 PUFAs. Two underlying mechanisms could be responsible for our findings. First, the soy IF diet was protective in attenuating bone resorption during acute estrogen deficiency and rapid bone loss as evidenced by the early (4 weeks) conservation of BMC in the tibia. In this case, the IFs may minimize osteoclast activity and a significant dietary interaction was responsible for the lower level of Pyd. Second, the LC n-3 PUFAs, which have been reported to support bone formation, resulted in higher BMC at 12 weeks. The n-3 PUFAs may act through osteoblasts to maintain greater bone mass, which is consistent with our work in rats [5] and osteoblasts [7]. These findings indicate a complementary action of the dietary combination for attenuating bone mineral reduction in OVX rats. Significant research is needed to understand the relationships of these food components on bone health; however, success of the dietary intervention may be associated with administering the treatments at the time of estrogen decline in OVX rats.

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