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Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats

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Abstract

Hypoestrogenic states escalate bone loss in animals and humans. This study evaluated the effects of the amount and ratio of dietary n-6 and n-3 polyunsaturated fatty acids (PUFAs) on bone mineral in 3-month-old sexually mature ovariectomized (OVX) Sprague–Dawley rats. For 12 weeks, the rats were fed either a high-PUFA (HP) or a low-PUFA (LP) diet with a ratio of n-6/n-3 PUFAs of 5:1 (HP5 and LP5) or 10:1 (HP10 and LP10). All diets (modified AIN-93G) provided 110.4 g/kg of fat from safflower oil and/or high-oleate safflower oil blended with n-3 PUFAs (DHASCO oil) as a source of docosahexaenoic acid (DHA). Fatty acid analyses confirmed that the dietary ratio of 5:1 significantly elevated the amount of DHA in the periosteum, marrow and cortical and trabecular bones of the femur. Dual-energy X-ray absorptiometry measurements for femur and tibia bone mineral content (BMC) and bone mineral density showed that the DHA-rich diets (HP5 and LP5) resulted in a significantly lower bone loss among the OVX rats at 12 weeks. Rats fed the LP diets displayed the lowest overall serum concentrations of the bone resorption biomarkers pyridinoline (Pyd) and deoxypyridinoline, whereas the bone formation marker osteocalcin was lowest in the HP groups. Regardless of the dietary PUFA content, DHA in the 5:1 diets (HP5 and LP5) preserved rat femur BMC in the absence of estrogen. This study indicates that the dietary ratio of n-6/n-3 PUFAs (LP5 and HP5) and bone tissue concentration of total long-chain n-3 PUFAs (DHA) minimize femur bone loss as evidenced by a higher BMC in OVX rats. These findings show that dietary DHA lowers the ratio of 18:2n-6 (linoleic acid)/n-3 in bone compartments and that this ratio in tissue correlates with reduced Pyd but higher bone alkaline phosphatase activity and BMC values that favor bone conservation in OVX rats.

Keywords: Docosahexaenoic acid; Ovariectomy; Rat; BMC; Ratio of n-6/n-3 PUFAs

1. Introduction

Osteoporosis is a condition of decreased bone mass that is prevalent in postmenopausal women and places them at risk of fractures. In women, increased bone turnover with a relative increase in bone resorption results in a sharp decline in bone mass with the loss of estrogen after menopause. In female rats, ovariectomy artificially induces a marked reduction in endogenous estrogen concentrations subsequently causing a negative bone remodeling balance that augments bone loss and increases the incidence of osteopenia [1]. The similarities in the pathophysiology of bone loss caused by ovariectomy in this animal model and postmenopausal osteoporosis in women have been extensively examined such that ovariectomized (OVX) rats are a well-accepted model for investigating agents that could help in the prevention of this disease [2,3].

During menopause, the osteoprotective effect of estrogen is compromised due to diminishing levels of this hormone that lead to elevated secretions of bone-resorptive agents such as interleukin (IL)-1, tumor necrosis factor-α (TNFα) and prostaglandins [prostaglandin E_2 (PGE_2)], which are strong promoters of osteoclastogenesis and osteoclastic activity [4–6]. Moreover, attenuation of PGE_2 production

Abbreviations: AA, arachidonic acid; ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; COX, cyclooxygenase; DHA, docosahexaenoic acid; Dpd, deoxypyridinoline; DXA, dual-energy X-ray absorptiometry; EPA, eicosapentaenoic acid; IL, interleukin; LA, linoleic acid; OVX, ovariectomized; PGE_2, prostaglandin E_2; PUFAs, polyunsaturated fatty acids; Pyd, pyridinoline; TNFα, tumor necrosis factor-α.

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in osteoblastic-like cell cultures is associated with increased bone formation markers [7–9]. Among the factors that have been tested to modulate bone cell functions, certain families of the polyunsaturated fatty acids (PUFAs) have been found to be effective in altering the activities of both osteoblasts and osteoclasts, thus affecting bone formation and bone resorption. As the structural components of cell membrane bilayers as well as the precursors of a series of potent biologically active eicosanoids, n-3 PUFAs change the fatty acid composition of membrane phospholipids to influence cellular metabolism, specifically the biosynthesis of bone resorptive PGE_2 [10]. n-3 PUFAs also decrease the secretion of noninduced IL-6 and TNFα from cultured bone marrow cells and IL-1β levels in vivo (i.e., in bone marrow plasma). Another important effect of n-3 PUFAs is the significant elevation in the secretion of IL-10, which is a known anti-inflammatory cytokine [11]. Therefore, the biochemical and physiological actions of n-3 PUFAs on prostaglandin metabolism and cytokine production could explain their beneficial effects on preserving bone mass during the estrogen-deficient state.

Our laboratory and others have reported that long-chain (LC) n-3 PUFAs are effective in promoting bone formation in growing animals. 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 PGE_2 production and increased bone formation rates in the tibia and femur [10,12]. Our data also indicate that higher intakes of n-6 PUFAs lead to increased ex vivo bone PGE_2 production, which is associated with an elevated ratio of AA/EPA in bone and reduced bone formation rate [10]. A high dietary ratio of n-6/n-3 PUFAs is therefore believed to reduce bone formation capacity and cause greater bone resorption activity [13] through increased endogenous production of PGE_2. Hence, these findings on reducing the capacity for PGE_2 biosynthesis in bone and in osteoblast-like cell cultures [13,14] are evidence for the premise that dietary LC n-3 PUFAs support bone cell activities for sustaining bone formation. In contrast, the level of urinary pyridinium cross-links (markers of bone resorption) was significantly higher in rats fed an n-6 PUFA diet compared with those fed an n-3 PUFA diet [15]. Different dietary ratios of n-6/n-3 PUFAs were also tested in piglets for their effects on growth and bone metabolism, revealing that higher n-3 PUFA levels in the blood were associated with lower bone resorption [16]. The protective effect of n-3 PUFAs on bone loss was also shown in OVX rodents. For example, an EPA-enriched diet was effective in minimizing bone loss induced by estrogen deficiency, which prevented the decline of bone weight and strength in OVX rats [17], and feeding fish oil to OVX mice also attenuated bone loss [5].

The purpose of the current investigation was to test the hypothesis that a dietary source of LC n-3 PUFAs and lower ratio of n-6/n-3 PUFAs would reduce bone loss in estrogen-deficient rats. A 12-week study was undertaken to examine the effect of dietary DHA and total PUFAs (and total n-6 PUFAs) levels on femur and tibia bone density measurements in OVX rats. The fatty acid compositions of femoral bone tissue compartments and biomarkers in circulation were also assessed to confirm dual-energy X-ray absorptiometry (DXA) data.

2. Materials and methods

2.1. Animals and diets

Three-month-old virgin female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) initially weighing 221±6 g were either OVX (n=40) or sham operated (sham, n=10). Success of ovariectomy was confirmed by determination of wet weights of uterine horns compared with sham rats at the time of tissue collection (mean uterine weight for the sham group, 0.409±0.045 g; range for OVX groups, 0.089±0.005–0.114±0.007 g; P<.0001) [18]. The 40 OVX rats were randomly assigned to one of the four dietary treatments and the sham rats were given the control diet (n=10/group; Table 1). For 12 weeks, all rats were fed a casein-based semipurified basal diet (AIN-93G, Dyets, Bethlehem, PA, USA) with 25% of energy derived from fat. The treatments included two levels for the ratio of n-6/n-3 PUFAs and two levels of total PUFAs in the diets following a 2×2 factorial design. The diets with a high-PUFA (HP) level at two ratios of n-6/n-3 PUFAs (5:1 or 10:1), designated as HP5 and HP10, were formulated by mixing safflower oil with DHASCO oil (DHA-rich single-cell oil, Martek Biosciences, Baltimore, MD, USA); the low-PUFA diets (LP5 and LP10) were formulated by addition of a high-oleate safflower oil (Oil Seeds International, San Francisco, CA, USA) and DHASCO oil as shown in Table 1. Sham rats were fed a control diet (AIN-93G) containing 100% safflower oil [rich in linoleic acid (LA)]. 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.

2.2. Sample collections

After 12 weeks of feeding, rats were anesthetized with pentobarbital (60 mg/kg, Nembutal, Abbot Laboratories, North Chicago, IL, USA) and exsanguination by cardiac puncture to collect blood followed by cervical dislocation. Blood was immediately placed on ice to clot before the serum was separated by centrifugation. Right hindlimbs and lumbar vertebrae were harvested for ex vivo bone mineral and density measurements, and left femurs were used for fatty acid analyses. All samples were kept on ice at the time of collection and promptly frozen at −80°C.

Table 1
Fatty acid composition and ingredients of diets fed to rats

Fatty acid (wt%)	Dietary treatment[a,b,c,d]				
	Sham	HP5	HP10	LP5	LP10
12:0	0.5	0.3	0.3	0.2	0.5
14:0	0.1	2.5	1.4	1.4	0.8
16:0	6.5	7.4	7.1	6.1	5.9
16:1n-7	0.1	0.5	0.3	0.3	0.2
18:0	2.3	2.0	2.1	2.0	2.0
18:1n-9	16.2	18.7	17.7	50.8	52.7
18:2n-6	72.9	56.4	63.6	31.9	34.7
18:3n-3	0.2	0.1	0.1	0.1	0.1
20:0	0.3	0.3	0.3	0.3	0.4
20:1n-9	0.2	0.2	0.2	0.2	0.2
22:0	0.3	0.3	0.3	0.3	0.3
22:1n-9	0.3	0.2	0.3	0.1	0.1
22:6n-3	0.1	10.5	5.9	6.0	3.2
24:1n-9	0.2	0.2	0.2	0.2	0.2
SATs	9.7	13.1	11.7	10.6	9.6
MONOs	16.8	19.6	18.5	51.4	53.3
PUFAs	73.1	67.0	69.7	37.9	38.0
n-6 PUFAs	72.9	56.4	63.6	31.9	34.7
n-3 PUFAs	0.2	10.6	6.0	6.1	3.3
n-6/n-3 PUFAs	298.0	5.3	10.5	5.3	10.6

SATs indicate total saturated fatty acids; MONOs, total monounsaturated fatty acids.

[a] The semipurified 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.

[b] The salt mix provided the following (mg/kg diet): $CaCO_3$, 12,495; K_2HPO_4, 6860; $C_6H_5O_7K_3 \cdot H_2O$, 2477; NaCl, 2590; K_2SO_4, 1631; MgO, 840; $C_6H_5O_7Fe$, U.S.P., 212.1; $ZnCO_3$, 57.75; $MnCO_3$, 22.05; $CuCO_3$, 10.5; KIO_3, 0.35; Na_2SeO_4, 0.359; $(NH_4)_2MoO_4 \cdot H_2O$, 0.278; $Na_2O_3Si \cdot 9H_2O$, 50.75; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; LiCl, 0.609; H_3BO_3, 2.853; NaF, 2.223; $NiCO_3$, 1.113; and NH_4VO_3, 0.231.

[c] The vitamin mix provided the following (mg/kg diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 17; 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; and vitamin K_1, 0.75.

[d] Dietary fat treatments included safflower oil (control), 76.4% safflower oil+23.6% DHASCO oil (Martek Biosciences) (HP5), 86.8% safflower oil+13.2% DHASCO oil (HP10), 86.6% SAFMIX [a mixture of 37.7% safflower oil plus 62.3% high-oleate safflower oil (Oil Seeds International) that contains 50% linoleate compared with the regular safflower oil]+13.4% DHASCO oil (LP5) and 92.9% SAFMIX+7.1% DHASCO oil (LP10). The total fat content in each diet was 110.4 g/kg of diet.

2.3. DXA analysis

Initial (1 week after ovariectomy, designated as 0 week) and at 4-week intervals thereafter (i.e., 4, 8 and 12 weeks), in vivo two-dimensional bone mineral density (BMD) and bone mineral content (BMC) were assessed using peripheral 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 a supine position with the right hindlimb held at a right angle in external rotation and routinely scanned for the BMC and BMD measurements on the femur and tibia [18].

Excised femurs and tibiae devoid of musculature were scanned. These bones were uniformly positioned and immersed in a saline water bath at a depth of 1.5 cm [19] to enhance scanning resolution. The lumbar spine containing L1–L4 vertebrae was excised with minimal disruption to its associated musculature and scanned for BMD and BMC in the L4 and L1–L4 regions [18].

2.4. Serum biomarkers

Serum biomarkers of bone formation and resorption were assessed at 12 weeks. Alkaline phosphatase (ALP) isoenzyme activity (bone specific), an indicator of osteoblast activity in bones, was measured as previously described for rats [18]. De novo serum carboxylated and decarboxylated rat osteocalcin, the unbound specific protein products released into circulation by osteoblast activity, were measured directly using a sandwich ELISA Kit for rats (Biomedical Technologies, Stoughton, MA, USA). The bone resorption markers pyridinoline (Pyd, a bone and collagen degradation product) and deoxypyridinoline (Dpd, a bone-specific Type 1 collagen degradation product) were quantified using commercial rat kits (Metra Serum Pyd EIA Kit and Metra Total Dpd EIA Kit, Quidel, USA).

2.5. Fatty acid analysis of femoral bone tissues

Lipids in the diet and in various tissue compartments of rat femurs (periosteum, trabecular bone, marrow and cortical bone) were extracted with chloroform/methanol (2:1, vol/vol). At the time of specimen collection, femurs were harvested with their associated musculature intact and frozen to preserve the periosteal tissue and bone lipids during storage (−80°C). 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 [5]. Bone marrow was flushed out and harvested with methanol after the proximal and distal ends of the femur were transected. Lipids from the diets and rat bone compartments were saponified, and the fatty acid methyl esters were prepared using boron trifluoride in methanol (10% wt/vol, Supelco, Bellefonte, PA, USA) prior to gas chromatographic analysis [10,18].

2.6. Statistical analyses

Evaluation of the data in the tables and in Fig. 1 was performed by two-way analysis of variance (ANOVA), and the data in Fig. 2 were analyzed by Pearson's correlation procedure using the SAS data analysis system. Results for variations between treatment groups are expressed as means±the pooled standard deviation or mean±S.D., where applicable. When significant differences ($P \leq .05$) were identified, a Tukey's Studentized Range Test was performed.

3. Results

3.1. Body weight, feed intake and ovariectomy

Rat final body weights were not different among the OVX groups (from 326.0±7.3 to 335.2±9.4 g), but they

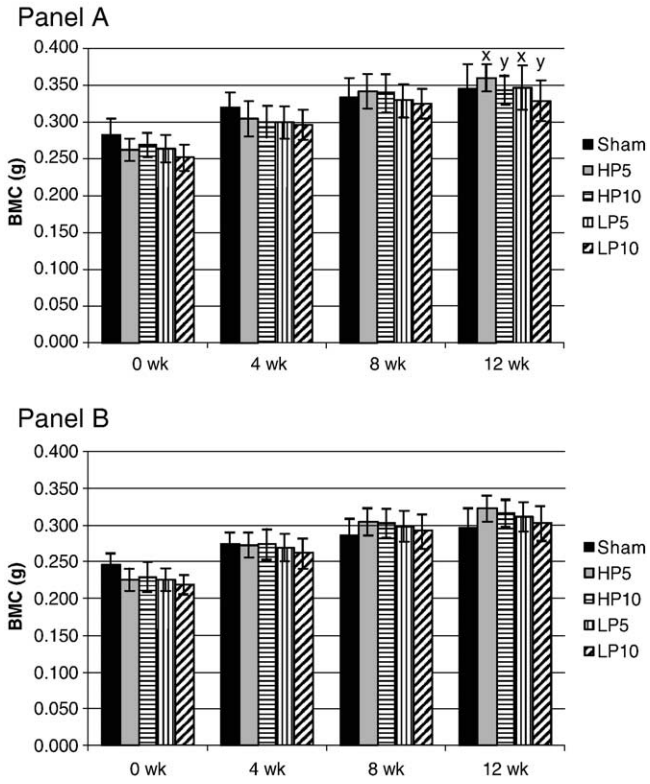

Fig. 1. BMC (g) of the Right femur (A) and tibia (B) from in vivo DXA scans over the feeding period of 12 weeks. The ratio of n-6/n-3 PUFAs had a significant effect on femur BMC at the 12-week scan (P=.03). Values within each period having different letters (x and y for ratio of n-6/n-3 PUFA effect) are significantly different by two-way ANOVA and Tukey's Studentized Range Test (α=.05).

were all higher compared with the sham control group (291.9±9.3 g) (P=.01). There was no difference in average weekly feed consumption among the treatment groups (sham control, 92.1±6.0 g; OVX control, 94.0±5.8 g; HP5, 98.6±8.9 g; HP10, 93.4±7.6 g; LP5, 95.8±7.4 g; and LP10, 92.6±3.6 g); however, the feed efficiency (total g weight gain/total g feed consumed) was higher in the OVX rats (from 0.09±0.01 to 0.11±0.02) compared with the sham rats (0.05±0.02) (P<.0001).

3.2. Bone DXA measurements

Among the OVX rats fed the HP or LP diets, rats in the 5:1 ratio of n-6/n-3 PUFA groups (HP5 and LP5) had significantly higher in vivo femur BMC than rats in the 10:1 ratio of n-6/n-3 PUFA groups (HP10 and LP10) at 12 weeks (P=.03), indicating that the ratio of n-6/n-3 PUFAs may play a significant role in minimizing bone mineral loss in femur (Fig. 1A). No difference in BMC of tibia bone was detected during the entire period of the feeding study (Fig. 1B). No significant difference was found for the in vivo BMD measurements among the OVX groups. The DXA measurements for excised (ex vivo) femur bone confirmed the in vivo findings that the BMC was greatly affected by the ratio of n-6/n-3 PUFAs (HP5 and LP5 had higher BMC than HP10 and LP10, P=.04). In contrast, the DXA data for tibia and lumbar vertebra L4 did not show any treatment effect (the BMD ranged from 0.170 to 0.181 g/cm^2 for excised tibia and from 0.157 to 0.187 g/cm^2 for L4; the BMC ranged from 0.277 to 0.292 g for excised tibia and from 0.104 to 0.123 g for L4).

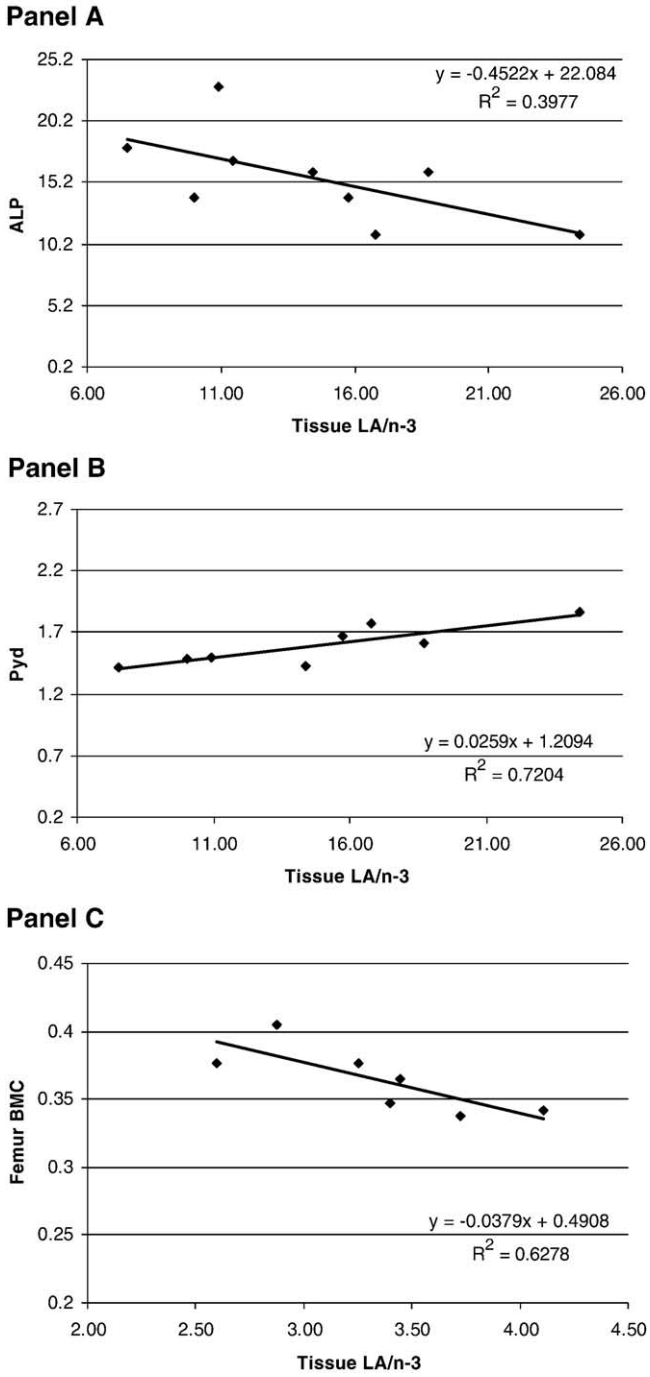

Fig. 2. Correlation analyses between selected bone parameters and tissue fatty acid ratios of LA/n-3 PUFAs. Panel A shows the femoral marrow fatty acid ratio (in LP groups) versus bone ALP (P=.07). Panel B shows the femoral marrow fatty acid ratio (in LP groups) versus Pyd (P=.008). Panel C shows the femoral periosteum fatty acid ratio (in n-6/n-3 ratio 5:1 groups) versus femur BMC (P=.03).

Table 2
Serum bone biomarker measurements in rats after 12 weeks of feeding different dietary lipid treatments

Measurement	Dietary treatment					Pooled S.D.	*P* (ANOVA)		
	Sham	HP5	HP10	LP5	LP10		PUFAs	Ratio	P×R
Osteocalcin (nmol/L)	7.40	7.94^{b}	8.08^{b}	8.94^{a}	9.70^{a}	1.41	.03	.4	.6
ALP (U/L)	11.11	12.58^{B}	13.78^{AB}	16.30^{A}	13.00^{B}	2.80	.08	.4	.03
Serum Pyd (nmol/L)	2.32	2.01^{a}	1.97^{a}	1.54^{b}	1.79^{b}	0.35	.02	.4	.3
Serum Dpd (nmol/L)	5.82	6.32^{a}	6.11^{a}	5.10^{b}	4.79^{b}	1.43	.01	.6	.9

Mean values for bone turnover biomarkers (for osteocalcin, $n=7$; for Pyd, $n=7$; for Dpd, $n=10$ for HP5, $n=9$ for sham, HP10 and LP10 and $n=8$ for LP5; for ALP, $n=9$ for sham and HP10, $n=12$ for HP5, $n=8$ for LP10 and $n=10$ for LP5) within a row having different superscripts (*a* and *b* for PUFA effect and *A* and *B* for interaction effect) are significantly different by two-way ANOVA and Tukey's Studentized Range Test ($\alpha=.05$).
Dietary fat treatments included safflower oil (control for sham), 76.4% safflower oil+23.6% DHASCO oil (Martek Biosciences; HP5), 86.8% safflower oil+13.2% DHASCO oil (HP10), 86.6% SAFMIX [a mixture of 37.7% safflower oil plus 62.3% high-oleate safflower oil (Oil Seeds International) that contains 50% linoleate compared with the regular safflower oil]+13.4% DHASCO oil (LP5) and 92.9% SAFMIX+7.1% DHASCO oil (LP10). Total fat content in each diet was 110.4 g/kg of diet.

3.3. Serum bone biomarkers

Bone biomarker measurements after 12 weeks of dietary treatment revealed that osteocalcin was higher in rats given the LP diets ($P=.03$) and that serum Pyd and Dpd were both higher in the groups of rats fed the HP diets ($P=.02$). These data indicate that diets with lower PUFA levels would be better in promoting bone formation

Table 3
Fatty acid composition of femoral bone periosteum from rats fed different dietary lipid treatments

Fatty acid (wt%)	Dietary treatment					Pooled S.D.	*P* (ANOVA)		
	Sham	HP5	HP10	LP5	LP10		PUFAs	Ratio	P×R
12:0	0.1	0.1^{ax}	0.1^{ay}	0.1^{bx}	0.1^{by}	0.01	.001	.001	.2
14:0	0.8	1.6^{ax}	1.5^{ay}	1.4^{bx}	1.3^{by}	0.1	.001	.02	.8
14:1n-5	0.02	0.1	0.1	0.1	0.1	0.04	.7	.4	.9
15:0	0.1	0.2^{a}	0.1^{a}	0.1^{b}	0.1^{b}	0.01	.04	.06	.6
16:0	18.5	20.7^{a}	20.4^{a}	18.5^{b}	19.2^{b}	0.8	.0004	.5	.3
t16:1	0.3	0.4^{b}	0.4^{b}	0.7^{a}	0.7^{a}	0.1	<.0001	.3	.4
16:1n-7	1.8	2.8	3.1	2.5	2.8	0.8	.4	.5	1.0
17:0	0.2	0.2^{a}	0.2^{a}	0.2^{b}	0.1^{b}	0.02	.02	.1	.5
18:0	8.3	7.0	6.8	5.8	6.3	1.0	.09	.7	.4
18:1n-9	13.2	15.7^{b}	15.3^{b}	33.1^{a}	31.6^{a}	2.3	<.0001	.4	.6
18:1n-7	2.3	1.8	1.8	1.8	1.8	0.1	.8	.96	.5
18:2n-6	35.0	32.9^{B}	35.3^{A}	23.2^{C}	22.4^{C}	1.5	<.0001	.4	.04
18:3n-6	0.2	0.1^{ay}	0.1^{ax}	0.1^{by}	0.1^{bx}	0.01	<.0001	.002	.08
18:3n-3	0.2	0.2	0.2	0.2	0.2	0.1	.2	.2	.7
20:1n-9	0.1	0.1^{b}	0.1^{b}	0.1^{a}	0.1^{a}	0.03	.02	.3	.2
20:2n-6	0.3	0.4^{a}	0.3^{a}	0.1^{b}	0.1^{b}	0.1	.002	.4	.4
20:3n-6	0.4	0.4^{a}	0.3^{a}	0.3^{b}	0.3^{b}	0.04	.008	.9	.4
20:4n-6	9.4	3.2^{y}	3.7^{x}	2.9^{y}	3.9^{x}	0.7	.9	.04	.4
20:5n-3	N.D.	0.3^{ax}	0.2^{ay}	0.2^{bx}	0.1^{by}	0.03	.007	<.0001	.3
22:4n-6	1.1	0.2^{y}	0.2^{x}	0.2^{y}	0.2^{x}	0.1	.6	.02	.6
22:5n-3	0.3	0.3	0.2	0.2	0.2	0.04	.07	.2	.2
22:6n-3	3.2	9.1^{a}	7.4^{a}	6.5^{b}	6.4^{b}	1.5	.04	.2	.7
SATs	27.9	29.8^{a}	29.1^{a}	26.1^{b}	27.1^{b}	1.2	.0002	.7	.2
MONOs	17.7	20.8^{b}	20.7^{b}	38.2^{a}	37.0^{a}	2.7	<.0001	.6	.7
PUFAs	51.8	47.0^{a}	47.9^{a}	33.8^{b}	33.9^{b}	1.8	<.0001	.6	.6
n-6_{LC}	12.2	3.3^{y}	3.9^{x}	3.0^{y}	4.1^{x}	0.7	.9	.03	.5
n-3_{LC}	3.5	9.7^{a}	7.8^{a}	6.9^{b}	6.7^{b}	1.6	.04	.2	.3
n-6_{LC}/n-3_{LC}	3.6	0.3^{by}	0.5^{bx}	0.5^{ay}	0.6^{ax}	0.1	.007	<.0001	.9
n-6	48.1	37.1^{ay}	40.0^{ax}	26.7^{by}	27.0^{bx}	1.3	<.0001	.03	.06
n-3	3.6	9.9^{a}	8.0^{a}	7.1^{b}	6.9^{b}	1.5	.03	.2	.3
n-6/n-3	13.5	3.8	5.2	3.8	4.1	0.9	.1	.08	.2

Mean values for fatty acid composition ($n=6$ for sham, HP10 and LP10; $n=3$ for HP5; and $n=4$ for LP5) of rat femoral bone periosteum within a row having different superscripts (*a* and *b* for PUFA effect; *x* and *y* for ratio of n-6/n-3 PUFA effect; and *A*, *B* and *C* for interaction effect) are significantly different by two-way ANOVA and Tukey's Studentized Range Test ($\alpha=.05$). The sham group was not included in the statistical analysis.
Dietary fat treatments included safflower oil (control for sham), 76.4% safflower oil+23.6% DHASCO oil (Martek Biosciences; HP5), 86.8% safflower oil+13.2% DHASCO oil (HP10), 86.6% SAFMIX [a mixture of 37.7% safflower oil plus 62.3% high-oleate safflower oil (Oil Seeds International) that contains 50% linoleate compared with the regular safflower oil]+13.4% DHASCO oil (LP5) and 92.9% SAFMIX+7.1% DHASCO oil (LP10). Total fat content in each diet was 110.4 g/kg of diet.
N.D. indicates not detected (peak detection at 10 ng); n-6_{LC}=20:4n-6+22:4n-6+22:5n-6; n-3_{LC}=20:5n-3+22:5n-3+22:6n-3.

and reducing bone resorption in the OVX rat model (Table 2).

3.4. Bone tissue fatty acid analysis

The fatty acid composition of rat femoral periosteum reflected the dietary lipid treatments (Table 3). The rats fed the HP diets had significantly higher amounts of 18:2n-6 (LA), 18:3n-6, 20:2n-6, 20:3n-6, 20:5n-3, 22:6n-3, total saturated, total PUFAs, total n-6 and total n-3 PUFAs. The amounts of 18:1n-9 and total monounsaturates were significantly higher in the LP groups presumably due to the use of the high-oleate safflower oil, which reduced the total amount of PUFAs (LA) in these diets (Table 1). The dietary ratio of n-6/n-3 PUFAs (Table 1) greatly affected the amounts of 20:4n-6, 20:5n-3, 22:4n-6, n-6_{LC}, ratio of n-6_{LC}/n-3_{LC} and total n-6 PUFAs in accord with the dietary treatments (Table 3). In Table 4, femoral proximal trabecular bone fatty acid composition was affected by dietary treatments similarly as in the periosteum, except that the amounts of 22:5n-3 and 22:6n-3 were significantly altered by both the dietary level of PUFAs and the ratio of n-6/n-3 PUFAs. The ratios of n-6/n-3 and n-6_{LC}/n-3_{LC} in the femoral proximal trabecular bone were not different probably because of the higher variation in these measurements.

The femoral bone marrow and cortical bone fatty acid compositions were similarly affected as in the other bone compartments by the dietary lipid treatments. Interestingly, the amounts of DHA (for marrow, 1.1–3.6%; for cortical bone, 1.1–3.5%; across treatment groups) and total n-3 PUFAs (for marrow, 1.4–4.2%; for cortical bone, 1.3–4.2%) were far less compared with the amounts found in the periosteum (6.4–9.1% for DHA and 6.9–9.9% for total n-3). These data indicate that the dietary source of DHA influences the fatty acid composition of bone compartments and that periosteum contains the highest amount of DHA in these tissues.

Table 4
Fatty acid composition of femoral proximal trabecular bone from rats fed different dietary lipid treatments

Fatty acid (wt%)	Dietary treatment					Pooled S.D.	*P* (ANOVA)		
	Sham	HP5	HP10	LP5	LP10		PUFAs	Ratio	P×R
12:0	0.1	0.2^{ax}	0.2^{ay}	0.2^{bx}	0.1^{by}	0.01	<.0001	<.0001	.5
14:0	1.1	2.2^{ax}	1.9^{ay}	1.8^{bx}	1.5^{by}	0.1	<.0001	.0009	.7
14:1n-5	0.1	0.1	0.1	0.1	0.1	0.1	.7	.9	.4
15:0	0.1	0.2	0.2	0.2	0.2	0.01	.08	.99	.5
16:0	16.0	18.9^{a}	19.0^{a}	17.5^{b}	17.2^{b}	0.6	<.0001	.7	.4
t16:1	0.6	0.6^{b}	0.6^{b}	0.9^{a}	0.9^{a}	0.1	<.0001	.4	.7
16:1n-7	2.2	3.9	4.3	3.7	3.5	0.7	.06	.7	.2
17:0	0.2	0.1	0.1	0.1	0.1	0.03	.5	.06	.2
18:0	4.2	3.6	3.6	3.3	3.6	0.2	.2	.3	.2
18:1n-9	22.7	20.6^{b}	20.1^{b}	39.5^{a}	36.6^{a}	4.6	<.0001	.4	.6
18:1n-7	2.2	1.9	1.9	2.0	2.0	0.1	.5	.8	.7
18:2n-6	43.5	38.6^{a}	40.2^{a}	24.1^{b}	28.3^{b}	4.8	<.0001	.2	.6
18:3n-6	0.3	0.2^{ay}	0.3^{ax}	0.1^{by}	0.2^{bx}	0.04	.002	.007	.6
18:3n-3	0.2	0.2	0.2	0.2	0.2	0.1	.09	.7	.8
20:1n-9	0.03	0.1^{b}	0.04^{b}	0.2^{a}	0.2^{a}	0.1	.0003	.2	.6
20:2n-6	0.4	0.5^{a}	0.5^{a}	0.2^{b}	0.3^{b}	0.1	<.0001	.6	.95
20:3n-6	0.3	0.3^{a}	0.3^{a}	0.2^{b}	0.2^{b}	0.1	.0009	.6	.9
20:4n-6	3.3	1.9^{y}	2.3^{x}	1.8^{y}	2.4^{x}	0.4	.7	.01	.7
20:5n-3	0.01	0.3^{ax}	0.1^{ay}	0.2^{bx}	N.D.^{by}	0.04	<.0001	<.0001	.9
22:4n-6	0.8	0.2	0.3	0.2	0.4	0.1	.2	.05	.5
22:5n-6	0.4	N.D.	N.D.	N.D.	0.1	0.1	.3	.4	.4
22:5n-3	0.02	0.4^{ax}	0.2^{ay}	0.2^{bx}	0.1^{by}	0.1	.0003	.0002	.96
22:6n-3	0.5	4.3^{ax}	2.6^{ay}	2.6^{bx}	1.4^{by}	0.5	<.0001	<.0001	.2
SATs	21.7	25.2^{a}	25.0^{a}	23.0^{b}	22.7^{b}	0.7	<.0001	.4	.8
MONOs	27.7	27.1^{b}	27.0^{b}	46.3^{a}	43.0^{a}	4.9	<.0001	.5	.5
PUFAs	49.7	46.8^{a}	47.0^{a}	29.8^{b}	33.4^{b}	5.2	<.0001	.4	.5
n-6_{LC}	4.4	2.0^{y}	2.5^{x}	2.0^{y}	2.8^{x}	0.6	.5	.03	.6
n-3_{LC}	0.6	4.9^{ax}	3.0^{ay}	2.9^{bx}	1.5^{by}	0.6	<.0001	<.0001	.3
n-6_{LC}/n-3_{LC}	12.4	0.4	0.9	0.7	4.8	4.6	.2	.3	.4
n-6	48.9	41.6^{a}	43.8^{a}	26.7^{b}	31.7^{b}	5.5	<.0001	.2	.6
n-3	0.8	5.2^{ax}	3.2^{ay}	3.1^{bx}	1.7^{by}	0.6	<.0001	<.0001	.3
n-6/n-3	77.8	8.2	14.0	8.6	31.2	22.5	.3	.2	.4

Mean values for fatty acid composition ($n=6$ for sham, HP10 and LP10; $n=5$ for HP5; and $n=4$ for LP5) of rat femoral proximal trabecular bone within a row having different superscripts (*a* and *b* for PUFA effect; *x* and *y* for ratio of n-6/n-3 PUFA effect) are significantly different by two-way ANOVA and Tukey's Studentized Range Test ($\alpha=.05$). The sham group was not included in the statistical analysis.

Dietary fat treatments included safflower oil (control for sham), 76.4% safflower oil+23.6% DHASCO oil (Martek Biosciences; HP5), 86.8% safflower oil+13.2% DHASCO oil (HP10), 86.6% SAFMIX [a mixture of 37.7% safflower oil plus 62.3% high-oleate safflower oil (Oil Seeds International) that contains 50% linoleate compared with the regular safflower oil]+13.4% DHASCO oil (LP5) and 92.9% SAFMIX+7.1% DHASCO oil (LP10). Total fat content in each diet was 110.4 g/kg of diet.

3.5. Correlations between bone fatty acids and bone measurements

Data on bone markers (ALP and Pyd), femur BMC and femoral fatty acid values were analyzed by correlation regression analysis to evaluate possible relationships between dietary lipid effects on bone parameters. Fig. 2A shows that serum ALP was negatively correlated with the bone marrow ratio of LA/n-3 ($P=.07$). In Fig. 2B, serum Pyd was positively correlated with the marrow ratio of LA/n-3 ($P=.008$); in Fig. 2C, femur BMC was negatively correlated with the periosteum ratio of LA/n-3 ($P=.03$; Fig. 2). These correlations illustrate that bone formation markers are negatively affected by an increasing ratio of LA/n-3 and that the opposite was true for bone resorption markers. Hence, a diet containing LC n-3 PUFAs (DHA) moderates the actions of LA, an n-6 PUFA, to support a bone biomarker profile that is associated with greater BMC in the femur of OVX rats.

4. Discussion

In this experiment, the comparison of the OVX group with the sham group does not reflect a positive control as with estrogen but the intact female rat represents a point of reference for estrogen deficiency in ovariectomy. The higher body weights of OVX rats compared with the sham rats are expected; however, the fact that dietary lipid treatments (ratio of n-6/n-3 PUFAs and total PUFA level) did not influence body weights of OVX rats indicates that bone density was not a result of a change in body mass. Thus, the distinction of estrogen status as a physiological end point is maintained (sham vs. OVX) while the dietary PUFA comparisons during estrogen deficiency are examined in this investigation.

The response of rat bone fatty acid composition to the dietary lipid treatments in the OVX groups is consistent with our previous studies on dietary PUFAs in this rat model [18]. The higher level of DHA in the rat femoral periosteum relative to the bone marrow in the OVX model was observed in male growing rats [10,20]. The fatty acid composition of femur bone compartments in the control group (sham-operated rats fed safflower oil) is practically identical to the values we reported in studies evaluating dietary PUFAs [20] and soy protein [18] in OVX rats.

Feeding DHA to OVX rats minimized the decline of BMC caused by estrogen deficiency in this investigation. The result is in agreement with a similar study we reported recently that feeding both n-3 PUFAs (from menhaden oil) and soy protein to OVX rats together attenuated bone mineral decline in the tibia [18]. These findings in the OVX rat model are also consistent with our previous investigations in growing rats where LC n-3 PUFAs improved bone formation rates and reduced ex vivo PGE_2 production in bone organ cultures [10]. In addition, we observed a protective effect of maintenance of bone mineral in mature OVX rats fed LC n-3 PUFAs compared with the sham rats [13]. Evidence from cell culture studies supports the underlying mechanism that LC n-3 PUFAs reduced cyclooxygenase (COX)-2 protein expression and PGE_2 production but increased ALP activity in cultured osteoblast-like MC3T3-E1 cells [13].

The prominent phenomenon during the course of postmenopausal osteoporosis is an elevated bone resorption activity relative to bone formation rate, which is believed to be mediated by an increased production of osteoclastogenic cytokines and prostaglandins [4–6]. We speculate that the target for the bone-sparing actions of n-3 PUFAs is directed on the regulation of prostanoid formation as well as the metabolism of various cytokines that are produced in bone post menopause. It is well known that COX-2-dependent PG synthesis is necessary for osteoclastogenesis and bone resorption induced by cytokines such as IL-1β, TNFα or lipopolysaccharides [21]. The elevation of these cytokines and PGE_2 during menopause aggravates the symptoms of estrogen deficiency in the bone [6]. Although DHA is not a direct precursor of prostaglandins, it is likely that DHA, as well as other LC n-3 PUFAs, not only reduces PGE_2 production but also depresses the actions of cytokines upstream from prostanoid biosynthesis.

The bone-conserving effect of LC n-3 PUFAs may also be partly attributed to their suppression of bone resorption activity that is linked to anti-inflammatory properties. In comparison with n-3 PUFAs, an excess of n-6 PUFAs has been shown to increase the production of proinflammatory cytokines (TNFα and IL-6) and reactive oxygen species such as the inducible nitric oxide (iNO) that can potentially mediate some of the deleterious effects associated with cytokines on bone resorption during disease [22]. In addition, an increase in the concentration of iNO under inflammatory conditions has been linked to enhanced osteoclastic activity and bone resorption in inflammation-induced osteoporotic rats [23]. In a human osteoblast-like cell line MG-63 culture, gene expression (mRNA level) of the proinflammatory cytokines IL-1α, IL-1β and TNFα was significantly increased by AA treatment while EPA caused a significant inhibition of their expression [24]. Supplementation of n-3 PUFAs (18:3n-3 and EPA) to cultures of human OA cartilage explants abolished the expression of mRNA for several mediators of inflammation (COX-2, 5-lipoxygenase, TNFα and IL-1) [25].

At present, one large epidemiological investigation confirmed that a higher dietary ratio of n-6/n-3 PUFAs was associated with lower BMD in the hips of men and women [26]. In the human study, LA was used as the dietary factor for calculating the fatty acid ratio with the BMD correlations. Likewise, in our OVX rats, we used the ratio of LA/n-3 PUFAs in the bone and observed a similar relationship with femur BMC. In addition, we found a higher femur BMC with a lower dietary ratio of n-6/n-3 PUFAs of 5. Other published investigations performed in a limited number of human subjects revealed the following. Two studies showed positive correlations between a low dietary ratio of n-6/n-3 PUFAs and bone formation markers (serum calcium and osteocalcin)

[27] and bone density [28]; another showed no effect on the bone [29]. Although no study has evaluated the effects of DHA alone on bone status and bone biomarkers in humans, our work provides useful information about LC n-3 PUFAs and their role for improving the dietary ratio of n-6/n-3 PUFAs to be protective against bone loss in older men and women.

In the current study, we showed that as LC n-3 PUFAs accumulated in femur bone compartments of rats fed DHA, the ratio of LA/n-3 PUFAs decreased and correlated with more favorable levels of bone biomarkers, higher ALP (bone formation) and reduced Pyd (bone resorption), leading to a conservation of BMC. The dietary level of PUFAs and ratio of n-6/n-3 PUFAs appear to be important determinates in maintaining BMC during estrogen-induced accelerated bone loss in OVX rats. The effects of LC n-3 PUFAs on the OVX rat model may be applicable to humans as estrogen declines; however, research must now delineate the actions of these different PUFAs on osteoblast and osteoclast functions as well as in the bone during estrogen deficiency.

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