

Docosahexaenoic Acid and 17 β -Estradiol Co-Treatment Is More Effective Than 17 β -Estradiol Alone in Maintaining Bone Post-Ovariectomy

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Bone-protective effects of combined treatment with long chain polyunsaturated fatty acids (LCPUFAs) and estrogenic compounds following ovariectomy have previously been reported. Recent evidence suggests the n-3 LCPUFA docosahexaenoic acid (DHA, 22:6n-3) is particularly bone-protective. The aim of this study was to determine whether combined treatment with DHA and estrogenic compounds has a beneficial effect on bone mass in ovariectomized (OVX) rats. Rats were randomized into 9 groups and either ovariectomized (8 groups) or sham-operated (1 group). Using a 2 $\times$ 4 factorial design approach, OVX animals received either no estrogenic compound, genistein (20 mg/kg body weight/day), daidzein, (20 mg/kg body weight/day) or 17 β -estradiol (1 μ g/day) with or without DHA (0.5 g/kg body weight/day) for 18 weeks. Bone mineral content (BMC), area (BA), and density (BMD), plasma osteocalcin and IL-6 concentrations, and red blood cell (RBC) fatty acid composition were measured. Femur BMC was significantly greater in animals treated with DHA or 17 β -estradiol than in ovariectomized controls. Plasma carboxylated osteocalcin was significantly higher in DHA-treated animals and total osteocalcin significantly lower in 17 β -estradiol-treated animals compared with ovariectomized controls. There were significant interactions between treatment with estrogenic compounds and DHA for femur BMC, plasma IL-6 concentration, and RBC fatty acid composition. Combined treatment with 17 β -estradiol+DHA was more effective than either treatment alone at preserving femur BMC and lowering circulating concentrations of pro-inflammatory IL-6. The per-

centage of n-3 LCPUFAs in RBCs was significantly greater in animals receiving 17 β -estradiol+DHA compared with either treatment alone. There was no beneficial effect of combined DHA and phytoestrogen treatment on bone. Results from this study raise the possibility that co-treatment with 17 β -estradiol and DHA may allow a lower dose of 17 β -estradiol to be used to provide the same bone-protective effects as when 17 β -estradiol is administered alone. *Exp Biol Med* 233:592–602, 2008

Key words: isoflavones; n-3 fat; long chain polyunsaturated fatty acids; estrogen; phytoestrogens

Introduction

Estrogen decline because of natural or surgical menopause results in loss of bone mass and can lead to the development of osteoporosis. Although hormone replacement therapy (HRT) is an effective means of preventing post-menopausal bone loss, HRT has been associated with increased risk of other chronic diseases, such as some forms of cancer (1). There is a need to develop alternative anti-osteoporotic treatments for the management of post-menopausal bone loss.

One of the consequences of estrogen deficiency is disruption of long chain polyunsaturated fatty acid (LCPUFA) metabolism such that tissue and blood concentrations of very long chain PUFAs decline (2, 3). Dietary supplementation with n-3 LCPUFAs increases their concentration in tissue and blood and may assist in maintaining bone mass post-ovariectomy in rodents (4) and post-menopause in women (5).

In 1999, Schlemmer *et al.* reported a possible synergistic effect of treatment with 17 β -estradiol in conjunction with dietary supplementation with two LCPUFAs, gamma-linolenic acid (GLA, 18:3n-6), and eicosapentanoic acid (EPA, 20:5n-3) in ameliorating ovariectomy-induced bone mineral loss in rats (6). One possible means to reduce the risks associated with HRT may be to reduce the dose of estrogen used in the treatment. The results of

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Schlemmer *et al.* (1999) suggest that combining LCPUFAs with estrogen-replacement therapy may allow a lower dose of estrogen to be used whilst still maintaining the efficacy of the treatment on bone.

An alternative to conventional HRT are phytoestrogens. Phytoestrogens are compounds produced by plants that have similar, albeit much weaker, estrogenic activity to mammalian estrogens. Safety and toxicity studies indicate few health risks associated with phytoestrogen consumption (7–9). Although some studies have reported weak protective effects of phytoestrogens on bone mass post-menopause (10–13), others have found no effect (14–16). As a result, the efficacy of phytoestrogens as a means of combating post-menopausal bone loss remains in doubt. A reduction in the rate of bone resorption in ovariectomized rats receiving a phytoestrogen-containing diet supplemented with menhaden oil (rich in n-3 LCPUFAs) has been reported (17). It is possible that combined LCPUFA and phytoestrogen treatment may have greater efficacy as a treatment for post-menopausal bone loss than either treatment alone.

Recent evidence suggests the n-3 LCPUFA docosahexaenoic acid (DHA, 22:6n-3) may have more potent bioactivity in bone than eicosapentanoic acid (EPA, 20:5n-3) (18–21). As supplements containing a mixture of LCPUFAs were used in the two previous studies that examined the interaction between LCPUFAs and estrogenic compounds on bone mass, the aim of the present study was to compare the effect of dietary DHA supplementation with and without 17 β -estradiol treatment or phytoestrogen supplementation on bone mass in the ovariectomized rat.

Method

Study Design. One hundred and four female Sprague-Dawley rats were obtained from the Small Animal Production Unit, Massey University. At age 7 months (week 0), animals were randomly assigned to one of nine groups and either ovariectomized (OVX) (1 group, $n = 10$; 7 groups, $n = 12$) or sham operated (1 group, $n = 10$). The operations were performed under general anesthetic (isoflurane). Sham-operated animals were anesthetized and an incision made but the ovaries left intact. The ovaries were removed from the OVX animals.

The ovariectomized animals were allocated to one of eight treatment groups based on a 4×2 factorial design. Treatments consisted of an estrogenic compound (genistein (GEN), daidzein (DAI), 17 β -estradiol [OES] or none) with or without DHA as shown in Table 1. Study duration was 18 weeks.

Diets and Treatments. Throughout the study period, animals were fed a nutritionally balanced, semi-synthetic diet based on AIN93M (Table 2). Animals treated with genistein, daidzein, and/or DHA received modified versions of this diet as described below. Diets were randomly sampled and analyzed to confirm nutrient quantity.

Sham-operated animals were fed *ad libitum*. As ovariectomy leads to increased appetite and weight gain, the food intake of ovariectomized animals was limited to that of the sham controls. Animals were maintained in individual shoebox cages at 22°C ($\pm 2^\circ\text{C}$) with a 12/12-hr light/dark cycle in a dedicated room in the Small Animal Production Unit at Massey University. All animals had *ad libitum* access to deionized water. The study was approved by the Massey University Animal Ethics Committee (Approval number 03/102).

Genistein, Daidzein, and DHA Treatments. Genistein, daidzein, and DHA were added to the base AIN-93M diet. In order to maintain the same macronutrient and caloric content of all diets, the amount of corn oil in DHA-containing diets and the amount of cellulose in phytoestrogen-containing diets were reduced.

Genistein (95%) and daidzein (96%) were purchased from LC Laboratories, PKC Pharmaceuticals Inc., Woburn, MA, USA. Based on previous studies that have shown a positive effect of phytoestrogens on bone mass in ovariectomized rats, a dose of 20 mg of genistein or daidzein per kg rat body weight per day was chosen for use in the present study (22, 23). DHA ethyl ester (80%, 01177B-E80) was purchased from SanMark LLC, China. The dose of DHA ethyl ester used was 0.5 g per kg rat body weight per day. This dose was chosen based on results of a previous study, which demonstrated significant bone-protective effects of DHA on bone mass in rats post-ovariectomy (18).

At the beginning of the trial, the AIN-93M diet was manufactured in bulk; however, not all of the cellulose or corn oil was initially added to allow changes in the amount of genistein, daidzein, and DHA in the diets over the course of the trial in keeping with animal body weight changes. Food intake was recorded daily, and the average food intake of sham-operated animals was determined weekly. Ovariectomized animals were offered the same amount of food per day as sham-operated animals were habitually consuming. Animals were weighed weekly, and an average rat weight was obtained for all genistein-treated, daidzein-treated, and DHA-treated animals. The average body weight for animals receiving each respective treatment was used to calculate the amount of genistein, daidzein, or DHA to be added to the diet. Batches of the treatment diets sufficient to feed the animals for one week were made by blending the required amount of phytoestrogen, cellulose, DHA, and corn oil in to the base diet. As the rats gained body weight over the study period, the amount of phytoestrogens and DHA in the diets gradually increased. Negligible changes to the total amount of cellulose in the diet were required in order to compensate for the increases in phytoestrogen content as shown in Table 2. All DHA-containing diets contained at least 2% corn oil, an amount in excess of the minimum amount required to prevent n-6 essential fatty acid deficiency (1%).

Estradiol Treatment. At the time of ovariectomy, a 90-day slow-release 17 β -estradiol pellet (providing 1 μg

Table 1. Allocation of Treatments to Study Groups

Group name	Operation	Estrogenic compound	DHA
SHAM	Sham	None	No
OVX	Ovariectomy	None	No
DHA	Ovariectomy	None	Yes
GEN	Ovariectomy	Genistein	No
GENDHA	Ovariectomy	Genistein	Yes
DAI	Ovariectomy	Daidzein	No
DAIDHA	Ovariectomy	Daidzein	Yes
OES	Ovariectomy	17 β -estradiol	No
OESDHA	Ovariectomy	17 β -estradiol	Yes

17 β -estradiol/day, NE-121, Innovative Research of America, USA) was inserted under the skin at the nape of the neck in animals allocated to the estradiol treatment groups. All other non-estradiol treated animals received a placebo pellet (NC-111; Innovative Research of America) inserted in the same way. To ensure continual estradiol/placebo dosing throughout the remainder of the 18-week trial, 60-day release pellets were inserted in the OES and OESDHA animals at study week 12. The dose of 1 μ g 17 β -estradiol/day was chosen to match that previously used by Schlemmer *et al.* (1999) in the original investigation, which demonstrated a possible beneficial effect of combined LCPUFA and 17 β -estradiol treatments on bone mass post-ovariectomy in rats (6).

Dual Energy X-Ray Absorptiometry (DEXA)

Scans. Bone mineral contents and densities were determined with a Hologic QDR4000 bone densitometer using a pencil beam unit (Bedford, MA, USA). Prior to scanning animals were anesthetized with a mixture of 0.2 ml Acepromazine (ACP) + 0.5 ml Ketamine + 0.1 ml Xylazine + 0.2 ml sterile water, at a dosage of 0.05 ml/100 g body weight administered intra-peritoneally via a 25 G $\times$ 15.875 mm needle. A suitable level of anesthesia was attained after five to ten minutes and was maintained for up to 2 hours.

Anesthetized rats were placed in a supine position on an acrylic platform of uniform 38.1 mm thickness so that the femur was at right angles to the long axis of the spine and similarly, at right angles to the tibia ("frog-leg" position). Regional high-resolution scans of both femurs and the lumbar spine were performed using a 1.524 mm diameter collimator with 0.30516 mm point resolution and 0.64516 mm line spacing. Scans were made at weeks -2 (baseline), 5, and 18. A daily quality control (QC) scan was made to ensure precision met with the required coefficient of variation.

Blood Sampling. At week 8, rats were placed in a purpose-built restrainer, which was then placed on top of a heat pad under a heat lamp. A single blood sample of approximately 1 ml was withdrawn from the lateral tail vein, using a 23 G $\times$ 15.875 mm hypodermic needle and 1 ml syringe. Blood samples were collected into vacutainers containing heparin and centrifuged at 750 g for 10 minutes. The plasma was removed, snap-frozen with liquid nitrogen, and stored at -80°C for later analysis of 17 β -estradiol.

Euthanasia and Dissection. At week 18 after final DEXA scans, animals were anesthetized. The animals were subsequently exsanguinated by cardiac puncture with a 19

Table 2. Ingredient Composition (% Air-Dry Weight) of Control and Experimental Diets

	SHAM	OVX	Dai	Gen	Oes	DHA	Dai + DHA	Gen + DHA	Oes + DHA
Cornstarch	59.7	59.7	59.674	59.674	59.7	59.7	59.674	59.674	59.7
Sodium caseinate	14	14	14	14	14	14	14	14	14
Sucrose	6	6	6	6	6	6	6	6	6
Cellulose	5	5	5	5	5	5	5	5	5
AIN 93M vitamin mix	5	5	5	5	5	5	5	5	5
AIN93M mineral mix (excluding calcium)	5	5	5	5	5	5	5	5	5
Calcium carbonate	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Corn oil ^{a,b}	4	4	4	4	4	3.3	3.3	3.3	3.3
Docosahexaenoic acid ethyl ester ^a	0	0	0	0	0	0.7	0.7	0.7	0.7
Genistein	0	0	0	0.026	0	0	0	0.026	0
Daidzein	0	0	0.026	0	0	0	0.026	0	0

^a DHA dose was 0.5 g/kg mean rat body weight/day. The percentages of DHA and corn oil in the diet shown in this table are the percentages at trial commencement. The percentage of DHA in diets increased and percentage of corn oil decreased as the body weight of animals increased over the trial period. At trial completion the percentage of DHA in the diet was 0.9% and the percentage of corn oil was 3.1%.

^b Fatty acid composition of corn oil: 58% linoleic acid, 28% oleic acid, 11% palmitic acid, and 2% stearic acid.

G × 38.1 mm needle and 5 ml syringe. Two blood samples were collected from each animal, one into a vacutainer containing heparin and one into a vacutainer containing EDTA. Samples were centrifuged at 500 *g* at 4°C for 10 minutes. Plasma was collected from heparinized samples and stored at –80°C for later analysis. Plasma was discarded from samples in EDTA, and the remaining red blood cells washed with isotonic saline and stored at –80°C pending analysis of fatty acid composition.

The uteri and adnexae were removed, and their wet weight determined as a quality control measure to confirm success of ovariectomy. Both rear legs and the spine were removed and stored in phosphate buffered saline at –20°C pending biomechanical testing and CT analysis.

Red Blood Cell Fatty Acid Composition. Red blood cell fatty acid composition was determined by direct transmethylation followed by gas chromatography. To each sample, 1 ml of internal standard (2.5 mg/ml tricosanoic acid methyl ester [C23:0, Sigma-Aldrich Chemicals] dissolved in chloroform), 2 ml of toluene and 5 ml of 5% sulfuric acid in methanol was added. Tubes were sealed, shaken, and fatty acid methyl esters (FAMES) formed by heating at 80°C for 1 hour. Samples were then cooled and shaken with 5 ml of saturated NaCl, then centrifuged at 2000 RCF for 10 minutes at 10°C. The upper toluene layer was collected and 1 µl (with 1:100 split) injected into a Gas Chromatograph (Agilent 6890, Agilent Technologies, Santa Clara, CA, USA) with auto sampler and flame ionization detector. A SGHE Sol Gel Wax column was used with a column length of 30 meters, internal diameter 0.25 mm, and film thickness 0.25 µm. Hydrogen flow rate was 1.5 ml/minute, and constant flow and average linear velocity were 50 cm/sec. The initial injection temperature was 170°C, and temperature was ramped at 1°C/minute to 225°C. Fatty acids in the samples were determined by comparison with known standards supplied by Sigma-Aldrich Chemicals (37-component FAME mixture C4:0 – C24:0, PUFA 1 and PUFA 3) and Restek, Bellefonte, PA, USA (NLEA FAME Mix, 28 components).

Plasma Concentrations of 17β-Estradiol, IL-6 and Osteocalcin. Concentration of 17β-estradiol in rat plasma was determined at week 8 post-ovariectomy using a commercially available RIA kit purchased from Diasorin, Saluggia, Italy. Plasma concentrations of IL-6, carboxylated and under-carboxylated osteocalcin were determined at trial completion using commercially available ELISA kits as follows: Quantikine Rat IL-6 Immunoassay ELISA kit (Cat.# SR6000B, R&D Systems, Minneapolis, MN, USA), Rat Gla-Oc (Cat.# MK122), and Rat Glu-Oc (Cat.# MK121) competitive EIA Kits (Takara Bio Inc., Otsu, Shiga, Japan). The antibody used to measure carboxylated osteocalcin detected osteocalcin with a γ-carboxylated glutamic acid residue at position 17. The antibody used to detect under-carboxylated osteocalcin was specific for non-carboxylated glutamic acid at positions 21 and 24. Both antibodies detect

intact osteocalcin as well as fragments of the polypeptide chain containing these specific residues.

Computed Tomography (CT). Following completion of the trial, the opportunity arose to access a pQCT scanner. As both femurs had previously been subjected to destructive testing (biomechanical testing and bone marrow fatty acid analysis), the right tibia was used for pQCT analysis. After removal of skin and disarticulation, tibia length was determined manually with callipers, and the tibia was positioned for scanning on a plastic cradle. Scans were made with a XCT2000 pQCT scanner (Stratec, Pforzheim, Germany) 5 mm from the proximal end of the tibia (at a constant site in the proximal metaphysis) and at 50% of the length of the tibia (mid-diaphysis). Voxel size was 0.1 mm, and scan speed was 5 mm per second. Scans were analyzed using the manufacturer's software; the contour threshold was 280 mg/cm³. Main outcome measures were trabecular BMC and BMD (determined in the 5 mm slice) and cortical BMC, BA, and BMD, and endosteal and periosteal circumferences (determined at 50% of tibia length).

Biomechanical Testing. Right femurs were scraped free of adhering flesh and maintained in PBS at room temperature for 1 hour prior to testing. The length of each femur was measured with an electronic calliper and the midpoint marked. Both the anterior-posterior and latero-medial diameter at the midpoint of the femur were similarly determined. The maximum load, breaking load, maximum deformity (stroke length), breaking stress, breaking strain (the percent deformation of the femur just prior to the time of breaking), the breaking energy (the amount of energy required to break the femur), and elastic modulus (force required to bend the bone in the elastic phase of deformation) were determined using a Shimadzu Ezi-test (Kyoto, Japan) materials-testing machine. The femurs were subjected to a three-point bending test with the application point of the upper fulcrum positioned midway between the two supporting rods of the testing jig; the supporting rods were 15 mm apart. Load was applied at a constant deformation rate of 50 mm/min at the midpoint of the anterior surface of the femur.

Statistical Analysis. Bone densitometry data were analyzed by repeated measures mixed model analysis (Proc Mixed) using SAS 9.1 (SAS Institute Incorporated, Cary, NC, USA). In all cases the Toeplitz model was found to give the best fit to the data. All other data conformed to the requirements of the general linear model and were analyzed by two-way ANOVA (Proc GLM) with factorial-design analysis using SAS 9.1 (SAS Institute Incorporated). For the overall ANOVA, *P* < 0.05 was considered statistically significant.

Selected contrasts of interest were included in the original ANOVA in keeping with the available degrees of freedom. Main effects (i.e., the effects of treatment with daidzein, genistein, 17β-estradiol and DHA) were contrasted with untreated ovariectomy. The effects of combined treatment with DHA and each estrogenic compound were

contrasted with the effect of treatment with either compound alone (i.e., contrasts were made as follows: OESDHA vs. DHA, OESDHA vs. OES, GENDHA vs. DHA, GENDHA vs. GEN, DAIDHA vs. DHA, DAIDHA vs. DAI). A Bonferroni correction was made to protect the overall error rate and as a result $P < 0.01$ was considered statistically significant for all contrasts.

Results

Diets. The formulated nutrient compositions of the diets were confirmed by the conduct of proximate analysis on random diet samples (data not shown). The digestibility, metabolism, and bioavailability of the genistein and daidzein supplements used were assessed in a separate study involving ovariectomized rats of the same age as used in the present study (unpublished results). In brief, mean plasma concentrations of genistein, daidzein, and equol for the same dosage rate as used in the present study were 24 nmol/L, 17 nmol/L and 18 nmol/L, respectively. Inclusion of DHA in the diet did not affect genistein or daidzein metabolism, digestibility, or bioavailability.

Animal Body Weights and Food Intake. Baseline animal body weights were not significantly different between groups (mean weight 313.5 ± 2.93 g). Final rat body weight was significantly higher in ovariectomized compared to sham-operated animals ($P = 0.0003$) (Table 3). Dietary DHA supplementation had no statistically significant effect on either average daily food intake or final rat body weight. There was no statistically significant effect of any estrogenic compound on mean daily food intake. However, treatment with 17β -estradiol or daidzein was associated with significantly lower final rat body weight compared with no estrogenic treatment or treatment with genistein ($P = 0.0005$ and $P = 0.0015$, respectively). There was no statistically significant effect of combined treatment with DHA and any of the three estrogenic compounds on mean daily food intake or final animal body weight. Uterus weight was significantly higher in 17β -estradiol treated animals, but not phytoestrogen-treated animals compared with untreated ovariectomized controls (Table 3).

Osteocalcin. Ovariectomy had no statistically significant effect on plasma concentrations of total, carboxylated or undercarboxylated osteocalcin (Table 3). However, dietary DHA supplementation was associated with a higher plasma concentration of carboxylated osteocalcin and a lower plasma concentration of undercarboxylated osteocalcin compared with untreated ovariectomy ($P = 0.02$). Treatment with 17β -estradiol was associated with lower total plasma osteocalcin concentration compared to untreated ovariectomy ($P = 0.01$).

Interleukin-6. There was a significant interaction between treatment with estrogenic compounds and DHA supplementation on plasma IL-6 concentrations ($P = 0.03$). Treatment with 17β -estradiol in conjunction with DHA supplementation resulted in significantly lower plasma IL-6

concentrations compared with 17β -estradiol treatment without DHA supplementation ($P = 0.01$) (Table 3).

Red Blood Cell Fatty Acid Composition. The ratio of AA relative to DHA in RBCs was significantly higher in ovariectomized animals compared with sham controls ($P = 0.001$, Table 4). Treatment with genistein or daidzein was associated with a significantly higher ratio of AA relative to DHA in RBCs compared with untreated ovariectomy ($P < 0.0001$). In contrast, DHA supplementation was associated with a significantly lower ratio of AA relative to DHA in RBCs compared with untreated ovariectomy ($P < 0.0001$). The proportion of EPA in RBCs was also significantly influenced by DHA and estrogenic treatment, and there was a statistically significant interaction between the two treatments for the percentage of EPA in RBCs ($P = 0.0009$). In the absence of estrogenic compounds, DHA treatment was associated with a significantly higher percentage of EPA in RBCs compared with untreated ovariectomy ($P < 0.0001$). The proportion of EPA in RBCs was greater in animals treated with 17β -estradiol and DHA compared with either DHA or 17β -estradiol treatment alone ($P < 0.0001$). Similarly, the proportion of DHA in RBCs was also significantly higher in animals treated with both 17β -estradiol and DHA compared with either treatment alone ($P < 0.0001$). Overall, the percentage of both n-3 and n-6 LCPUFAs was significantly higher, and the ratio of AA relative to DHA significantly lower, in animals treated with both 17β -estradiol and DHA compared with animals receiving DHA alone ($P < 0.0001$). In contrast, the percentage of n-6 LCPUFAs in RBCs was significantly higher in animals treated with a combination of daidzein and DHA compared with DHA alone ($P = 0.01$).

Bone Densitometry—Lumbar Spine and Femur. Ovariectomy resulted in significantly lower LS BMC and BMD at week 18, and F BMC and BMD at weeks 5 and 18, compared with sham (Fig. 1).

Over time, LS BMC was significantly influenced by treatment with estrogenic compounds and DHA ($P < 0.0001$ and $P = 0.04$, respectively). There was a significant interaction between estrogenic and DHA treatments on LS BMC ($P = 0.04$). Compared with untreated ovariectomy, final LS BMC was significantly higher in animals treated with 17β -estradiol ($P = 0.0002$). Final LS BMC in animals treated with both 17β -estradiol and DHA was not significantly different from animals treated with 17β -estradiol alone ($P = 0.07$). LS BMD was significantly greater in animals treated with 17β -estradiol compared with untreated ovariectomy at both week 5 ($P = 0.007$) and week 18 post-surgery ($P < 0.0001$) (Fig. 1).

At week 5, F BMC in animals treated with genistein, 17β -estradiol or DHA was significantly greater than in untreated ovariectomized animals ($P = 0.01$, $P = 0.0006$, and $P = 0.01$ respectively). However at week 18, F BMC was greater than untreated ovariectomized animals only in animals receiving the 17β -estradiol or DHA treatments ($P < 0.0001$ and $P = 0.01$, respectively). There was a statistically

significant interaction between treatment with estrogenic compounds and DHA ($P = 0.02$) and week 18 F BMC was significantly greater in animals treated with 17 β -estradiol and DHA compared with either 17 β -estradiol ($P < 0.0001$) or DHA alone ($P = 0.01$). Final F BMD was significantly greater in animals treated with 17 β -estradiol compared to untreated ovariectomy ($P < 0.0001$) (Fig. 1).

Trabecular and Cortical Bone Mineral Content, Area, and Density. Ovariectomy was associated with significantly lower trabecular BMC and BMD compared to sham-operation ($P < 0.0001$) (Table 5). Final trabecular BMC was significantly higher in DHA and 17 β -estradiol treated animals compared with untreated ovariectomized controls ($P = 0.008$ and $P < 0.0001$, respectively). Neither genistein nor daidzein treatment were associated with any significant effect on trabecular or cortical BMC, BMD, or BA.

There was no evidence of an interaction between DHA supplementation and treatment with estrogenic compounds on trabecular BMC, BMD, or BA. Cortical BMC and BA tended to be higher in the group supplemented with 17 β -estradiol and DHA than the group receiving 17 β -estradiol alone ($P = 0.05$ and $P = 0.02$, respectively); however, the difference failed to reach the level of significance required for the contrast following Bonferroni correction ($P < 0.01$). Periosteal circumference was significantly greater in the group receiving 17 β -estradiol and DHA than in the group receiving 17 β -estradiol alone ($P = 0.01$) (Table 5).

Biomechanics. Femurs from ovariectomized animals were significantly less elastic ($P = 0.01$) but not significantly weaker in terms of energy absorbed prior to breaking upon loading in the anterior-posterior direction than femurs of sham-operated animals (Fig. 2). Treatment with 17 β -estradiol had no statistically significant effect on elastic modulus but was associated with increased femur strength in terms of the amount of energy able to be absorbed prior to breaking ($P = 0.01$). The increased femur strength observed in 17 β -estradiol-treated animals was also apparent in animals treated with 17 β -estradiol and DHA.

Discussion

Ovariectomy resulted in decreased BMC and BMD in both the lumbar spine and femur. The effects of ovariectomy were particularly apparent in trabecular bone. Treatment with either genistein or daidzein had no beneficial effect on bone mass in the present study. However, treatment with 17 β -estradiol and to a lesser extent, DHA, provided some protection against the ovariectomy-induced decline in BMC.

The effect of phytoestrogens on bone post-ovariectomy is controversial. Although many studies have been published in this field, considerable heterogeneity in terms of the composition and dose of phytoestrogen supplements that have been administered and the type of animal model used have likely contributed to the considerable inconsistency in

Table 3. Mean Daily Food Intake, Final Uterus and Body Weights, and Plasma Concentrations of 17 β -Estradiol, IL-6, and Biochemical Markers of Bone Turnover

	Sham		Ovariectomized										P-value for interaction	
	No est.		Dai		Gen		Oes		No est.		Combined treatment			
	No DHA	No DHA	No DHA	No DHA	No DHA	No DHA	No DHA	No DHA	Dai+DHA	Gen+DHA	Oes+DHA	SE	Est. × DHA	
Mean daily food intake ^a (g)	18.3	18.4	18.0	18.8	17.4	18.9	18.9	18.0	18.9	18.9	18.2	0.52	0.81	
Final rat body weight (g)	341.9*	408.0	363.3*	410.5	363.9*	412.4	412.4	378.3	399.8	399.8	369.8	12.8	0.80	
Final uterus weight (mg)	749.6*	159.9	204.3	169.1	452.2*	191.0	191.0	199.3	179.2	179.2	431.8 ^c	20.1	0.60	
17β-estradiol (measured at week 8) (pmol/L)	77.5*	BD	BD	BD	40.3*	BD	BD	BD	BD	BD	42.3	6.7	0.93	
Final plasma IL-6 (pg/ml)	19.6	24.4	19.9	22.1	26.7	21.3	21.3	25.7	23.7	23.7	19.6 ^d	0.9	0.03	
Final carboxylated osteocalcin ^b (ng/ml)	1762	1765	1767	1766	1754	1773*	1773*	1787	1780	1780	1762	16.8	0.88	
Final under-carboxylated osteocalcin ^b (ng/ml)	478	475	473	474	485	467*	467*	453	460	460	478	16.7	0.88	
Final total osteocalcin ^b (ng/ml)	2313	2285	2309	2311	2246*	2310	2310	2279	2293	2293	2283	44.8	0.21	

^a Food intake of ovariectomized animals was limited to that of sham controls to minimize ovariectomy-induced weight gain.

^b Represents intact and fragmented osteocalcin. All osteocalcin measurements were made in plasma; BD, below detectable limits of assay.

^c Significantly different from DHA alone at $P < 0.01$ (contrast made for combined treatment only).

^d Significantly different from estrogenic compound alone at $P < 0.01$ (contrast made for combined treatment only).

* Significantly different from OVX at $P < 0.01$ (contrast made for main effects only).

Table 4. Percentage of Long Chain Polyunsaturated Fatty Acids in Total Red Blood Cell Lipids in Female Rats Following Ovariectomy (Ovx) or Sham Operation and Treatment with Either Daidzein (DAI, 20 mg/kg Body Weight/Day), Genistein (GEN, 20 mg/kg Body Weight/Day), 17 β -Estradiol (OES, 1 μ g/Day), or Docosahexaenoic Acid (DHA, 0.5 g/kg Body Weight/Day) or a Combination of Estrogenic Compound and DHA for 18 Weeks

	Sham	Ovariectomized								<i>P</i> -value for interaction	
	No est.	No est.	Dai	Gen	Oes	No est.	Combined treatment				
	No	No	No	No	No		Dai+	Gen+	Oes+		
	DHA	DHA	DHA	DHA	DHA		DHA	DHA	DHA	DHA	SE
AA	21.3	19.8	20.7	20.4	21.6*	16.4*	18.3 ^{a,b}	16.4 ^b	18.4 ^{a,b}	0.39	0.05
EPA	0.49	0.54	0.45	0.44	0.67*	1.04*	0.90 ^b	0.90 ^b	1.36 ^{a,b}	0.04	0.0009
DHA	5.8*	5.5	5.4	5.4	6.1	7.7*	7.5 ^b	7.5 ^b	8.3 ^{a,b}	0.22	0.70
AA:DHA	6.2*	6.4	7.1*	7.7*	6.5	2.1*	2.4 ^b	2.1 ^b	2.1 ^b	0.24	0.002
Total n-3	6.8	6.6	6.3	6.3	7.4*	9.6*	9.2 ^b	9.1 ^b	10.6 ^{a,b}	0.30	0.09
Total n-6	21.7	20.3	21.1	20.9	22.0*	17.9*	18.9 ^{a,b}	16.9 ^b	18.9 ^{a,b}	0.38	0.04
Ratio n-3:n-6	0.35	0.35	0.32	0.35	0.37	0.56*	0.50 ^{a,b}	0.57 ^b	0.58 ^b	0.02	0.08

^a Significantly different from DHA alone at $P < 0.01$ (contrast made for combined treatment only).

^b Significantly different from estrogenic compound alone at $P < 0.01$ (contrast made for combined treatment only).

* Significantly different from OVX at $P < 0.01$ (contrast made for main effects only).

results obtained. In the present study, genistein supplementation had a weak protective effect against ovariectomy-induced loss of LS BMC in the first 5 weeks following ovariectomy. However, the effect was transient, and no beneficial effect of genistein on bone mass was evident at week 18 following ovariectomy. Three short-term studies have reported bone mass-preserving effects of genistein and equol (the main metabolite of daidzein) in the first 2–3 weeks following ovariectomy (24–26). Beneficial effects on bone mass of daidzein and/or genistein administered in similar doses to those in the present study have been reported up to 22 weeks post-ovariectomy in animals fed a low calcium diet (23, 27, 28). It is possible that when a calcium-adequate diet is fed, such as in the present study, the effects of genistein and daidzein on bone mass are minimal in the longer term. In the present study, daidzein supplementation was associated with reduced body weight gain post-ovariectomy. A similar result has previously been shown following soy isoflavone consumption and is believed to be a result of a reduction in food utilization rate (29).

Unlike phytoestrogens, the effect of 17 β -estradiol on bone mass post-ovariectomy is well-established. In the present study, femurs of 17 β -estradiol-treated animals were stronger and had greater BMC than those of untreated ovariectomized controls. Treatment with 17 β -estradiol was associated with lower total plasma osteocalcin concentration compared with ovariectomized controls. Osteocalcin is a bone matrix protein produced by osteoblasts, odontoblasts and hypertrophic chondrocytes (30). It is released into the bloodstream during new bone formation (31), and fragments of osteocalcin are released during osteoclastic bone resorption (32). Osteocalcin is activated by γ -carboxylation

of glutamic acid at positions 17, 21, and 24. In the present study, the antibodies used to measure carboxylated and undercarboxylated osteocalcin detected intact as well as fragmented osteocalcin. The total circulating osteocalcin concentration obtained serves as a marker of bone remodeling rate with a lower concentration indicative of a lower rate of bone turnover (32). The lower circulating concentration of total osteocalcin observed with 17 β -estradiol treatment both in the present study in ovariectomized rats and previously in post-menopausal women (33) is consistent with a reduction in the rate of bone turnover following 17 β -estradiol treatment.

In women, circulating IL-6 concentration increases post-menopause and decreases following estrogen replacement therapy (34). In the present study, plasma IL-6 concentration was not significantly different in ovariectomized rats compared with sham, or in 17 β -estradiol-treated animals compared with untreated ovariectomy. In rats, responsiveness of IL-6 to estrogenic compounds is tissue specific (35, 36). IL-6 expression therefore may need to change by a greater amount in rats compared to humans before the change is reflected in circulating IL-6 concentration.

In the present study, both 17 β -estradiol and DHA treatments were associated with significantly higher trabecular BMC and femur BMC and BMD at week 18 compared with untreated ovariectomy. Unlike femurs from 17 β -estradiol-treated animals, however, femurs from DHA-treated animals were not significantly stronger than those from untreated ovariectomized animals. As neither endosteal circumference nor total plasma osteocalcin concentration were significantly different in DHA-treated animals compared with ovariectomized controls, DHA may have

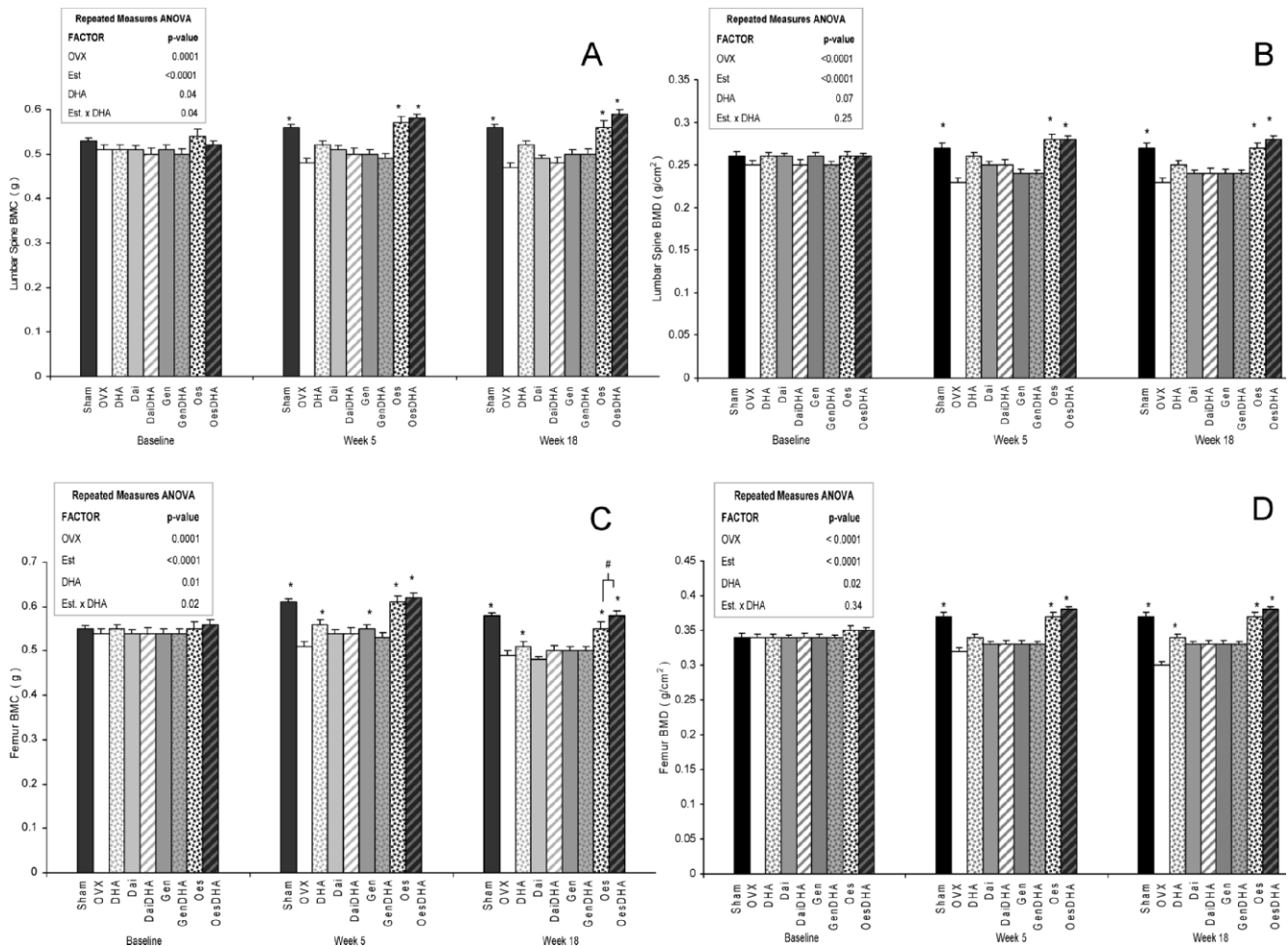


Figure 1. Comparison of (A) lumbar spine bone mineral content, (B) lumbar spine bone mineral density, (C) femur bone mineral content, and (D) femur bone mineral density between untreated ovariectomized animals and sham-operated or ovariectomized animals treated with estrogenic compounds and/or DHA at trial commencement (baseline) and study weeks 5 and 18. Measurements were made *in vivo* by DEXA. Results were analyzed by repeated measures factorial analysis. P-values for main effects of treatment and the interaction between treatments are provided. * Significantly different from untreated ovariectomized controls. # Significant difference between treatment with estrogenic compound alone and estrogenic compound + DHA.

maintained bone mass by a different mechanism than that of 17β -estradiol. Protective effects of DHA on bone mass have previously been reported in studies comparing the effects of supplementation with high-DHA and low-DHA oils on bone mass in ovariectomized rodents (20, 37). However, the mechanism of action of DHA remains unclear. In the present study, total plasma osteocalcin concentration was unchanged with DHA treatment. However, mean plasma concentration of carboxylated osteocalcin was greater, and concentration of undercarboxylated osteocalcin was lower in DHA-treated compared with untreated ovariectomized controls. Gamma-carboxylation of osteocalcin is essential for hydroxyapatite binding to bone matrix and hence mineralization of bone. Phospholipids form an integral part of the gamma-carboxylase enzyme and are essential for carboxylase activity (38). The effect of the type of fat incorporated into the phospholipids within gamma-carboxylase on enzyme activity is unknown. However consump-

tion of n-3 fatty acids has previously been shown to affect the levels of vitamin K-dependent coagulation factors in rats (39), suggesting a modulatory effect of n-3 fatty acids on vitamin K-dependent enzyme activity.

In the present study, combined supplementation with genistein or daidzein and DHA had no beneficial effect on BMC above that of DHA treatment alone. Watkins *et al.* (2005) observed a reduction in serum concentration of pyridinoline, a marker of bone resorption, in growing ovariectomized rats fed a low calcium diet (0.11% of diet) supplemented with menhaden oil (a source of n-3 LCPUFAs) and a soy isoflavone-containing protein supplement (17). Although treatment with n-3 LCPUFAs alone had a beneficial effect on bone mass in the Watkins *et al.* (2005) study, combined soy isoflavone/n-3 LCPUFA supplementation failed to further augment bone mass (17). Results of the Watkins *et al.* (2005) study as well as of the present study do not support a beneficial effect of combined phytoestrogen

Table 5. Trabecular and Cortical Bone Mineral Content, Area, and Density of Right Tibias from Female Rats as Measured by pQCT Following Ovariectomy or Sham Operation and Treatment with Either Daidzein (DAI, 20 mg/kg Body Weight/Day), Genistein (GEN, 20 mg/kg Body Weight/Day), 17 β -Estradiol (OES, 1 μ g/Day), or Docosahexaenoic Acid (DHA, 0.5 g/kg Body Weight/Day) or a Combination of Estrogenic Compound and DHA for 18 Weeks

	Sham	Ovariectomized								SE	<i>P</i> -value for interaction
	No est.	No est.	Dai	Gen	Oes	No est.	Combined treatment				
	No DHA	No DHA	No DHA	No DHA	No DHA		Dai+ DHA	Gen+ DHA	Oes+ DHA		
Trab BMC (mg/mm)	5.69	3.47	4.11	4.16	5.80*	4.84*	4.58	4.34	6.13	0.13	0.45
Trab BA (mm ²)	9.5	10.2	9.8	9.5	10.2	10.1	10.3	9.4	10.5	0.15	0.63
Trab BMD (mg/cm ³)	593	338	421	437	570*	482	453	459	586	11.4	0.29
Cort BMC (mg/mm)	6.9	6.8	6.7	6.9	7.0	6.9	6.7	6.8	7.2	0.06	0.23
Cort BA (mm ²)	5.07	5.12	5.00	5.10	5.08	5.15	5.00	5.07	5.28	0.04	0.27
Cort BMD (mg/cm ³)	1322	1331	1314	1322	1341	1326	1320	1315	1337	3.6	0.61
Periosteal circ. (mm)	10.05	10.13	10.05	10.11	10.01	10.16	10.08	10.05	10.21 ^a	0.04	0.15
Endosteal circ. (mm)	5.99	6.24	6.14	6.12	5.96*	6.13	6.12	6.05	6.08	0.04	0.42

^a Significantly different from estrogenic compound alone at $P < 0.01$ (contrast made for combined treatment only).

* Significantly different from OVX at $P < 0.01$ (contrast made for main effects only).

and LCPUFA supplementation on ovariectomy-induced bone loss additional to that of LCPUFAs alone.

Combined treatment with 17 β -estradiol and DHA resulted in greater F BMC at week 18 compared with either treatment alone in the present study. There was a statistically significant interaction between estrogenic compound and DHA treatments for F BMC. This may indicate DHA

and 17 β -estradiol acted synergistically to promote greater BMC. Alternatively, it may reflect a divergent effect of co-treatment with DHA and phytoestrogens as opposed to 17 β -estradiol as combined treatment with DHA and either daidzein or genistein resulted in slightly, but not significantly, lower BMC than DHA treatment alone. There was also evidence of possible interactions between estrogenic

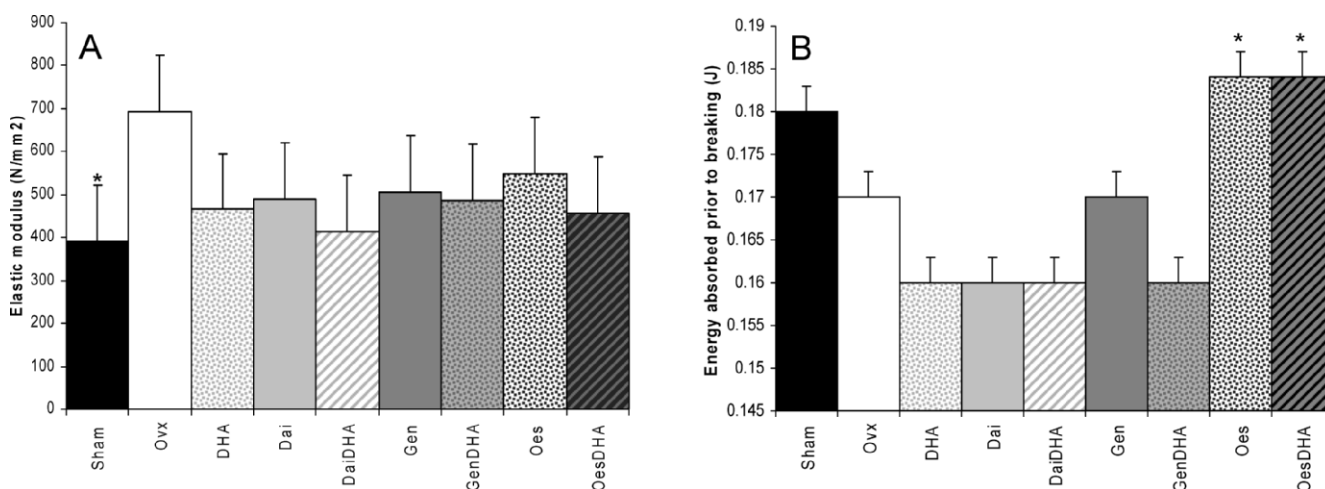


Figure 2. Biomechanical properties of femurs from untreated ovariectomized rats compared with sham-operated animals and ovariectomized animals treated with estrogenic compounds and/or DHA. (A) Elastic modulus and (B) energy absorbed prior to breaking. Measurements were made in the right femur by 3-point bending 18 weeks following ovariectomy or sham-operation. * Significantly different from untreated ovariectomized controls.

compounds and DHA on both plasma IL-6 concentration and the percentage of n-3 LCPUFAs in RBCs, which again may be a result of divergent effects of phytoestrogens compared with 17 β -estradiol when co-administered with DHA. As co-treatment with 17 β -estradiol and DHA was associated with a lower plasma IL-6 concentration and a higher percentage of n-3 LCPUFA in RBC, combined treatment with 17 β -estradiol and DHA may have a beneficial effect in reducing inflammatory status. This may have implications for the prevention of other inflammatory diseases.

In conclusion, combined DHA and 17 β -estradiol treatment was more effective than either treatment alone in countering the effects of ovariectomy on BMC. There was no evidence of a beneficial effect of combined DHA and daidzein or genistein treatment on BMC post-ovariectomy in the present study. Treatment with 17 β -estradiol in a lower dose than in conventional HRT in conjunction with DHA supplementation may potentially reduce the health risks currently associated with HRT use. Further work is required in order to determine the mechanism of action of DHA on bone and to clarify the nature of the interaction between 17 β -estradiol and DHA.

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