

## Fish Oil with Higher DHA Content and Voluntary Exercise Decreases Postmenopausal Bone Loss

Jameela Banu<sup>1\*</sup> and Gabriel Fernandes<sup>2</sup>

<sup>1</sup>Department of Health and Biomedical Sciences and Department of Biology, University of Texas Rio Grande Valley, Edinburg, Texas, USA

<sup>2</sup>Department of Medicine, University of Texas Health Science Center, San Antonio, Texas, USA

### Abstract

There is increasing evidence suggesting that fish oil (FO) decreases bone resorption by reducing osteoclastogenesis and regular exercise (EX) increases bone mass. EX is associated with increasing bone formation. Therefore, the combined effects of FO intake and EX may have additive effects by both increasing bone formation and decreasing bone resorption. To demonstrate this, we studied the effects of FO and EX on the bone of mice after an ovariectomy-induced bone loss.

Twelve months old C57BL/6 female mice were either sham operated or ovariectomized, divided into different dietary and EX group and maintained for 3 months before sacrifice.

The distal femoral metaphysis (DFM) showed significantly higher total BMC in both the FO sham sedentary groups. 30/20 ovariectomized sedentary mice had higher total bone mineral content (BMC). EX significantly increased BMC in the control and one of the FO ovariectomized (18/12) groups. The total bone mineral density (BMD) was higher in both the FO ovariectomized mice. EX significantly increased BMD in the control and combination with 18/12 ovariectomized groups. Cortical BMC increased after EX with both FO diets.

The DFM showed no significant changes in the trabecular thickness. However, the trabecular number increased in mice fed both FO diet and on EX. Trabecular separation was decreased in the control and 30/20 FO-fed animals but increased in the 18/12 FO-fed mice.

In conclusion, in the distal femoral metaphysis, FO-fed mice showed increased BMD. In combination with EX, the 30/20 FO-fed mice showed higher trabecular number and cortical bone mass as well as decreased trabecular separation.

**Keywords:** Fish oil; Postmenopausal bone loss, pQCT;  $\mu$ CT densitometry

### Introduction

Osteoporosis is a disease primarily associated with aging in men and menopause in women. The hallmark of this medical condition is that minor trauma causes fractures as the bones are very fragile. Bones are dynamic organs that undergo modeling and remodeling as vertebrate organisms grow, mature and age, via specialized cells called osteoblasts and osteoclasts [1]. Bone remodeling maintains the skeleton by adding to growing bone, repairing damaged portions and removing old bone. Bones also are the body's reservoir depots for calcium and phosphorus, and both can be released into the blood to maintain ionic homeostasis. Bone loss occurs when there is an imbalance in the process of bone remodeling. This imbalance is because there is increased bone resorption alone or this is combined with decreased bone formation.

Therefore, treating and/or preventing bone loss will be more effective if it is focused on the reduction of bone resorption (osteoclast activity) and in increasing bone formation (by reactivation or maintenance of osteoblasts). Currently, several therapeutics are available to treat and prevent osteoporosis. However, the side effects of these drugs can outweigh the benefits in some patients [2-5] which has increased interest in finding alternative medicines to treat and prevent osteoporosis.

Essential fatty acids like omega 3 fatty acids have been shown to protect bones from postmenopausal bone loss [6-9]. Salt water fish is a major source of omega-3 fatty acid. Fish oil (FO) inhibits osteoclastogenesis, thereby, decreasing bone resorption. Two of the

long chain omega-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have shown different effects on bone [10] with DHA showing more bone protective properties than EPA. In this study, we tested two different combinations of EPA/DHA to determine their effects on bone after ovariectomy.

As age advances, there is a reduction in physical activity and this may also contribute to increased bone loss. It is very well established that increase in physical activity or exercise has beneficial effects on bone mainly by stimulation of bone formation [11]. However, moderate voluntary exercise has the most benefits on bone. A common form of voluntary exercise used on rodent animal models is treadmill running, as this can produce high bone strains of normal coordinated activity [12-14]. In the present study, we used both FO (two different EPA/DHA ratios) and flatbed treadmill exercise to determine the effects on the distal femur of middle-aged ovariectomized mice.

**\*Corresponding author:** Jameela B, Department of Health and Biomedical Sciences and Department of Biology, College of Health Affairs and College of Sciences, University of Texas Rio Grande Valley, Edinburg, TX, USA, Tel: 9566653222; Fax: 9566655253; E-mail: [jameela.banu@utrgv.edu](mailto:jameela.banu@utrgv.edu)

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## Materials and Methods

### Animals

Twelve months old C57BL/6 female mice, were bought from Jackson Laboratory (Bar Harbor, ME). After two weeks of acclimatization, they were either sham-operated or ovariectomized and divided into the following groups: Group 1 Lab chow sham sedentary (LC S Sed); Group 2 LC S exercise (LC S EX); Group 3 LC ovariectomized S (LS O Sed); Group 4 LC O EX; Group 5 18/12 S Sed; Group 6 18/12 S EX; Group 7 18/12 O Sed; Group 8 18/12 O EX; Group 9 30/20 S Sed; Group 10 30/20 S EX; Group 11 30/20 O Sed; Group 12 30/20 O EX. Control group was fed and maintained on Lab Chow diet (AIN 93) and the treatment groups were fed experimental diets containing two different types of 5% fish oil (18/12 and 30/20), containing either EPA 18% and DHA 12% or 30% EPA and 20% DHA, added to the lab chow and mixed well.

Mice were on their respective diets and a treadmill exercise regimen three days after surgery. The mice were on their treatments for 3 months and then sacrificed. The femur was removed and stored for pQCT and  $\mu$ CT densitometry. The experiments were approved by UTHSCSA's IACUC committee. All the procedures conducted were in accordance with the ethical standards of NIH and the University at which the studies were conducted.

### Body weight and organ weights

At the beginning of the experiment, mice were weight matched using a CS 200 (Ohaus, Pine Brook NJ) balance. They were also weighed at the time of sacrifice. At the time of euthanasia, several organs including the uterus, adipose tissue, quadriceps and gastrocnemius, liver, spleen and kidneys were weighed using a Mettler Balance (Columbus, OH), at the time of sacrifice.

### Peripheral quantitative computerized tomography densitometry (pQCT)

Cortical and cancellous bones of the distal femoral metaphysis (DFM) were analyzed using pQCT densitometry (XCT Research M system, Norland Stratec, Birkenfeld, Germany), as described previously [11,15]. Five slices were scanned including the growth plate. Three slices 1mm proximal to the knee joint were analyzed. The following parameters were determined: cancellous bone mineral content (Cn BMC), cancellous bone mineral density (Cn BMD), cortical bone area (Ct Ar), cortical BMC (Ct BMC), cortical BMD (Ct BMD), cortical thickness (Ct Th), periosteal perimeter (Peri PM) and endocortical perimeter (Endo PM).

### Micro computed tomography ( $\mu$ CT)

The distal femoral metaphysis was scanned (n=6 from each group) using a high resolution  $\mu$ CT scanner Xradia  $\mu$ XT200 (Xradia Inc, Concord, CA) at 20  $\mu$ . All images were acquired using standard parameters: resolution of 9.1  $\mu$ m, x-ray source of 90 KV, power of 4.0 W and current of 44.0  $\mu$ A with an exposure time of 12 s per slice. The scans were analyzed using Tri/3D Bon (Ratoc, Inc., Japan) for the following parameters: Total Volume (TV), Bone Volume (BV), BV/TV, Trabecular Number (Tb N), Trabecular Thickness (Tb TH), Trabecular Separation (Tb S), connectivity density (Conn Den).

### Statistical analysis

Data were analyzed by one-way ANOVA and unpaired t-test using GraphPad Prism 4 (GraphPad Software Inc, San Diego, CA, USA). Results are expressed as Mean  $\pm$  SE.  $P \leq 0.05$  was considered

to be significant. Newman-Keuls multiple comparison test was used to analyze the differences between groups for significance.

## Results

### Body weight and organ weights

**Effects of fish oil:** Final body weight did not change significantly although the animals in the FO groups were heavier than those in the lab chow group (Figure 1ii). Mice in the FO-fed groups had significantly increased adipose tissue when compared to that of the LC fed mice (Figure 1iii). Ovariectomized mice from all groups had significantly smaller uterus (Figure 1iv).

**Effects of exercise:** Exercise did not change the body weight in all the groups studied (Figure 1ii). Although exercise decreased adipose tissue in all the exercise groups, this decrease was significant only in the 18/12 S EX and 30/20 S EX groups (Figure 1iii). The weight of the uterus was further decreased in the EX groups (Figure 1iv).

### pQCT densitometry

**Effects of fish oil:** Total BMC was significantly higher in the LC O EX and 30/20 O Sed groups when compared to that of LC O Sed groups (Figure 2i). Both the FO groups showed significantly higher total BMC in the sham Sed groups. Higher total BMD was seen in the 30/20 S Sed and 30/20 O Sed groups, but this increase was not statistically significant (Figure 2ii). Significant increases in the cortical BMC were seen in the 30/20 S Sed group when compared to that of LC S Sed group (Figure 2iii). However, there was no significant change in the cortical BMD (Figure 2iv), cortical thickness, periosteal perimeter and endocortical perimeter in any of the groups studied (Figure 3i-3iii).

**Effects of exercise:** Significant increase in the total BMC was seen in the LC O EX, 30/20 S Sed, and the LC O EX groups (Figure 2i). The total BMD increased in mice from 18/12 O EX ( $p < 0.08$ ) (Figure 2ii). Mice from the LC O EX group showed significantly higher cortical BMC when compared to that of LC O Sed mice (Figure 2iii). Similarly, significantly higher cortical BMC was observed in mice from the 18/12 O EX group, when compared to that of 18/12 O Sed. Cortical BMD did not change significantly in any of the groups studied (Figure 2iv). Cortical thickness increased in the 30/20 S EX animals than LC S EX group (Figure 3i). Similarly, 18/12 S EX mice showed higher cortical thickness, when compared to that of 18/12 S Sed and barely increased ( $p < 0.06$ ), than LC S EX group mice (Figure 3i).

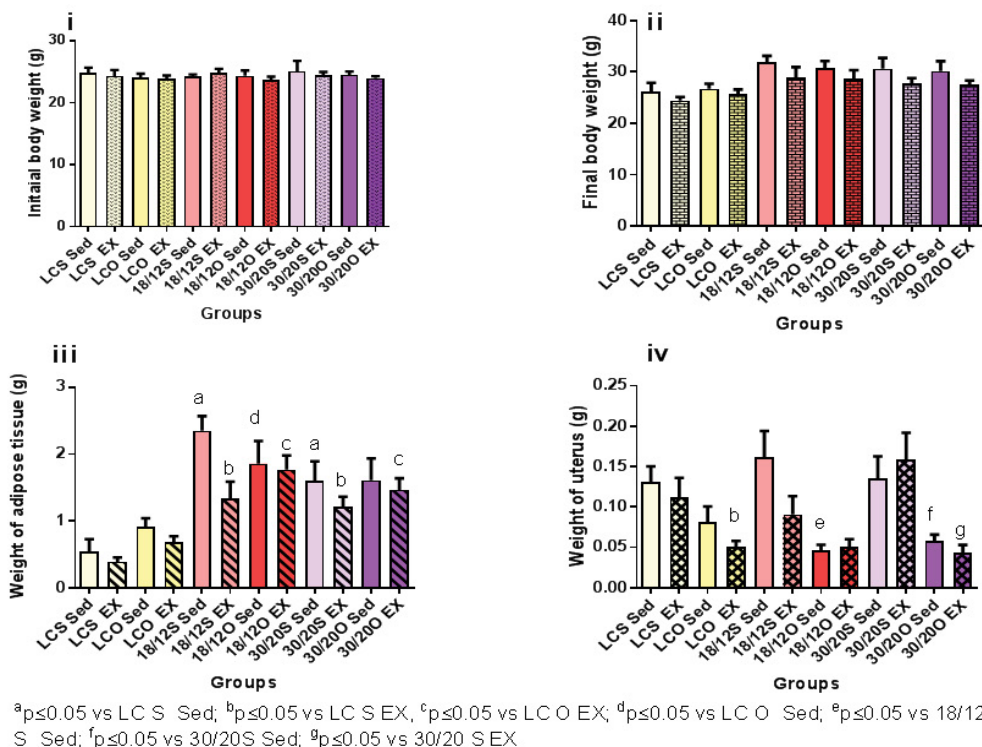
### $\mu$ CT densitometry

**Effects of fish oil:** Higher trabecular number was seen in mice from 18/12 S EX group, while those in the 30/20 fed mice, showed increased trabecular number in the OVX groups (Figure 4i). There were no changes in the trabecular thickness was observed in any of the groups studied. Mice from 18/12 fed group showed higher trabecular separation, while those from 30/20 group showed less trabecular separation (Figure 4iii).

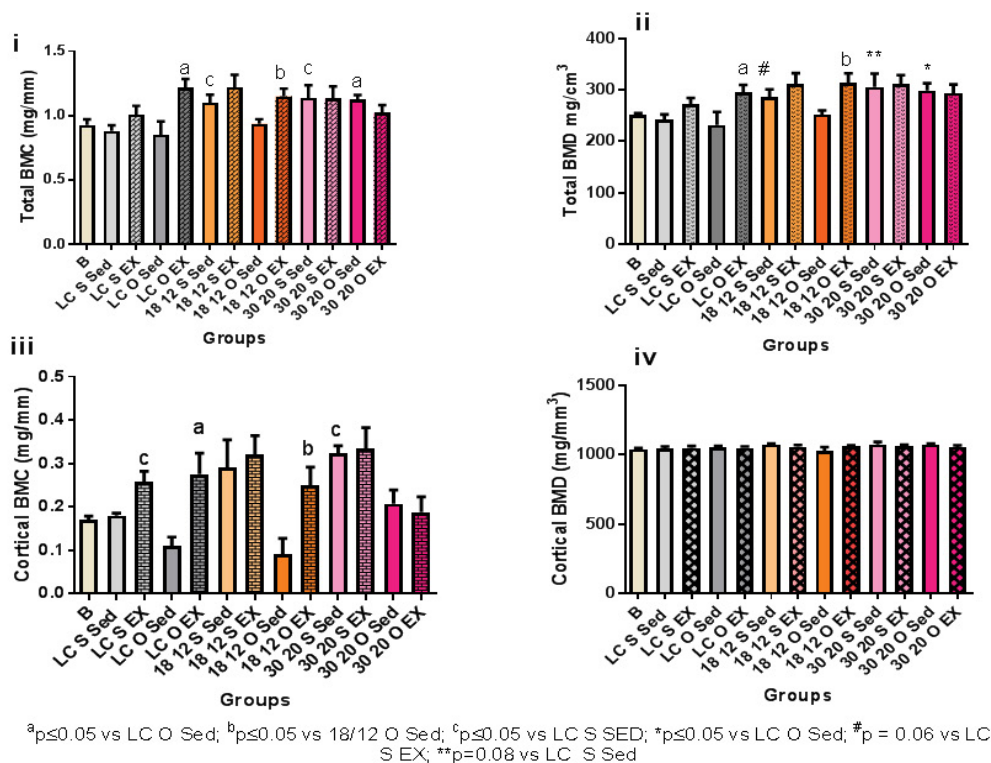
**Effects of exercise:** Significantly higher trabecular number was seen in the 18/12 S EX as compared to 18/12 S Sed and LC S EX (Figure 4ii). There was increased trabecular number in the 30/20 O EX group, but this increase was not statistically significant (Figure 4ii). No significant difference in the trabecular thickness was seen in any of the groups studied (Figure 4i). EX decreased trabecular separation in the FO treated sham and OVX groups (Figure 4iii).

## Discussion

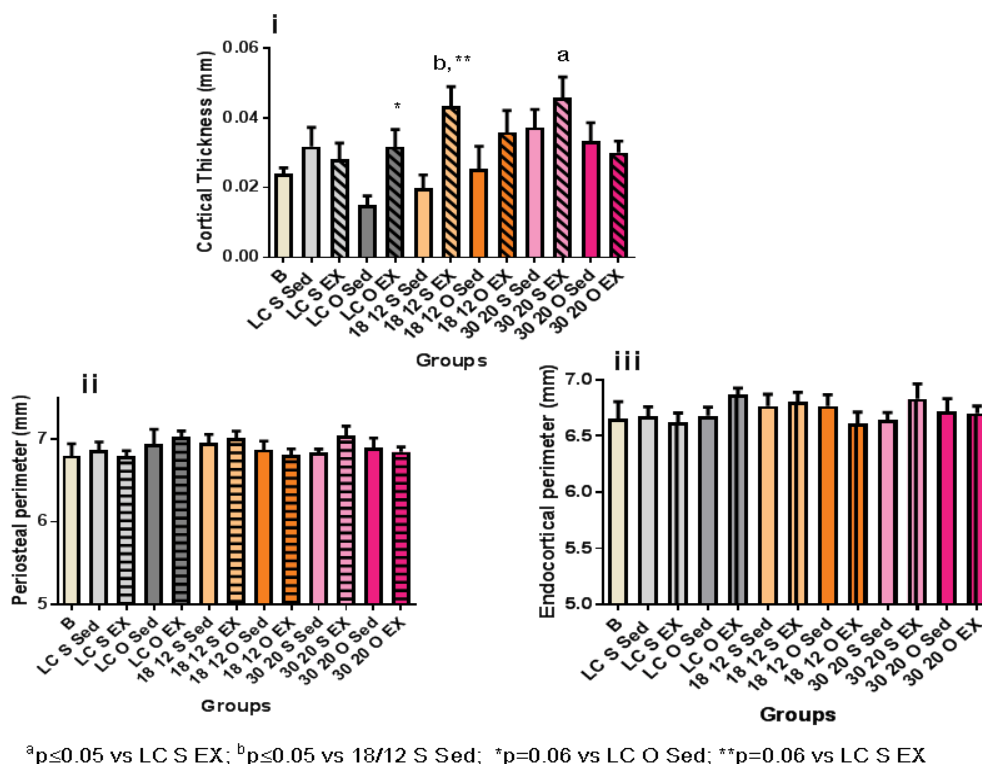
Dietary fatty acids have significant beneficial effects on bone [16].



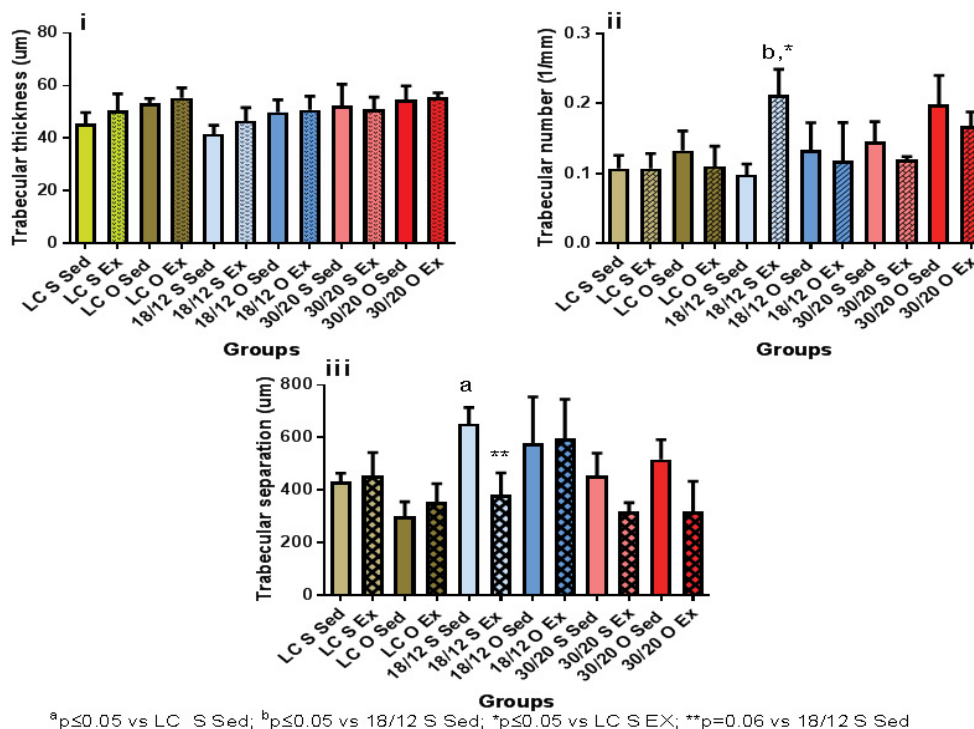
**Figure 1:** Effects of fish oil on body weight and organ weights of middle aged female C57Bl/6 Mice: (i) Initial body weight; (ii) Final body weight; (iii) Weight of adipose tissue; (iv) Weight of uterus (ap ≤ 0.05 vs. LC S Sed; bp ≤ 0.05 vs. LC S EX, cp ≤ 0.05 vs. LC O EX; dp ≤ 0.05 vs. LC O Sed; ep ≤ 0.05 vs. 18/12 S Sed; fp ≤ 0.05 vs. 30/20S Sed; gp ≤ 0.05 vs. 30/20 S EX).



**Figure 2:** Effects of Fish Oil on the Total and Cortical Bone Parameters of the Distal Femoral Metaphysis of Middle Aged C57Bl/6 Mice (pQCT): (i) Total BMC; (ii) Total BMD; (iii) Cortical BMC (iv) Cortical BMD (ap ≤ 0.05 vs. LC O Sed; bp ≤ 0.05 vs. 18/12 O Sed; cp ≤ 0.05 vs. LC S SED; <sup>\*</sup>p ≤ 0.05 vs. LC O Sed; <sup>#</sup>p=0.06 vs. LC S EX; <sup>\*\*</sup>p=0.08 vs. LC S Sed).



**Figure 3:** Effects of fish oil on the cortical thickness and perimeters of the distal femoral metaphysis of middle aged C57Bl/6 mice (pQCT): (i) cortical thickness; (ii) Periosteal perimeter; (iii) Endocortical perimeter (ap ≤ 0.05 vs. LC S EX; bp ≤ 0.05 vs. 18/12 S Sed; \*p=0.06 vs. LC O Sed; \*\*p=0.06 vs. LC S EX).



**Figure 4:** Effects of fish oil on the trabecular parameters of the distal femoral metaphysis of middle aged C57Bl/6 mice (μCT): (i) trabecular thickness; (ii) trabecular number; (iii) Trabecular separation. (ap ≤ 0.05 vs. LC S Sed; bp ≤ 0.05 vs. 18/12 S Sed; \*p ≤ 0.05 vs. LC S EX; \*\*p=0.06 vs. 18/12 S Sed).

Whether the fatty acid is beneficial or detrimental to bone health is dependent on the type of fatty acid that is consumed [17]. Fatty acids may influence bone using different pathways including inflammatory and oxidative stress related pathways. Some fatty acids like n-6 fatty acids enhance inflammation, and others such as n-3 fatty acids can reduce inflammation and counter oxidative stress [18,19]. However, the ratio of n-6/n-3 fatty acids has a major effect and can protect bone in animal models and humans [20-27]. The major fatty acids in FO, EPA and DHA are both beneficial to bone but the ratio of these n-3 fatty acids plays an important role in determining the extent of the benefits.

In addition to FO, EX also is implicated in helping increased bone mass by stimulating bone formation and reducing bone resorption [11]. In the present study, we tested two ratios of EPA/DHA along with exercise. We observed that FO 30/20 with higher concentrations of EPA and DHA could reduce bone loss mainly in the trabecular bone compartment of the distal femur. Exercise further reduced bone loss in the presence of 30/20. Cortical BMC was also increased in 30/20 FO-fed mice. This is in line with several reports that support the beneficial effects of FO on bone [6-10,24], although, this is the first report that tested two concentrations of EPA and DHA.

In conclusion, FO-fed mice had increased bone mass in the distal femur; both fish oils showed higher trabecular number while 30/20 FO showed more decreased trabecular separation. It is probable that higher concentrations of EPA/DHA along with exercise may be more beneficial to bone. More extensive work must be conducted to understand the mechanism behind this influence of FO on bone.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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