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The effect of docosahexaenoic acid on bone microstructure in young mice and bone fracture in neonates

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ABSTRACT

Background: As low bone mineral density is a risk factor for fracture in childhood, optimizing age appropriate bone mass is recommended and might lower the impact of bone loss related to age. Consumption of omega-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic and docosahexaenoic (DHA) acids have been shown to beneficially modulate bone metabolism. The objective of this study was to determine the incidence of fracture in neonates receiving a fish compared with soybean oil-based intravenous lipid emulsion and evaluate the effect of varying dietary omega-3 PUFA consumption on growing bone in young mice.

Materials and methods: Eligibility criteria for the clinical study included gestational age ≤ 37 wk and parenteral nutrition-dependence for ≥ 4 wk. Radiographs were reviewed after lipid initiation to identify radiologic bone fracture. The animal study evaluated female C57/Bl6 mice randomized into one of five groups from age 3–12 wk, at which time femurs were harvested for micro-computed tomography and light microscopy analysis.

Results: A lower incidence of bone fracture was found in neonates maintained on fish compared with soybean oil. In the animal study, findings suggest the DHA diet provides the best protection against trabecular bone loss as evidenced by increased bone volume fraction, increased trabecular number, and decreased trabecular separation on micro-computed tomography. These protective effects appeared to affect the bone microstructure alone.

Conclusions: The lower fracture risk observed in fish oil fed neonates in combination with the protective effects of DHA observed in the femurs of young C57/BL6 mice suggest an important role for omega-3 PUFAs on bone growth.

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1. Introduction

At least 90% of peak bone mass is obtained by the age of 18 y [1]. As low bone mineral density is a risk factor for fracture in childhood, optimizing age appropriate bone mass is recommended and might lower the impact of bone loss related to age. Consumption of omega-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are important biomediators and have been shown to beneficially modulate bone metabolism. Murine studies have shown reduced bone loss after dietary omega-3s in ovariectomized and aged animals [2–4] and beneficial effects on bone development and metabolism in young growing animals [5–11]. In human studies, it has been shown that dietary fat modulates the formation of bone and remodeling during development, especially over the first year of life. Growth failure and metabolic bone disease are commonly seen in parenteral nutrition (PN)-dependent neonates and those born prematurely, as the greatest mineral accumulation occurs during the third trimester. In caring for PN-dependent neonates at our institution, the administration of intravenous fish oil appeared to lessen the frequency of bone fracture and fragility compared with soybean oil. This was purely an observation regarding the type of lipid administration and warranted further investigation. Therefore, we hypothesize that omega-3 PUFA fortified diets provide the best protection against trabecular bone loss. To that end, the objective of this study was two-fold: to determine the incidence of fracture in neonates receiving a fish oil compared with a soybean oil-based intravenous lipid emulsion and subsequently to evaluate the effect of varying dietary omega-3 PUFA consumption on growing bone in young C57/BL6 mice.

2. Materials and methods

2.1. Clinical neonatal review

A research study protocol (No IRB-P00004009) was approved by the Institutional Review Board at Boston Children's Hospital to conduct a single-center retrospective review of prospectively collected data to evaluate the fracture risk of PN-dependent neonates on fish versus soybean oil-based lipid emulsions. Neonates with cholestasis (direct bilirubin ≥ 2 mg/dL) due to congenital or acquired gastrointestinal disease who were enrolled in an open-label treatment protocol with a fish oil-based lipid emulsion at 1 g/kg/d (Omegaven; Fresenius Kabi AG, Bad Homburg v.d.H., Germany) from 2005–2012, were compared with a historical cohort of PN-dependent cholestatic neonates who received a soybean oil-based lipid emulsion at 1–4 g/kg/d (Intralipid; Fresenius Kabi, Uppsala, Sweden) from 1999–2007 [12,13]. The comparison cohort preceded the introduction of fish oil and received the standard of care dose and type of lipid emulsion at the time. Neonates that were included in the control cohort had two consecutive direct bilirubin values ≥ 2 mg/dL while on PN that could not be attributed to another cause of hepatic disease. Eligibility criteria for the present study included a gestational age ≤ 37 wk and PN-dependence for ≥ 4 wk. There were 131

neonates on fish oil and 50 neonates on soybean oil who met study criteria. Radiographs were taken as clinically indicated and reviewed from lipid start until stop date or 4 mo after lipid initiation to identify radiologic bone fracture. Radiological reports reviewed by an attending radiologist were used to confirm bone fracture. The incidence and type of fracture was evaluated between the fish and soybean oil cohorts.

2.2. Murine dietary protocol

Female mice (aged 21 d) were obtained from Jackson Laboratories (Bar Harbor, ME). These animals were fed standard rodent chow (AIN-93M Purified Rodent Diet No. 110900; Dyets Inc, Bethlehem, PA) and were randomized and housed in groups of five in regular vented cages within a barrier room with a 12-h light cycle. Male mice were not used as preliminary studies showed no difference in the outcomes evaluated. For the present study, mice were randomized into one of five dietary groups ($n = 10$ per group), which varied by the lipid type. Group 1 (Soy) was fed standard rodent chow (AIN-93M; Dyets No. 110900) with soybean oil, group 2 (HCO) was fed hydrogenated coconut oil (HCO), an essential fatty acid free diet (Dyets No. 102328), and groups 3–5 were fed modified AIN-93M diets. Group 3 (Menhaden) was fed menhaden oil as the sole source of fat (Dyets No. 102332), group 4 (20:1 DHA:arachidonic acid [AA]) was fed a 20:1 ratio of DHA:AA consisting of 2.0% calories from DHA, 0.1% from AA, and 7.9% from HCO (Dyets No. 102536), and group 5 (DHA) was fed a DHA-rich diet consisting of 2.1% calories from DHA and 7.9% from HCO (Dyets No. 102681). The selection and composition of these diets were based on previous research in our laboratory [14–17]. All diets contained equal caloric and food weight components with total fat calories at 5%. HCO and AA (98% grade) were purchased from Cayman Chemical (Ann Arbor, MI). Esterified DHA (87.4% DHA, 12.6% sterols) was provided by Martek (Columbia, MD). HCO, AA, and DHA were stored at -60°C .

After randomization into respective groups, animals were earmarked and fed their assigned diets for nine consecutive weeks (aged 3–12 wk), a period of rapid growth. Animals had full and uninhibited access to food and water. Animals were individually weighed every third day, and growth and appearance were closely monitored and documented. Food was checked daily and refreshed every 2–3 d.

The animal protocol (No. 10-03-1620) complied with the National Institutes of Health Animal Research Advisory Committee guidelines and was approved by the Boston Children's Hospital Animal Care and Use Committee.

2.3. Murine femur harvest

At 12 wk, mice were euthanized, and femurs were harvested for micro-computed tomography (μCT) and descriptive histomorphometric analyses using light microscopy. For imaging analysis, cleaned femurs were placed in normal saline and frozen at -20°C until imaging was performed. For histology, femurs were placed in 10% formalin for 24 h followed by Bouin solution for 24 h, then 70% ethanol in phosphate buffered saline. Samples were embedded in paraffin and were cut

Table 1 – Demographics and diagnoses of neonates.

	Fish oil, n (%)	Fish oil fracture, n (%)	Soybean oil, n (%)	Soybean oil fracture, n (%)
Sex (male)	75 (57)	3 (42.9)	31 (62)	5 (83.3)
GA* (wk)	31.0 ± 4.3	26.0 ± 1.9	30.6 ± 4.6	24.3 ± 1.2
Diagnosis				
Esophageal atresia/tracheoesophageal fistula	5 (3.8)	0	2 (4.0)	0
Gastroschisis	26 (19.8)	0	8 (16.0)	0
Hirschsprung disease	3 (2.3)	0	2 (4.0)	0
Intestinal atresia	22 (16.8)	0	8 (16.0)	0
Microvillus inclusion disease	4 (3.1)	0	1 (2.0)	0
Necrotizing enterocolitis	57 (43.5)	7 (100)	24 (48.0)	6 (100)
Omphalocele	3 (2.3)	0	2 (4.0)	0
Pseudo-obstruction	2 (1.5)	0	1 (2.0)	0
Spontaneous intestinal perforation	3 (2.3)	0	1 (2.0)	0
Volvulus	15 (11.5)	0	6 (12.0)	0
Other	6 (4.6)	0	2 (4.0)	0

* Mean ± standard deviation. Several patients have >1 diagnosis.

transversely at the mid-femur to obtain appropriate bone cross-section before staining with hematoxylin and eosin.

2.4. Murine bone morphometric and densitometric assessment

Bone morphology and microarchitecture were assessed using a high-resolution μ CT imaging (μ CT40; Scanco Medical, Brüttisellen, Switzerland) as described previously [18]. Transaxial images through distal metaphyseal (200 slices) and mid-diaphyseal (100 slices) segments of mouse femurs were obtained at 12- μ m isotropic voxel size using x-ray tube energy and current settings of 55 kVp and 145 mA, respectively, and integration time of 250 ms, while applying a 1200 mg/cm³ hydroxyapatite beam-hardening correction. The images were binarized to separate bone from background using an established adaptive thresholding procedure [19]. A three-dimensional Gaussian filter ($\sigma = 0.8$) with a limited finite filter support (support = 1) was used to suppress the noise in the volumes.

For the trabecular (distal metaphyseal) segment, bone volume fraction (BV/total volume [TV], mm³/mm³), bone surface density (BS/BV), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th, millimeter), connectivity density (Conn.D, 1/mm³), and Structure Model Index (SMI) were assessed. For the cortical (mid-diaphyseal) segment, cortical BV, TV, marrow volume (MV), cortical bone volume fraction (BV/MV), and cortical thickness (Ct.Th, millimeter) were assessed. Trabecular and cortical volumetric apparent and bone mineral densities (ρ_{APP} and ρ_{MIN} , milligram per cubic centimeter) were also measured by the μ CT using a hydroxyapatite phantom, supplied by the manufacturer, to convert x-ray attenuation coefficient (micro) to mineral density. The variability of μ CT assessment of three-dimensional microstructural and densitometric indices of excised bone samples is <0.5% in our laboratory.

2.5. Statistical analysis

Normality of continuous data was assessed by using the Kolmogorov–Smirnov test. Morphometric and densitometric

indices served as dependent variables and were compared across the five diet groups with the use of one-way analysis of variance with post hoc Tukey correction for multiple comparisons. Data analysis was performed on the SPSS statistical package (version 21.0; IBM, NY). All reported P values are two-tailed with P < 0.05 considered statistically significant.

3. Results

3.1. Clinical neonatal review

Fractures were identified in seven of 131 neonates (5.3%) receiving the fish oil–based lipid emulsion and in six of 50 neonates (12%) maintained on the soybean oil–based emulsion (Table 1). In the fish oil group, there were one upper extremity and six rib fractures identified compared with the soybean oil group, in which there were three upper extremity, one femur, one multiple rib, and one upper extremity and rib fractures identified. Within the time period evaluated, four of six neonates (67%) in the soybean oil group had a subsequent fracture compared with two of seven neonates (29%) receiving fish oil.

3.2. Murine protocol

3.2.1. μ CT: trabecular bone specimens

Bones from the DHA diet exhibited increased bone volume fraction (BV/TV) values compared with the bones from the Soy and DHA:AA diets (P = 0.04 and 0.0001, respectively). Additionally, BV/TV values of bones from the Menhaden group were greater than those from the DHA:AA diet group (P = 0.02). No differences in BV/TV were observed among the other groups (Fig. 1A). Bones from the DHA diet exhibited increased bone surface density (BS/BV) values compared with the bones from the DHA:AA diet group (P = 0.003). No other changes in bone surface density were observed. Similarly, bones from the DHA diet exhibited increased Tb.N values compared with the bones from the DHA:AA diet group (P = 0.03). No other changes

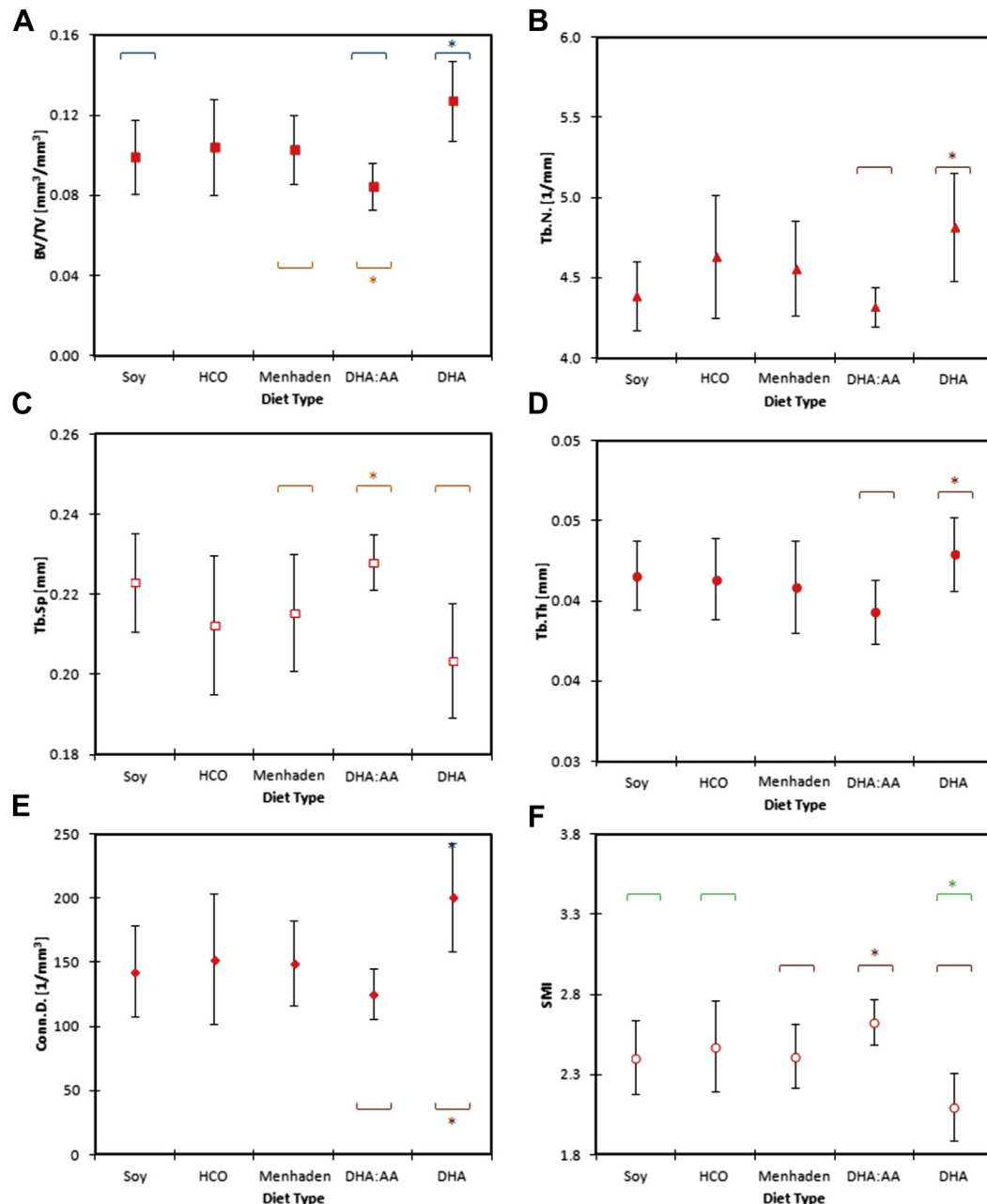


Fig. 1 – Presentation of the effects of different diets on trabecular bone specimen: (A) BV, (B) Tb.N, (C) Tb.Sp, (D) Tb.Th, (E) Conn.D and (F) SMI values. The color-matched brackets delineate significance between groups, with the asterisk denoting the group which is significantly higher than the other group(s).

in Tb.N were observed (Fig. 1B). Bones from the DHA:AA diet exhibited increased Tb.Sp values compared with the bones from the Menhaden and DHA diets ($P = 0.03$ and 0.01 , respectively; Fig. 1C). Additionally, bones from the DHA diet exhibited increased Tb.Th and Conn.D values compared with the bones from the DHA:AA diet group ($P = 0.03$ and 0.02 , respectively; Fig. 1D and 1E). SMI values of the bones from the DHA group were smaller than those from the Soy and HCO groups ($P = 0.014$ and 0.007 , respectively). Also, SMI values of the bones from the DHA:AA group were larger than those from the Menhaden and DHA groups ($P = 0.03$ and 0.001 , respectively; Fig. 1F).

Bones from the DHA group have higher apparent bone density values than those from the DHA:AA group ($P = 0.004$ and 0.001 , respectively). Likewise, bones in the Menhaden group had higher apparent density values than those in the DHA:AA group ($P = 0.007$). Bone mineral density values were not different between any of the diet groups ($P > 0.05$ for all; Fig. 2).

3.2.2. μ CT: cortical bone specimens

BV, TV, and MV were not different between any of the diet groups. Cortical bone volume fraction of the bones from the Soy group was greater than those from the DHA:AA group

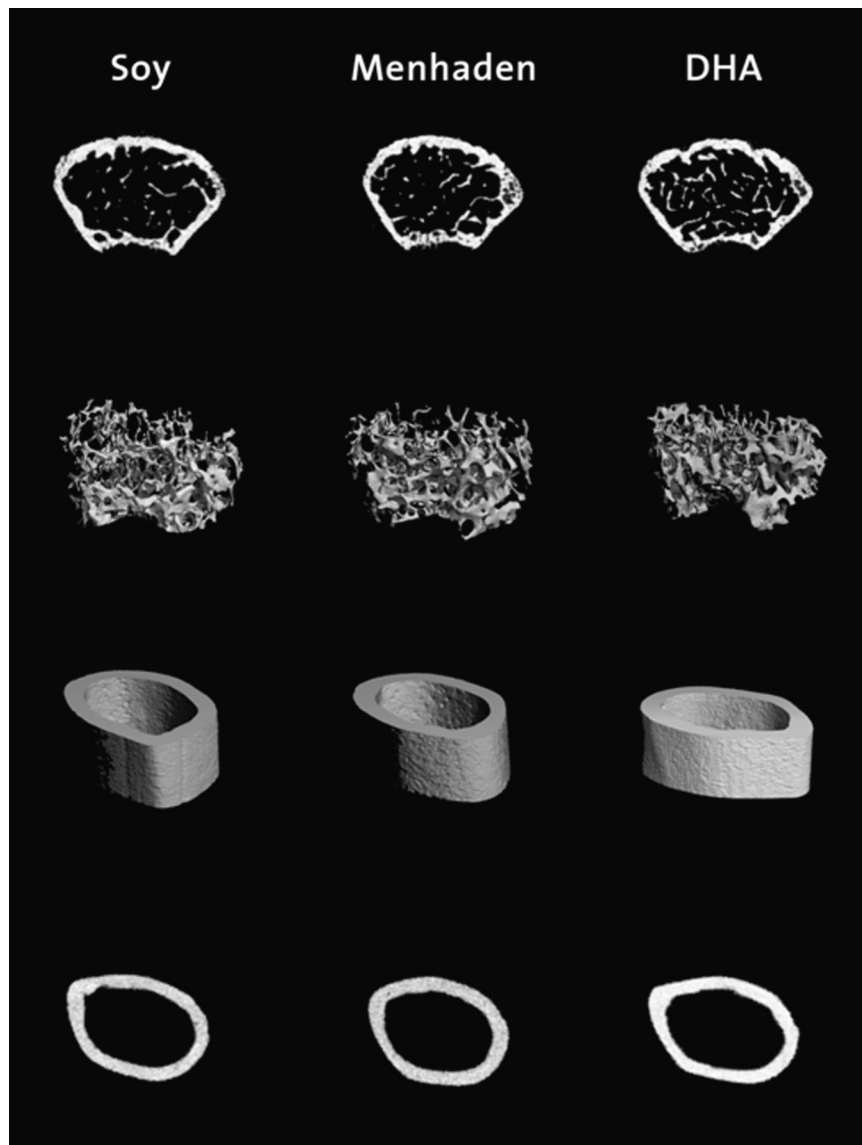


Fig. 2 – Representative images from Soy, Menhaden, and DHA groups highlighting cortical and trabecular bone compartments. The first two rows provide a visual confirmation of the changes in the trabecular microarchitecture, with DHA showing greater trabecular presence and connectivity. In turn, cortical changes are significantly more subtle, thereby not evident at gross-level presentations in rows 3 and 4.

($P = 0.04$). Ct.Th of the bones from the HCO group are greater than those from the Menhaden and DHA groups ($P = 0.04$ for both). Bone mineral density values of the DHA group were greater than those of the Soy and HCO groups ($P = 0.004$ and 0.02 , respectively). See [Table 2](#).

3.3. Bone histology

Osteoblasts were absent to rare in femurs from animals in the Soy group compared with the 20:1 DHA:AA and DHA groups, where osteoblasts lined up along lamellar bone ([Fig. 3](#)). Trabeculae were scant in the Soy group and localized to beneath the supracondylar bone compared with well-developed and prominent bone trabeculae lined by osteoblasts in the 20:1 DHA:AA and DHA groups.

4. Discussion

Results from the retrospective clinical review demonstrated a lower incidence of bone fracture in neonates maintained on intravenous fish oil compared to soybean oil. These findings support prior studies, which have demonstrated beneficial effects of omega-3 PUFAs on bone strength [\[20–23\]](#).

The results from the animal study indicate that the DHA diet provides the best protection against trabecular bone loss. This is achieved by increased bone volume fraction, increased bone surface density, increased Tb.N, increased Tb.Th, increased Conn.D, and decreased Tb.Sp. These findings are supported by Li *et al.* [\[6\]](#) who found that DHA accumulates in the osteoblast-rich and nerve-abundant periosteum of femur

Table 2 – Morphometric and densitometric indices of the mid-diaphyseal cortical bone specimens.

Group	BV (mm ³)	TV (mm ³)	MV (mm ³)	BV/TV (mm ³ /mm ³)	Ct.Th (mm)	Bone density (mg/cm ³)
Soy	0.480	1.132	0.652	0.424	1.7623	1186.70
SD	0.026	0.051	0.034	0.013	0.0811	8.84
HCO	0.455	1.093	0.638	0.417	1.8144	1189.84
SD	0.025	0.062	0.047	0.017	0.0541	14.47
Menhaden	0.476	1.133	0.657	0.420	1.6823	1194.69
SD	0.042	0.077	0.041	0.015	0.1089	12.81
DHA:AA	0.467	1.146	0.679	0.408	1.7654	1196.92
SD	0.025	0.052	0.028	0.005	0.0718	8.45
DHA	0.488	1.163	0.675	0.420	1.6840	1204.80
SD	0.032	0.055	0.026	0.010	0.0585	6.34

SD = standard deviation.

in growing rats and appears to be a vital constituent of healthy modeling bone. Similar results of improved long-bone microarchitecture were obtained by Lukas *et al.* [11], although in female rats administered high-fat (12% by weight) diets containing menhaden or flaxseed oil compared with soybean and/or corn oil. In the present study, the increase in the Tb.N and Conn.D values suggests an actual increase in the number of trabecular elements and subsequent strengthening of the trabecular network in the animals maintained on the DHA diet. This is further verified by the decrease in the SMI value of the DHA group, suggesting the shifting of the trabeculae from rod like to plate like elements, as opposed to a fenestration process, which involves increased Conn.D and SMI values. It is possible that these dietary-induced changes may be protective against age-related bone loss. Sun *et al.* [24] found that growing female mice fed 5% fish oil for 12 wk had reduced ovariectomized-induced bone mineral density loss after ovariectomy, and Watkins *et al.* [25] similarly found a positive response between omega-3 PUFAs and bone mineral content in femur bones of ovariectomized rats.

In the present study, the DHA:AA group exhibits a drastically different effect on bone morphometric indices by causing the sharpest decline in the BV/TV, Tb.N, Tb.Th, and Conn.D values and the highest increases in the SMI and Tb.Sp values compared with the control Soy group and other groups. The DHA:AA group is the weakest group, as demonstrated by the lowest bone volume fraction, Tb.N, Tb.Th and Conn.D

values in addition to the highest Tb.Sp, and SMI values. The reduction in BV/TV suggests loss of bone through reduced thickness, lower Tb.Th, and increased SMI (conversion of plates to rods), with concomitant increase in Tb.Sp, and reduced Tb.N as evidenced by decreased Tb.N and Conn.D.

These changes correspond to decreased bone stiffness in comparison with the control (Soy) group. The Menhaden diet exhibits some degree of protection against trabecular bone loss by increased bone volume fraction, Tb.N and Conn.D, and decreased Tb.Sp. This trend is similar to the one observed in the DHA group, yet to a smaller degree. The HCO group does not differ from the control Soy group in any of the morphometric indices. These results suggest that there are differences between the type and quantity of omega-3 PUFAs administered on the resultant bone quality and structure. It is well known that different omega-3 PUFAs have different biological properties and physiological effects [26,27]. The protective effects of the diets used in this study appear to affect bone microstructure alone, as no change in bone mineral density values was found in trabecular bone. It is possible that the diets could affect the bone matrix density measures; however, relevant data to address this area were not collected in the present study. The selection and composition of diets were chosen based on previous research in the laboratory.

The diets seem to cause little change in cortical morphometric indices of mouse femurs. Ct.Th of femurs in the HCO group was reported to be larger than most other groups.

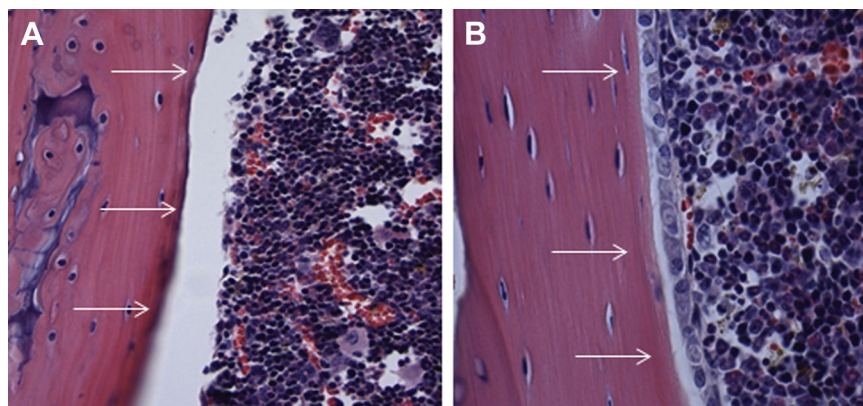


Fig. 3 – Representative images of osteoblasts in femurs from soybean oil (A) and DHA (B) groups. Osteoblasts line up adjacent to the bone marrow in the DHA group (see arrows). No osteoblasts are seen in femurs from the soybean oil group. Femurs are stained with hematoxylin and eosin. Magnification $\times 60$.

Interestingly, bone mineral density of the DHA group was higher than that of the Soy control group. It appears that DHA diet had a positive effect on cortical bone mineral density compared with HCO and Soy groups; however, it is unlikely that increased bone mineral density alone could play a significant protective role for cortical bones.

The lower fracture risk observed in fish oil fed neonates in combination with the protective effects of DHA observed in the femurs of young C57/BL6 mice suggest an important role for omega-3 PUFAs on bone development. These findings will be further investigated in animal studies focused on evaluating the underlying mechanism and will lay the groundwork for a clinical trial in neonates to evaluate the effect of dietary omega-3 fatty acids on bone growth and development.

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All authors reviewed the article and were involved in the final approval of version to be published.

Disclosure

A license agreement for the use of Omegaven has been signed by Boston Children's Hospital and Fresenius Kabi and a patent has been submitted by Boston Children's Hospital on behalf of Mark Puder and Kathleen Gura.

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