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Exposure to omega-3 fatty acids at early age accelerate bone growth and improve bone quality☆

Netta Koren[a], Stav Simsa-Maziel[a], Ron Shahar[b], Betty Schwartz[a], Efrat Monsonego-Ornan[a,*]

[a]Institute of Biochemistry and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel
[b]Koret School of Veterinary, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

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Abstract

Omega-3 fatty acids (FAs) are essential nutritional components that must be obtained from foods. Increasing evidence validate that omega-3 FAs are beneficial for bone health, and several mechanisms have been suggested to mediate their effects on bone, including alterations in calcium absorption and urinary calcium loss, prostaglandin synthesis, lipid oxidation, osteoblast formation and inhibition of osteoclastogenesis. However, to date, there is scant information regarding the effect of omega-3 FAs on the developing skeleton during the rapid growth phase. In this study we aim to evaluate the effect of exposure to high levels of omega-3 FAs on bone development and quality during prenatal and early postnatal period. For this purpose, we used the *fat-1* transgenic mice that have the ability to convert omega-6 to omega-3 fatty acids and the ATDC5 chondrogenic cell line as models. We show that exposure to high concentrations of omega-3 FAs at a young age accelerates bone growth through alterations of the growth plate, associated with increased chondrocyte proliferation and differentiation. We further propose that those effects are mediated by the receptors G-protein coupled receptor 120 (GPR120) and hepatic nuclear factor 4α, which are expressed by chondrocytes in culture. Additionally, using a combined study on the structural and mechanical bone parameters, we show that high omega-3 levels contribute to superior trabecular and cortical structure, as well as to stiffer bones and improved bone quality. Most interestingly, the *fat-1* model allowed us to demonstrate the role of maternal high omega-3 concentration on bone growth during the gestation and postnatal period.
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1. Introduction

Adequate growth and intrinsic bone quality depend mainly on heredity, diet and physical activity. Nonetheless, total bone mass can be improved by modifications of the material properties of the tissue [1,2]. In recent years, it has become clear that proper nutrition and in particular consumption of certain foods directly affect bone material properties and are required to reach the full genetic potential during bone development.

Omega-3 (ω-3) is the common name for long-chain polyunsaturated fatty acids (LCPUFAs) that contain unsaturated carbon in the third position from their methyl terminal. ω-3 and ω-6 (another LCPUFA containing unsaturated carbon in the sixth position) originate from dietary α-linolenic acid and linoleic acid, respectively, two essential fatty acids [3] that are vital components of membrane phospholipids, as well as precursors for a range of metabolites [4,5]. These fatty acids are progressively desaturated and elongated by a shared enzyme system to form longer chain and more highly unsaturated FA, resulting in the formation of the ω-6 arachidonic acid [5] (n-6, 20:4) and ω-3 eicosapentaenoic (EPA) (n-3, 20:5) and docosahexaenoic acids (DHA) (n-3, 22:6). Mammals depend on dietary sources of long-chain ω-3 and ω-6 FA since they lack the enzymatic system synthesizing such fatty acids. In addition, mammalian cells cannot convert ω-6 to ω-3 fatty acids because they lack the converting enzyme named ω-3 desaturase.

In many nutritional studies, diet is commonly used to change the composition of nutrients in the tissues, creating inevitable differences between the various diets that could lead to inconsistent or conflicting results. This fact is particularly known for dietary studies in the field of lipids, in which fish oil, plant seeds and vegetable oil are used instead of pure ω-3 and ω-6 FA. However, these oils contain more bioactive components that can affect study results. Furthermore, the use of dietary supplements is also challenging because there is a possibility for interactions between nutrients and other components that exist inside the foods. Thus, there is a need to create a research model that is capable to revoke these confounding factors and can lead to an understanding of the health effects of specific nutrients such as ω-3. The FAT-1 mice were engineered to express the *Caenorhabditis elegans* fat-1 gene that encodes the enzyme ω-3 desaturase. This enzyme, which does not exist in mammals, converts

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* Corresponding author. Institute of Biochemistry and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel. Tel.: +972 89489712; fax: +972 89363208.
E-mail address: efrat.mo@mail.huji.ac.il (E. Monsonego-Ornan).

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ω-6 to ω-3 FA and is expressed in various tissues [6]. Conversion of ω-6 to ω-3 FA leads to an increase in endogenous levels of ω-3 and a reduction of ω-6 FA in the organs and tissues of FAT-1 mice, without the need for supplementation in the diet.

Over the past years, it has become clear that ω-3 PUFAs are beneficial for bone health [7]. Several mechanisms have been suggested to mediate the effects of dietary fats on bone, including alterations in calcium absorption and urinary calcium loss, prostaglandin synthesis, osteoblast formation and lipid oxidation [8,9]. In addition, ω-3 PUFA works synergistically with estrogen to exert a stimulatory effect on bone mineral deposition and an inhibitory effect on bone resorption. It was suggested that ω-3 FA maybe involved in down-regulation of osteoclastogenesis or an up-regulation of osteoblastogenesis [9–11] and thus may play a beneficial role in preventing osteoporosis.

Most studies conducted to date have focused on providing ω-3 as a means of secondary prevention, after the development of bone disease or in situation of high risk for fracture and osteoporosis. The aim of this study was to evaluate the influence of ω-3 FA on growth plate development and bone quality from birth and in the first period of life. To this end, we used both *in vivo* and *in vitro* models: the transgenic mice FAT-1 and the ATDC5 chondrogenic cell line which undergoes cell proliferation, hypertrophy, synthesis and mineralization of extracellular matrix, as occurs during longitudinal bone growth *in vivo*, thereby providing an excellent model for studying the molecular mechanisms underlying growth plate development [12–16]. We show that high concentrations of ω-3 FA during young age accelerate bone growth through increased chondrocytes proliferation and differentiation. In addition, structural and mechanical bone analyses indicate that ω-3 FA improves bone quality.

2. Methods and materials

2.1. Materials

Dulbecco's modified Eagle's medium and Ham's F-12, insulin–transferrin–sodium selenate and XTT proliferation assay were purchased from Biological Industries (Beit Haemek, Israel). TRI reagent, *cis*-5,8,11,14,17-EPA and *cis*-4,7,10,13,16,19-DHA were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Digoxigenin dUTP was purchased from Enzo (Mannheim, Germany). Digoxigenin-RNA labeling mix, 4-nitroblue tetrazolium (nbt) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Roche (Wiesbaden, Germany). Rabbit polyclonal anti-hepatic nuclear factor 4α (HNF4α) antibody was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal anti-GAPDH and rabbit polyclonal anti-G-protein coupled receptor 120 (GPR120) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals

Male and female *fat-1* mice (with the background of C57BL/6) were kindly received from J.X. Kang (Boston, MA, USA). For all analyses (unless described differently), heterozygous×heterozygous breeding was conducted, and heterozygous *fat-1* offspring were selected (homozygous offspring are lethal). C57BL/6 mice at the matching age were used as control. All mice were housed under specific pathogen-free conditions in an environmentally controlled clean room and fed normal chow diet. All procedures were approved by the Hebrew University Animal Care Committee. At 3 weeks of age, 2 mm of tail sample was taken from *fat-1* offspring for genotyping and FA composition analysis. Genotyping was performed by extraction and amplification of DNA (1 μg) with specific primers for *fat-1* gene (F: ATATTCTAGACAAGTTTGAGG-TATGGTCGC, R: ATATACTAGTAAGAGTTAT GGCTTTATGCA; NM_001028389). The presence of the 1500-bp product was identified on ethidium bromide gel. Fatty acid analysis of tissue lipids was performed from extraction of tail as described by Kang *et al.* [4,17]. Briefly, 1 cm of mouse tail was snap-frozen in liquid nitrogen. Frozen tissues were separately ground to rough powder using a crater and pestle, followed by adding 1 ml of GC-grade hexane and 1 ml of 14% boron trifluoride in methanol reagent. The mixture was flushed with nitrogen for about 30 s, incubated for 1 h at 100°C and then cooled on ice for 5–10 min. After adding 1 ml of H_2O, the extract was vortexed and centrifuged at 18,000*g* for 1 min. The upper phase, containing the methylated fatty acids, was concentrated under nitrogen. Fatty acid methyl esters were analyzed using an Agilent gas chromatograph (7890A) equipped with a flame ionization detector and capillary column (Agilent DB-23, 60 m, 0.25 mm, 0.25 μm). Samples were injected in pulsed split-less mode (pulsed pressure of 30 psi for 0.65 min). Conditions of analysis were the following: flow of carrier gas (hydrogen), 1 ml/min; temperature of injector, 270°C; temperature of detector, 250°C; temperature of column oven, 150°C (1 min); then ramped at 5°C/min to 230°C (held for 10 min). The concentrations of EPA [(n-3) 20:5], α-linolenic acid [(n-3) 18:3]), arachidonic acid [(n-6) 20:4] and linoleic acid [(n-6) 18:2] were evaluated using authentic standards (Sigma-Aldrich, Rehovot, Israel). After final identification, weight and tail length of *fat-1* and control mice were measured at 4, 6, 8, 10, 12, 14 and 16 weeks of age (n=10 at each age).

2.3. Histological staining and in situ hybridization of growth plate sections

Tibial growth plates from 2-, 5-, 12- and 24-week-old mice were fixed overnight in 4% paraformaldehyde (Sigma, USA) at 4°C followed by 3 weeks of decalcification in 0.5 M EDTA pH 7.4. The samples were then dehydrated, cleared in Histo-Clear (Bar-Naor, Ramat-Gan, Israel) and embedded in Paraplast, and 5-m sections were prepared. Masson's trichrome staining was performed as described by Simsa-Maziel *et al.* In brief: slides were stained in Weigert's iron hematoxylin solution for 10min, followed by rinsing in running warm tap water for 10 min, and washed with distilled water [18]. Next, stain in Biebrich scarlet-acid fuchsin solution for 15 min and wash in Distilled water (DW). Incubate in phosphomolybdic–phosphotungstic acid solution and transferred directly to aniline blue solution for 5min, followed by quick rinse in DW and 1% acetic acid solution for 2 min [19]. Hybridizations were preformed as described previously. In brief: the sections were deparaffinized in xylene, rehydrated and digested with proteinase K. After digestion, slides were fixed in 10% formaldehyde, blocked in 0.2% glycine and rapidly dehydrated. The sections were then hybridized with digoxigenin-labeled antisense probes for Col II or X (probes were kindly provided by Dr. Elazar Zelzer, the Weizmann Institute of Science, Rehovot, Israel). Probes were detected using a polyclonal antidigoxigenin antibody attached to alkaline phosphatase (ALP) that, upon reaction with its substrates NBT and BCIP, produces a color response. Endogenous ALP was inhibited with levamisole [16,19,20]. In all hybridizations, no signal was observed with sense probes which were used as controls.

2.4. Measurement of growth plate width and chondrocytes number

The width of the whole growth plate and of the proliferative, prehypertrophic and hypertrophic zones was measured at six different points along the plate, averaged for each plate and then averaged with measurements from 10 other plate samples in each group. The number of cells per column at each zone was counted and averaged in slides from 10 different mice in each group. In each slide, 10 different columns were counted [16,19].

2.5. Micro-computed tomography (CT)

The region of proximal to middiaphysis of all tibiae (n=5) isolated from *fat-1* and C57BL/6 mice at the ages of 2, 5, 12 and 24 weeks was scanned with a Skyscan 1174 X-ray computed microtomograph scanner (Skyscan, Aartselaar Belgium) with a CCD detector. Images were obtained using 50-kVp X-ray tube potential and 800-μA tube current. Specimens were scanned using a 0.25-mm aluminum filter. Integration time was 3500 ms, and isotropic voxel size was 8.6 μm^3. For each specimen, a series of 900 projection images was obtained with a rotation step of 0.4°, two-frame averaging, for a total 360° rotation. Flat field correction was performed at the beginning of each scan for a specific zoom and image format. A stack of two-dimensional (2D) X-ray shadow projections was reconstructed to obtain transverse images using NRecon software version 1.6.1.1 (Skyscan) and subjected to morphometric analysis using CTAn software version 1.9.2.3 (Skyscan). During reconstruction, dynamic image range, postalignment value, beam hardening and ring-artifact reduction were optimized for each experimental set. Cortical analysis was performed on a standardized region of interest (ROI) in the mid-diaphysis equidistant from the ends of the bone, containing 150 slices, corresponding to 1.29 mm. The trabecular ROI consisted of 70 slices, extending distally from the end of the proximal growth plate of each bone and corresponding to 0.602 mm. Global grayscale thresholding levels were used for the cortical region and adaptive grayscale thresholding levels for the trabecular region. Three-dimensional (3D) images (CTM file format) were constructed from cortical and trabecular ROIs utilizing Marching Cubes 33 algorithm in CTVol software (Skyscan) [19,21].

2.6. Mechanical testing

Mechanical properties of tibiae isolated from *fat-1* and C57BL/6 mice at the ages of 5, 12 and 24 weeks (n=6 for each group) were determined by three-point bending tests performed with a custom-made micromechanical testing device. The caudal aspect of each bone was placed on two supports with rounded profiles (0.5 mm diameter) such that the supports were located equidistant from the ends of the bone and at the maximum feasible distance from each other so that they contacted a reasonably tubular part of the diaphysis [19,22]. The optimal distance between the supports was found to be 8 mm. Each bone was loaded on its anterior aspect by a prong attached in series to a load cell and linear motor, such that the prong contacted the bone at the midpoint between the two supports. Monotonic loading was then conducted at a constant rate of 2 mm/min up to fracture. Force and displacement data were collected by the custom-made system at 10 Hz. The following whole-bone biomechanical parameters were derived from the load/displacement curves: area under the curve, ultimate load, failure load, whole bone stiffness and yield load.

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