Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Nutritional Biochemistry 16 (2005) 368–374
Journal of Nutritional Biochemistry

Flaxseed oil and inflammation-associated bone abnormalities in interleukin-10 knockout mice

Stacey L. Cohen[a], Aideen M. Moore[b], Wendy E. Ward[a,*]

[a]Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 3E2
[b]Department of Neonatology, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

Received 6 January 2005

Abstract

Interleukin-10$^{-/-}$ (IL-10) knockout (KO) mice develop an intestinal inflammation that closely mimics human inflammatory bowel disease (IBD) which is accompanied by inflammation-associated bone abnormalities and elevated serum proinflammatory cytokines. The objective of this study was to use the IL-10 KO mouse model to determine whether flaxseed oil (FO) diet, rich in α-linolenic acid (ALA), attenuates intestinal inflammation and inflammation-associated bone abnormalities, compared to a corn oil (CO) control diet. Male wild-type (WT) or IL-10 KO mice were fed a 10% CO or 10% FO diet from weaning (postnatal day 28) for 9 weeks. At necropsy, serum, intestine, femurs and lumbar vertebrae were collected and analyzed. IL-10 KO mice fed CO had lower femur bone mineral content (BMC; $P<.001$), bone mineral density (BMD; $P<.001$), peak load ($P=.033$) and lumbar vertebrae BMD ($P=.02$) compared to WT mice fed either diet. Flaxseed oil had a modest, favorable effect on IL-10 KO mice as femur BMC, BMD and peak load were similar to WT mice fed CO or FO. In addition, lumbar vertebra BMD was similar among IL-10 KO mice fed FO and WT mice fed CO or FO. The fact that FO attenuated serum tumor necrosis factor-α (TNF-α) among IL-10 KO mice suggests that the positive effects of FO on femur BMC, BMD, peak load and vertebral BMD in IL-10 KO mice may have been partly mediated by changes in serum TNF-α. In conclusion, these findings suggest that a dietary level of ALA attainable from a 10% flaxseed oil diet results in modest improvements in some bone outcomes but does not attenuate intestinal inflammation that is characteristic of IL-10 KO mice.

Keywords: Flaxseed oil; Inflammatory bowel disease; Bone mass; Interleukin-10 knockout mice

1. Introduction

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the intestinal mucosa and an uncontrolled production of the proinflammatory cytokines, interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [1,2]. These cytokines stimulate osteoclastogenesis [3] and therefore may play a role in the mechanism whereby inflammation-associated bone abnormalities frequently occur in men [4–9], women [4–9] and children [10–12] with IBD. Lower bone mineral density (BMD) at multiple sites is common [4–9,11,13]. Consequently, IBD patients have a 40% greater incidence of spine or hip fracture compared to the healthy general population [14,15].

Children and young adults with IBD are at a disadvantage in terms of reaching their set point for peak bone mass as early life experiences are important in achieving an individual's full genetic potential [16]. In addition, IBD is commonly treated with corticosteroids, which can induce bone loss [17]. Because of the detrimental effects of corticosteroids on bone, dietary strategies that treat IBD are of interest, the most common being the omega-3 (n-3) long-chain polyunsaturated fatty acids (LCPUFA) that are abundant in fish oil. Fish oil, a rich source of the n-3 LCPUFA eicosapentanoic acid (EPA), has been used to modulate the dietary omega-6 (n-6) to n-3 ratio, with the aim to reduce intestinal inflammation and treat IBD [18–20]. Recently, an in vivo study confirmed that bone is sensitive to changes in the n-6 to n-3 dietary ratio [21]. Moreover, EPA has been shown to play a positive regulatory role in bone metabolism [22]. As an alternative to fish oil, flaxseed oil (FO), which contains approximately 56% α-linolenic acid (ALA) [23], a precursor to EPA, has generated interest as a

* Corresponding author. Tel.: +1 416 946 7366; fax: +1 416 978 5882.
E-mail address: wendy.ward@utoronto.ca (W.E. Ward).

0955-2863/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2005.01.008

potential anti-inflammatory agent due to the ability of ALA to be converted to EPA in humans and animals [21,23–25]. While it is known that dietary EPA is more efficient than dietary ALA in raising tissue EPA concentrations [23], the extent to which ALA is converted to EPA is controversial. It has been suggested that the degree of ALA conversion to EPA and docosahexanoic acid (DHA) is 5–10% and 2–5%, respectively [26]. Thus, ALA has the potential to alter the n- 6 to n-3 ratio and may thereby modulate inflammatory mediators. Moreover, flaxseed oil is readily available in the diet as flaxseed is incorporated into many commonly consumed foods such as breads, muffins and cereals.

The n-3 PUFAs in flaxseed oil [27] and fish oil [28–33] have anti-inflammatory properties that are mediated by the production of anti-inflammatory eicosanoids, which in turn offset the production of proinflammatory eicosanoids through competitive inhibition within their common metabolic pathway. Limiting the production of proinflammatory eicosanoids, such as prostaglandin E_2 (PGE_2), may be an important factor in minimizing the production of IL-6, IL-1β and TNF-α [32], which may mediate inflammation-associated bone abnormalities. Flaxseed-derived ALA has been shown to decrease ex vivo PGE_2 concentrations in monkey plasma [34] and in rat bone [35]. In addition, flaxseed oil has been shown to decrease TNF-α and IL-1β in human peripheral blood mononuclear cells (PBMC) [23]. Modulation of the dietary n-6 to n-3 ratio has been shown to be beneficial in various clinical [19,36] and animal [18] inflammatory disease states and animal models of bone metabolism [3,31]. To date there are no published data reporting the response of bone metabolism during intestinal inflammation to a diet rich in flaxseed-oil-derived ALA.

Previous studies have demonstrated that the interleukin-10 (IL-10) knockout (KO) mouse develops an intestinal inflammation that mimics human IBD [1,37–39]. More recently, it has been shown that IL-10 KO mice also develop bone abnormalities such as lower bone mass and biomechanical strength properties compared to wild-type (WT) mice [1]. Thus, the overall objective of this study was to determine whether a 10% FO diet attenuates intestinal damage as well as abnormalities in bone mineral content (BMC), bone mineral density (BMD) and biomechanical strength properties of femurs and lumbar vertebrae in developing male IL-10 KO mice.

2. Methods and materials

2.1. Animals and diets

Male and female WT (129 Sv/Ev, Taconic, Germantown, NY) or IL-10 KO (129 Sv/Ev IL-$10^{-/-}$ KO, generously provided by Dr. R. Fedorak, University of Alberta) mice were bred to obtain 14–19 male mice per group. At 21 days of life, pups were adapted to solid food over a period of 1 week. Pups were then randomized to one of two modified AIN93G diets (Dyets, Bethlehem, PA) that contained either 10% corn oil (CO) or 10% FO (Omega Nutrition Canada, Vancouver, BC) from postnatal day (PND) 28 through PND 91 (Table 1). A 10% FO diet was studied as it represents a level that is attainable by diet alone, without supplementation. The total amount of fat in each diet was 100 g/kg. Diets were isocaloric and differed only in the source of fat. Fresh food was provided every second or third day, and food intakes were calculated at each feeding. Body weight was measured weekly. At PND 91, mice were asphyxiated with carbon dioxide, blood was collected by cardiac puncture and mice were then killed by cervical dislocation. Femurs, lumbar vertebrae (LV1–LV4) and colons were immediately removed.

This protocol was approved by the Faculty Advisory Committee on Animal Services at the University of Toronto and conducted according to the guidelines established by the Canadian Council of Animal Care [40].

2.2. Serum fatty acid profile

2.2.1. Sample preparation

Total lipids were extracted [41] with chloroform–methanol (2:1, v/v) and saponified with a potassium hydroxide methanol solution. Added to each 50-μl aliquot of serum sample was 200 μl of internal standard (heptodecanoic acid, 25 μg/ml in methanol), 4 ml of chloroform–methanol (2:1, v/v) and 1 ml of 0.9% saline solution. The mixtures were shaken continuously for approximately 15 min and centrifuged at 2000 rpm for

Table 1
Composition of experimental diets[1]

Component	Amount (g/kg)	
	Corn	Flax
Casein	200	200
Cornstarch	367.50	367.48
Dyetrose	132	132
Sucrose	100	100
Cellulose	50	50
Corn oil	100	0
Flaxseed oil	0	100
t-Butylhydroquinone	0	0.02
L-Cystine	3.0	3.0
Choline bitartrate	2.5	2.5
Mineral mix[2]	35	35
Vitamin mix[3]	10	10

[1] Both diets were modified AIN93G standard rodent diet and contained either 10% CO or 10% FO.

[2] Mineral mix provided (mg/kg of diet): $CaCO_3$, 12,495; K_2HP0_4, 6860; $K_3C_6H_5O_7 \cdot H_20$, 2477.3; NaCl, 2590; K_2SO_4, 1631; MgO, 840; $FeC_6H_5O_7$ USP, 212.1; $ZnCO_3$, 57.75; $MnCO_3$, 22.05; $CuCO_3$, 10.5; KIO_3, 0.35; Na_2SeO_4, 0.359; $(NH_4)_2MoO_4 \cdot H_20$, 0.278; $Na_2O_3Si \cdot 9H_2O$, 9.625; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; LiCl, 0.609; B_3O_3, 2.853; NaF, 2.223; $NiCO_3$, 1.113, NH_4VO_3, 0.231; sucrose, finely powdered, 7735.9.

[3] Vitamin mix provided (mg/kg of diet): niacin, 30; calcium pantothenate, 1.6; pyridoxine HCL, 7; thiamine HCL, 6; riboflavin, 6; folic acid, 2; biotin, 0.2; vitamin E acetate (500 IU/g), 150; vitamin B_{12} (0.1%), 25; vitamin A palmitate (500,000 IU/g), 8; vitamin D_3 (400,000 IU/g), 2.5; vitamin K1/dextrose mix (10 mg/g), 75; sucrose, 9672.3.

5 min. The lower chloroform layers were collected and evaporated under nitrogen gas flow. The residues were saponified with 1 mol/L potassium hydroxide methanol solution at 90°C for 1 h. The resultants were mixed with 0.9% saline solution and extracted with hexane to remove cholesterol. The aqueous phases were neutralized with concentrated hydrochloric acid and extracted with hexane. The collected hexane layers, which contained total free fatty acids, were dried under nitrogen gas flow. The free fatty acids were converted into their methyl esters using a mixture of 14% boron–trifluoride etherate and methanol and heated at 90°C for 15 min. The resultants were mixed with 0.9% saline solution and extracted with hexane to collect fatty acid methyl esters. Hexane layers were dried under nitrogen gas flow. The fatty acid methyl esters were redissolved in 200 μl of hexane prior to injection for gas chromatography-mass spectrometry (GC-MS).

2.2.2. Fatty acid analyses

The serum fatty acid profiles were determined using a Hewlett-Packard (Avondietle, PA, USA) GC-MS (Model 5890 gas chromatograph, Model 5971 mass-selective detector). A DB-23 fused-silica capillary column (30 m× 0.32 mm I.D., 0.25 μm film thickness) was used for separation. The flow rate of the carrier gas, helium, was set at 0.6 ml/min, and a splitless inlet was used. The GC oven temperature was programmed from 50 °C (2 min hold) to 170 °C at a rate of 20 °C/min for 1 min and then 212 °C at a rate of 3 °C/min for 10 min. The temperature of the detector and injector were 280 °C and 265 °C, respectively. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode, and the scan mode was used for quantifying 17 fatty acid methyl esters in serum [data shown for linoleic acid (LA), ALA, arachadonic acid (AA), EPA and DHA]. The injection volume was 1 μl.

2.3. Intestinal histological assessment

Colons were removed immediately at necropsy (PND 91), rinsed with ice cold saline, fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections (5 μm) from the proximal colon were stained with hematoxylin and eosin for light microscopy. The severity of intestinal inflammation was assessed using a scale of 0–4, with 0 representing no inflammation and 4 representing severe inflammation characterized by widespread infiltration with inflammatory cells, mucosal thickening, submucosal cell infiltration, a decrease in goblet cells and destruction of architecture as previously described [1,42–44]. A minimum of three sections per mouse was examined to determine the mean histological colonic inflammatory score. Intestinal inflammation was scored by one investigator (A.M.M.) who was blinded to the dietary interventions.

2.4. Serum cytokines

Serum was obtained by centrifuging whole blood at 10,000 rpm for 15 min and was stored at −70°C. Serum levels of three proinflammatory cytokines (IL-1β, IL-6, TNF-α) were quantified using commercially available enzyme-linked immunoassays that are specific for mice (Quantikine Murine; R&D Systems, Minneapolis, MN). The detectable limits for each ELISA were 3.0 pg/ml (IL-1β), 1.6 pg/ml (IL-6) and 5.1 pg/ml (TNF-α).

2.5. Femur dimensions

Femur weight was measured using a digital scale accurate to four decimal places, and femur length and width were measured using digital calipers accurate to 0.01 mm (Cedarlane Laboratories, Hornby, ON) [45]. Femur width was measured at the midpoint of the bone shaft in the mediolateral and anterior–posterior positions, as femurs are not cylindrical at the midpoint where three-point bending is performed. All measurements were obtained after femurs had rehydrated in phosphate buffered saline for 4 h in preparation for biomechanical strength testing (i.e., three-point bending).

2.6. Biomechanical bone strength testing

Three-point bending of femurs and compression testing of LV3 were conducted using a materials testing system (Model 4442, Instron, Canton, MA).

2.6.1. Three-point bending

Three-point bending was performed to test the structural properties of the right femurs at the midpoint as previously described [45]. Femurs were placed on two supports separated by a distance of 5 mm with the crosshead directly over the midpoint of the femur. Force was applied

Table 2
Daily food intake and body weight at PND 21 and 91[1]

	WT		IL-10 KO		*P*
	Corn[2]	Flax[2]	Corn	Flax	
Food consumed (g/day)	2.34±0.05ab	2.28±0.05ab	2.22±0.02b	2.37±0.02a	0.010
Body weight at PND 21[3] (g)	10.10±0.59	10.52±0.33	9.02±0.27	9.39±0.30	NS
Body weight at PND 91[4] (g)	24.01±0.59	23.67±0.40	23.61±0.76	24.93±0.49	NS

[1] All data are expressed as mean±S.E.M.; means within a row having different superscripts are statistically different, $P<.05$. NS, not significant, $P>.05$.
[2] Dietary treatments were formulated by modifying the total amount of dietary fat to 100 g of FO or CO per kilogram of diet.
[3] PND 21 represents age at weaning.
[4] PND 91 represents age at necropsy.

Table 3
Serum fatty acid profiles[1]

Fatty acid (ng fatty acid/200 μl serum)	WT		IL-10 KO		P
	Corn[2]	Flax[2]	Corn	Flax	
LA	39,779±3368a	25,896±6339b	36,502±2477a	21,705±1660b	<.001
ALA	536±141b	13,447±1065a	335±119b	11,701±1203a	<.001
AA	24,679±1319a	3889±265b	22,298±3103a	5479±1714b	<.001
EPA	347±66b	12,169±847a	174±83b	9384±879a	<.001
DHA	5310±318b	7999±633a	6010±448ab	7346±624a	<.001

[1] All data are expressed as mean±S.E.M.; means within a row having different letters are statistically different, $P<.05$. NS, $P>.05$.
[2] Dietary treatments were formulated by modifying the total amount of dietary fat to 100 g of FO or CO per kilogram of diet.

at a rate of 6 mm/min until the femur fractured. From the load–deformation curve generated by specialized software (Series IX Automated Materials Tester, version 8.15.00, Instron), several strength properties were determined: yield load (a measure of the elastic limit of the femur); peak load (a measure of the fracture threshold of the femur); resilience (a measure of energy absorbed by the femur up until the yield load was reached); toughness (a measure of the energy absorbed by the femur up until the peak load was reached); and stiffness (a measure of the extrinsic rigidity of the femur).

2.6.2. Compression testing

The strength of an individual vertebra, LV3, was determined by compression testing as previously described [45]. LV3 was isolated from the other vertebra and placed in anatomical position on the center of a stainless-steel plate. A second suspended steel plate descended at a constant rate of 2 mm/min until LV3 was compressed. Peak load was determined from the load–deformation curve.

2.7. Bone mineral content and BMD of femurs and lumbar vertebrae (LV1–LV4)

Bone mineral content and BMD of femurs and lumbar spine (LV1–LV4) were determined using PIXImus dual-energy X-ray absorptiometry (DEXA) (Lunar Corp., General Electric Medical Systems) and specialized software (Lunar Corp., Lunar Software, version 1.46). Femurs and LV1–LV4 were scanned on a plastic tray in air at room temperature.

2.8. Statistical analysis

Statistical analyses were performed by one-way ANOVA and Tukey's test was used to compare multiple means. Analyses were performed using SigmaStat 2.0 (Jandel, San Rafael, CA). Differences were considered significant if P was less than .05. All data are expressed as mean±S.E.M.

3. Results

3.1. Food intake and body weight

IL-10 KO mice fed FO consumed more ($P<.05$) food compared to IL-10 KO mice fed CO. Body weight at the start of the study intervention (PND 28) or necropsy (PND 91) did not differ among groups (Table 2).

3.2. Serum fatty acid profile

Both WT and IL-10 KO mice fed FO had higher serum ALA ($P<.001$) and EPA ($P<.001$) and lower serum LA ($P<.001$) and AA ($P<.001$) compared to WT and IL-10 KO mice fed CO (Table 3). Serum DHA was higher in WT and IL-10 KO mice fed FO compared to WT mice fed CO (Table 3).

3.3. Intestinal histological assessment

Intestinal scores were higher ($P<.001$) among IL-10 KO mice compared to WT mice, irrespective of the diet provided. Intestinal scores were the following: WT CO=0.73±0.25; WT FO=0.14±0.09; IL-10 KO CO=2.08±0.33; IL-10 KO FO=2.13±0.37. Severe intestinal inflammation, in particular inflammatory cell infiltration and destruction of architecture, was observed in all of the IL-10 KO mice.

3.4. Serum cytokines

Serum IL-6 was higher ($P=.004$) in IL-10 KO mice fed CO or FO compared to WT mice fed either diet (Table 4). Serum TNF-α of IL-10 KO mice fed CO was higher

Table 4
Serum cytokines[1]

Cytokine (pg/ml serum)	WT		IL-10 KO		P
	Corn[2]	Flax[2]	Corn	Flax	
IL-6	24.33±1.28b	24.02±2.60b	44.27±6.40a	42.43±6.40a	.004
IL-1β	6.84±1.80	7.97±0.75	13.35±3.71	12.32±1.52	NS
TNF-α	26.72±5.56b	17.54±4.87b	41.55±5.93a	31.64±4.87ab	.037

[1] All data are expressed as mean±S.E.M.; means within a row having different letters are statistically different, $P<.05$. NS, $P>.05$.
[2] Dietary treatments were formulated by modifying the total amount of dietary fat to 100 g of FO or CO per kilogram of diet.

Table 5
Femur weight, dimensions, biomechanical strength properties, BMC and BMD[1]

	WT		IL-10 KO		P
	Corn[2]	Flax[2]	Corn	Flax	
Femur weight (mg)	58.72±0.001	58.90±0.001	55.00±0.001	59.62±0.001	NS
Femur length (mm)	14.50±0.08	14.47±0.07	14.01±0.06	13.94±0.05	NS
Width A (mm)[3]	1.17±0.01	1.20±0.01	1.16±0.01	1.18±0.01	NS
Width B (mm)[4]	1.36±0.02	1.40±0.02	1.32±0.02	1.37±0.02	NS
Yield load (N)	14±0.6	13±0.5	12±0.4	12±0.4	NS
Resilience ($J\times10^{-3}$)	0.51±0.04a	0.40±0.02ab	0.37±0.11b	0.37±0.08b	.004
Stiffness (N/mm)	239±9	253±5	242±6	234±7	NS
Peak load (N)	25±1.0ab	26±0.6a	22±0.8b	25±0.8ab	.033
Toughness ($J\times10^{-3}$)	4.82±0.72	5.36±0.46	5.66±0.72	7.07±0.52	NS
BMC (mg)	24.18±0.87a	25.13±0.69a	21.10±0.52b	23.62±0.53a	<.001
BMD (mg/cm^2)	59.93±0.99a	60.71±0.69a	56.31±0.78b	58.70±0.66ab	<.001

[1] All data are expressed as mean±S.E.M.; means within a row having different letters are statistically different, $P<.05$. NS, $P>.05$.
[2] Dietary treatments were formulated by modifying the total amount of dietary fat to 100 g of FO or CO per kilogram of diet.
[3] The anterior–posterior width.
[4] The mediolateral width.

($P=.005$) than WT mice fed CO or FO, while IL-10 KO mice fed FO did not differ from WT mice. There were no significant differences in serum IL-1β among groups (Table 4).

3.5. Femur weight, dimensions, biomechanical strength properties, BMC and BMD

Femur weight, width and length did not differ among groups (Table 5). Femur resilience was higher ($P=.004$) among WT mice fed CO compared to IL-10 KO mice fed CO or FO diet. Femur peak load was higher among ($P=.033$) WT mice fed FO compared to IL-10 KO mice fed CO (Table 5). With respect to other femur biomechanical properties, yield load, stiffness and toughness did not differ among groups (Table 5). IL-10 KO mice fed FO and WT mice fed CO or FO had a higher ($P<.001$) femur BMC than IL-10 KO mice fed CO (Table 5). IL-10 KO mice fed CO had a lower ($P<.001$) femur BMD than WT mice fed CO or FO, while IL-10 KO mice fed FO had a femur BMD that was intermediary to WT mice fed CO or FO, and IL-10 KO mice fed CO (Table 5).

3.6. Lumbar vertebrae (LV3) peak load and LV1–LV4 BMC and BMD

The peak load of LV3 was lower ($P<.001$) among IL-10 KO mice compared to WT mice fed CO or FO diet (Table 6). BMC of LV1–LV4 did not differ among groups, but BMD of LV1–LV4 was lower ($P=.02$) among IL-10 KO mice fed CO compared to WT mice fed CO or FO (Table 6). IL-10 KO mice fed FO had a BMD that was intermediary to the IL-10 KO mice fed CO and WT mice fed either CO or FO (Table 6).

4. Discussion

It has previously been demonstrated that IL-10 KO mice develop an intestinal inflammation that is accompanied by elevated serum proinflammatory cytokines and bone abnormalities, including lower femur and lumbar vertebrae BMD and biomechanical bone strength properties such as vertebral peak load, compared to WT mice [1]. Results of the present study indicate that a dietary intervention consisting of 10% FO, a level that is attainable by diet alone, provides modest protection against some of the inflammation-associated bone abnormalities in IL-10 KO mice. For example, IL-10 KO mice fed FO had similar femur BMC, BMD, peak load and lumbar vertebra BMD to WT mice fed CO or FO. In contrast, IL-10 KO mice fed CO had lower femur BMC, BMD and peak load and lumbar vertebrae BMD compared to WT mice fed either diet. While these findings indicate that the FO diet attenuated the inflammation-associated bone abnormalities, FO diet did not result in significant improvements over CO diet, except for femur BMC in IL-10 KO mice.

Cortical bone, found largely in the femur compared to the lumbar vertebrae, is thought to be less vulnerable to changes in bone mass, because its primary role is to provide

Table 6
Peak load of LV3 and BMC and BMD of LV1–LV4[1]

	WT		IL-10 KO		P
	Corn[2]	Flax[2]	Corn	Flax	
Peak load (N)	44.00±2.00a	42.25±3.06a	26.92±2.62b	29.46±3.20b	<.001
BMC (mg)	31.68±0.94	31.13±1.04	29.34±0.71	31.23±1.22	NS
BMD (mg/cm^2)	59.14±0.75a	59.21±1.03a	56.05±0.70b	57.91±1.04ab	.02

[1] All data are expressed as mean±S.E.M.; means within a row having different letters are statistically different, $P<.05$. NS, $P>.05$.
[2] Dietary treatments were formulated by modifying the total amount of dietary fat to 100 g of FO or CO per kilogram of diet.

structural support rather than acting as a metabolic reservoir of minerals. Findings from our previous study reported that cortical bone is compromised in IL-10 KO mice with chronic intestinal inflammation [1]. In this study, bone abnormalities in femurs were attenuated to a greater extent than the lumbar vertebra as both femur BMD and peak load were similar among IL-10 KO mice fed FO and WT mice. Similarly, lumbar vertebrae BMD was similar among these same three groups, but the peak load of LV3 was significantly lower in both IL-10 KO mice compared to WT mice, regardless of diet intervention. Because peak load measures primarily the contribution of matrix proteins to bone strength, it is possible that the intestinal inflammation compromised the production of bone matrix proteins or stimulated the degradation of bone matrix proteins. It was recently reported that decreased bone formation is the mechanism of inflammation-associated bone abnormalities in IL-10 KO mice [46]. A suppression of bone formation was demonstrated by a smaller trabecular surface and mineralizing surface, lower trabecular number and lower levels of serum osteocalcin [46].

It has been hypothesized that the pathogenesis of bone abnormalities in IBD patients is due in part to increased circulating proinflammatory cytokines such as TNF-α and IL-6 [5,6,12]. From our findings, it appears that FO attenuated the rise in TNF-α that is characteristic of IL-10 KO mice as only IL-10 KO mice fed CO, but not FO, had a higher serum TNF-α than WT mice. It is possible that some of the improvements in bone outcomes (femur BMC, BMD and peak load; lumbar vertebra BMD) were mediated in part by lower serum TNF-α. In contrast, serum IL-6 was not attenuated among IL-10 KO mice fed FO, suggesting that the positive effects of FO on femur BMC, BMD, peak load and vertebral BMD in IL-10 KO mice were not mediated by changes in serum IL-6. It is possible that the paracrine action of circulating cytokines is not the primary mechanism or the only mechanism responsible for the pathogenesis of inflammation-associated bone abnormalities in IL-10 KO mice. Indeed, serum cytokines may not be representative of local effects in bone [47]. To determine the mechanism by which a diet rich in ALA modulates bone tissue, future studies should investigate whether a FO diet attenuates elevated proinflammatory cytokines in bone.

The intestinal injury scores of WT and IL-10 KO mice are similar to those previously reported [48]. It is possible that the FO diet did not attenuate the intestinal inflammation because of the genetic modification of the IL-10 KO mice. IL-10 is a potent regulatory cytokine, and while the deletion of IL-10 is effective in generating an inflammatory condition in the IL-10 KO mice, it is possible that the dysregulated immune response that results is too severe to be attenuated by a dietary intervention.

Based on serum analyses, the ratio of n-6 to n-3 fatty acids was altered by feeding the FO diet. As expected, mice fed the FO diet had higher serum ALA compared to mice fed the CO diet. Furthermore, IL-10 KO mice were shown to convert ALA to LCPUFAs such as EPA and docosahexaenoic acid (DHA). Although both EPA and DHA suppress the immune system and the production of proinflammatory cytokines [33], EPA but not DHA is a precursor for the production of anti-inflammatory eicosanoids [49]. In rats fed a diet rich in ALA, there is an accumulation of DHA in the femur marrow, with the intermediate EPA being virtually absent [21]. In contrast, in rats fed a diet rich in fish oil, a direct source of EPA, there is a significant increase in femur marrow EPA and no change in the DHA content [31]. It is therefore possible that a flaxseed oil diet may not contribute to eicosanoid production to as great an extent as a fish oil diet. However, it is also important to consider the fact that DHA has been shown to have the ability to retro-convert to EPA (9.4% in humans) and may thereby indirectly influence the production of eicosanoids [50].

In conclusion, this study has demonstrated that a level of ALA that is attainable from a 10% flaxseed oil diet does not attenuate intestinal inflammation but does have some positive effects on the skeleton of IL-10 KO mice. It is possible that a supplemental dose may be required to observe positive therapeutic effects on intestine and to achieve greater effects on bone. Altering the timing of the dietary intervention, such as exposure in utero or from birth throughout the life cycle, may prove more efficacious at attenuating intestinal inflammation and reducing the risk of developing inflammation-associated bone abnormalities.

Acknowledgments

We express our gratitude to Zhen Liu for his excellent technical assistance with the fatty acid analysis, Dr. R. Fedorak, University of Alberta, for providing the initial IL-10 KO breeding pairs and the Flax Council of Canada for funding this research.

References

[1] Cohen SL, Moore AM, Ward WE. Interleukin-10 knockout mouse: a model for studying bone metabolism during intestinal inflammation. Inflamm Bowel Dis 2004;10:557–63.
[2] Jenkins HR. Inflammatory bowel disease. Arch Dis Child 2001; 85:435–7.
[3] Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J Bone Miner Res 2003;18:1206–16.
[4] Abitbol V, Roux C, Chaussade S, et al. Metabolic bone assessment in patients with inflammatory bowel disease. Gastroenterology 1995; 108:417–22.
[5] Pollak RD, Karmeli F, Eliakim R, Ackerman Z, Tabb K, Rachmilewitz D. Femoral neck osteopenia in patients with inflammatory bowel disease. Am J Gastroenterol 1998;93:1483–90.
[6] Roux C, Abitbol V, Chaussade S, et al. Bone loss in patients with inflammatory bowel disease: a prospective study. Osteoporos Int 1995;5:156–60.
[7] Klaus J, Armbrecht G, Steinkamp M, et al. High prevalence of osteoporotic vertebral fractures in patients with Crohn's disease. Gut 2002;51:654–8.

[8] Habtezion A, Silverberg MS, Parkes R, Mikolainis S, Steinhart AH. Risk factors for low bone density in Crohn's disease. Inflamm Bowel Dis 2002;8:87–92.
[9] Bjarnason I, Macpherson A, Mackintosh C, Buxton-Thomas M, Forgacs I, Moniz C. Reduced bone density in patients with inflammatory bowel disease. Gut 1997;40:228–33.
[10] Thearle M, Horlick M, Bilezikian JP, et al. Osteoporosis: an unusual presentation of childhood Crohn's disease. J Clin Endocrinol Metab 2000;85:2122–6.
[11] Boot AM, Bouquet J, Krenning EP, de Muinck Keizer-Schrama SM. Bone mineral density and nutritional status in children with chronic inflammatory bowel disease. Gut 1998;42:188–94.
[12] Cowan FJ, Warner JT, Dunstan FD, Evans WD, Gregory JW, Jenkins HR. Inflammatory bowel disease and predisposition to osteopenia. Arch Dis Child 1997;76:325–9.
[13] Dinca M, Fries W, Luisetto G, et al. Evolution of osteopenia in inflammatory bowel disease. Am J Gastroenterol 1999;94:1292–7.
[14] American Gastroenterological Association medical position statement: guidelines on osteoporosis in gastrointestinal diseases. Gastroenterology 2003;124:791–4.
[15] Bernstein CN, Leslie WD, Taback SP. Bone density in a population-based cohort of premenopausal adult women with early onset inflammatory bowel disease. Am J Gastroenterol 2003;98:1094–100.
[16] Heaney RP, Abrams S, Dawson-Hughes B, et al. Peak bone mass. Osteoporos Int 2000;11:985–1009.
[17] Nishimura J, Ikuyama S. Glucocorticoid-induced osteoporosis: pathogenesis and management. J Bone Miner Metab 2000;18:350–2.
[18] Nieto N, Torres MI, Rios A, Gil A. Dietary polyunsaturated fatty acids improve histological and biochemical alterations in rats with experimental ulcerative colitis. J Nutr 2002;132:11–9.
[19] Stenson WF, Cort D, Rodgers J, et al. Dietary supplementation with fish oil in ulcerative colitis. Ann Intern Med 1992;116:609–14.
[20] Aslan A, Triadafilopoulos G. Fish oil fatty acid supplementation in active ulcerative colitis: a double-blind, placebo-controlled, crossover study. Am J Gastroenterol 1992;87:432–7.
[21] Li Y, Greiner RS, Salem Jr N, Watkins BA. Impact of dietary n-3 fatty acid deficiency on rat bone tissue fatty acid composition. Lipids 2003;38:683–6.
[22] Reinwald S, Li Y, Moriguchi T, Salem Jr N, Watkins BA. Repletion with (n-3) fatty acids reverses bone structural deficits in (n-3)-deficient rats. J Nutr 2004;134:388–94.
[23] Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am J Clin Nutr 1996;63:116–22.
[24] Cunnane SC, Hamadeh MJ, Liede AC, Thompson LU, Wolever TM, Jenkins DJ. Nutritional attributes of traditional flaxseed in healthy young adults. Am J Clin Nutr 1995;61:62–8.
[25] Kelley DS. Immunomodulatory effects of flaxseed and other oils rich in alpha-linolenic acid. In: Thompson LU, Cunnane SC, editors. Flaxseed in Human Nutrition. 1st ed. Champaign (Ill): AOCS; 1995. p. 152.
[26] Davis BC, Kris-Etherton PM. Achieving optimal essential fatty acid status in vegetarians: current knowledge and practical implications. Am J Clin Nutr 2003;78:640S–6S.
[27] Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89:679–89.
[28] Das UN, Ramos EJ, Meguid MM. Metabolic alterations during inflammation and its modulation by central actions of omega-3 fatty acids. Curr Opin Clin Nutr Metab Care 2003;6:413–9.
[29] Belluzzi A, Boschi S, Brignola C, Munarini A, Cariani G, Miglio F. Polyunsaturated fatty acids and inflammatory bowel disease. Am J Clin Nutr 2000;71:339S–42S.
[30] Teitelbaum JE, Allan Walker W. Review: the role of omega 3 fatty acids in intestinal inflammation. J Nutr Biochem 2001;12:21–32.
[31] Watkins BA, Li Y, Allen KG, Hoffmann WE, Seifert MF. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. J Nutr 2000;130:2274–84.
[32] Watkins BA, Li Y, Seifert MF. Nutraceutical fatty acids as biochemical and molecular modulators of skeletal biology. J Am Coll Nutr 2001;20:410S–6S [discussion 417S–420S].
[33] Watkins BA, Li Y, Lippman HE, Feng S. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot Essent Fatty Acids 2003;68:387–98.
[34] Wu D, Meydani SN, Meydani M, Hayek MG, Huth P, Nicolosi RJ. Immunologic effects of marine- and plant-derived n-3 polyunsaturated fatty acids in nonhuman primates. Am J Clin Nutr 1996;63:273–80.
[35] Weiler H, Kovacs H, Nitschmann E, Fitzpatrick Wong S, Bankovic-Calic N, Ogborn M. Elevated bone turnover in rat polycystic kidney disease is not due to prostaglandin E2. Pediatr Nephrol 2002;17:795–9.
[36] James MJ, Proudman SM, Cleland LG. Dietary n-3 fats as adjunctive therapy in a prototypic inflammatory disease: issues and obstacles for use in rheumatoid arthritis. Prostaglandins Leukot Essent Fatty Acids 2003;68:399–405.
[37] Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. J Gastroenterol 2002;37:409–17.
[38] Madsen KL. Inflammatory bowel disease: lessons from the IL-10 gene-deficient mouse. Clin Invest Med 2001;24:250–7.
[39] Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP, Fedorak RN. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. Gastroenterology 2000;118:1094–105.
[40] Canadian Council on Animal Care. Guide to the care and use of experimental animals. Ottawa: Institute of Laboratory Animal Resources Commission on Life Sciences; 1984.
[41] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.
[42] Bourreille A, Doubremelle M, de la Bletiere DR, et al. RDP58, a novel immunomodulatory peptide with anti-inflammatory effects. A pharmacological study in trinitrobenzene sulphonic acid colitis and Crohn disease. Scand J Gastroenterol 2003;38:526–32.
[43] Boismenu R, Chen Y. Insights from mouse models of colitis. J Leukoc Biol 2000;67:267–78.
[44] Rennick DM, Fort MM. Lessons from genetically engineered animal models: XII. IL-10-deficient (IL-10(−/−) mice and intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 2000;278:G829–33.
[45] Fonseca D, Ward WE. Daidzein together with high calcium preserve bone mass and biomechanical strength at multiple sites in ovariectomized mice. Bone 2004;35:489–97.
[46] Dresner-Pollak R, Gelb N, Rachmilewitz D, Karmeli F, Weinreb M. Interleukin 10-deficient mice develop osteopenia, decreased bone formation, and mechanical fragility of long bones. Gastroenterology 2004;127:792–801.
[47] Salamone LM, Whiteside T, Friberg D, Epstein RS, Kuller LH, Cauley JA. Cytokine production and bone mineral density at the lumbar spine and femoral neck in premenopausal women. Calcif Tissue Int 1998;63:466–70.
[48] Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for the development of spontaneous colitis and immune system activitation in IL-10 deficient mice. Infect Immun 1998;66:5224–31.
[49] Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res 2001;40:125–48.
[50] Conquer JA, Holub BJ. Dietary docosahexaenoic acid as a source of eicosapentaenoic acid in vegetarians and omnivores. Lipids 1997;32:341–5.