

Original Research

Inhibition of Osteoporosis in Autoimmune Disease Prone MRL/Mpj-*Fas*^{lpr} Mice by N-3 Fatty Acids

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Objective: Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease involving the breakdown of cartilage and juxta-articular bone, which is often accompanied by decreased bone mineral density (BMD) and increased risk of fracture. Anti-inflammatory omega-3 fatty acids may prevent arthritis and bone loss in MRL/lpr mice model of arthritis and in humans.

Methods: In this study, the effect of long term feeding of 10% dietary n-3 (fish oil (FO)) and n-6 (corn oil (CO)) fatty acids begun at 6 weeks of age on bone mineral density (BMD) in different bone regions in an MRL/lpr female mouse model of RA was measured at 6, 9, and 12 months of age by dual energy x-ray absorptiometry (DEXA). After sacrificing the mice at 12 months of age, antioxidant enzyme activities were measured in spleen, mRNA for receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) was measured by RT-PCR in lymph nodes, and synovitis was measured in leg joints.

Results: At 6, 9 and 12 months of age, BMD was significantly higher ($p < 0.05$) in distal femur, proximal tibia, and lumbar spine of FO fed mice than those of CO fed mice. Spleen catalase (CAT) and superoxide dismutase (SOD) activities were also significantly higher ($p < 0.01$) in FO fed mice than in CO fed mice. Histology of knee joints revealed mild synovitis in CO fed mice, which was not present in FO fed mice. RT-PCR analysis of lymph nodes revealed decreased RANKL mRNA ($p < 0.001$) expression and enhanced OPG mRNA expression ($p < 0.01$) in FO fed mice compared to CO fed mice.

Conclusions: These results suggest beneficial effects of long-term FO feeding in maintaining higher BMD and lower synovitis in this mouse model. These beneficial effects may be due, in part, to increased activity of antioxidant enzymes, decreased expression of RANKL, and increased expression of OPG in FO fed mice thereby altering the RANKL/OPG ratio. These significant beneficial effects on BMD suggest that FO may serve as an effective dietary supplement to prevent BMD loss in patients with RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease involving the breakdown of cartilage and juxta-articular bone which has been shown to be associated with decreased bone mineral density (BMD) and increased risk of fractures [1]. Skeletal complications associated with RA

consist of focal erosion of marginal and subchondral bone, juxta-articular osteoporosis, and generalized bone loss with reduced bone mass [2]. Localized, juxta-articular bone loss may be directly related to joint inflammation, whereas, generalized bone loss has been suggested to be an extra-articular manifestation of RA associated with reduced mobility and increased disease activity [3]. In addition, at later stages, the

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Abbreviations: BHT = butylated hydroxy toluene, BMD = bone mineral density, CAT = catalase, CDNB = 1-chloro-2,4-dinitrobenzene, CLF = central left femur, CLT = central left tibia, CO = corn oil, DEXA = dual energy x-ray absorptiometry, DHA = docosahexaenoic acid, DLF = distal end of left femur, EPA = eicosapentaenoic acid, FID = flame ionization detector, FO = fish oil, GPx = glutathione peroxidase, GST = glutathione S-transferase, MUFA = monounsaturated fatty acids, NOS = Nitric oxide synthetase, OPG = osteoprotegerin, PI = peroxidizability index, PLT = proximal left tibia, P/S = polyunsaturated/saturated fatty acid ratio, PUFA = polyunsaturated fatty acids, RA = Rheumatoid arthritis, RANKL = receptor activator of NF- κ B ligand, SLE = systemic lupus erythematosus, SOD = superoxide dismutase, TBHQ = tertiary butyl hydroxy quinone.

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immobilization induced by the joint disease of RA itself may accelerate the bone loss. There is also increasing evidence that immunosuppressive drugs such as corticosteroids, e.g. prednisone, used in therapy of RA are associated with decreases in BMD and have been implicated as a cause of secondary osteoporosis which may contribute to systemic bone loss [4–6].

Bone resorption is a major pathological factor in chronic inflammatory diseases like osteoporosis and arthritis. Deregulation of immune and inflammatory response is crucial in initiating the bone resorption associated with these conditions. Proinflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) regulate the onset and progression of bone loss by initiating a cascade of cellular signals resulting in differentiation and activation of bone-resorbing osteoclasts [7]. The ligand for receptor activator of NF- κ B (RANKL) and the secretory glycoprotein OPG (osteoprotegerin, member of the TNF- α receptor family) system have regulatory effects on bone metabolism and may play a crucial role in pathogenesis of bone erosions in RA. RANKL secretion by activated T-cells activates its receptor RANK, which in turn promotes osteoclast formation and activation, and prolongs osteoclast survival by suppressing apoptosis [8]. The effects of RANKL are blocked by OPG which acts as a decoy receptor for RANKL. RANKL secretion by activated T-cells contributes to the pathophysiology of inflammation and destruction in RA, as shown in murine models where OPG inhibits bone erosions but not inflammation [9]. The balance between RANKL and OPG is regulated by cytokines and hormones and determines osteoclast functions. Alterations in RANKL/OPG ratio are critical in the pathogenesis of bone diseases that result from bone resorption like RA [10].

Oxidative stress has also been implicated in the pathogenesis of inflammatory diseases like systemic lupus erythematosus (SLE), asthma and RA [11–13]. Several human studies have shown that antioxidant defense system is compromised in RA with increased generation of free radicals and lipid peroxidation, and decreased levels of both enzymatic and non-enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione, vitamins A and C, etc [14–16]. We previously showed that antioxidant defense system is compromised in MRL/lpr mice with lower activity and expression of SOD, CAT, GPX and higher lipid peroxidation compared to control MRL/++ mice [17]. Studies have also indicated a positive correlation between oxidative stress and increased bone turnover [18,19]. This may be one of the mechanisms by which bone resorption occurs in patients of RA similar to MRL/lpr mice.

In recent years, nutritional intervention for the treatment of autoimmune diseases like RA has received considerable interest. Clinical trials with fish oil (FO) suggest amelioration of clinical symptoms of RA [20–24] suggesting that ω -3 lipids

have potential therapeutic value for inflammatory diseases [25,26]. Although several studies have reported benefits against RA symptoms using various doses of FO as a dietary supplement, these studies have not measured changes in BMD in these patients. Nutritional intervention with FO, containing the n-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), has been found to increase life span and delay onset of autoimmune disease in autoimmune-prone NZB/W female mice, which, in part, is associated with increased expression and activity of antioxidant enzymes and decreased lipid peroxidation [27–31]. FO supplementation at high levels has been shown to delay onset of lymphoproliferation and renal disease and extend life span in autoimmune-prone MRL/lpr mice [32,33]. EPA decreased the rate of bone loss in a rat model of postmenopausal osteoporosis [34]. Dietary supplementation with EPA and gamma-linoleic acid decreased bone turnover and increased BMD in femurs and lumbar vertebrae in a pilot human study [35]. In a recent study from our laboratory, ovariectomized (Ovx) Balb C mice fed FO showed 50% decrease in loss of BMD compared to mice fed CO [36]. This effect was associated with increased RANKL expression in T-cells from CO fed Ovx mice.

The MRL/lpr female mouse model of RA is used to study the effect of diet and drugs on autoimmune disease [37,38]. These mice spontaneously develop autoantibodies, high levels of acute phase proteins and immune complex glomerulonephritis [39]. This mouse strain has a genetic predisposition to arthritis with characteristics similar to human RA including cellular infiltration of joints, pannus formation, bone and cartilage breakdown, and the presence of serum RF [39–41]. It has been proposed that the MRL strain of mice could serve as a valuable laboratory animal model for the study of skeletal changes in systemic lupus erythematosus (SLE) and the influence of the disease activity on skeletal metabolism [42]. MRL/lpr mice exhibited lower osteocalcin and alkaline phosphatase compared to control mice. Moreover, lower trabecular bone volume in femoral head and femoral neck, and smaller cortical and femoral areas in the mid-femoral shaft was also noticed [42]. Although, several studies of the effect of FO and/or calorie restriction on immune parameters and disease activity in MRL/lpr female mice [32,33,43] have been reported, none have focused so far on its effect on BMD and RANKL/OPG ratio. Our previous studies have shown the protective effect of FO in autoimmune prone mice [44,45]. Also, our recent study clearly indicates that FO and/or soy protein has a protective effect on BMD in ovariectomized mice [46]. Therefore, the aim of the present study was to evaluate whether fish oil, enriched in omega-3 fatty acids would prevent bone loss associated with arthritis in MRL/lpr mice compared to corn oil feeding as a source of omega-6 fatty acids and whether this effect was associated with decreased RANKL/OPG ratio and increased antioxidant enzyme activity.

MATERIALS AND METHODS

Animals and Experimental Diets

Four-week-old female MRL/MpJ-*Fas^{lpr}* (MRL/lpr) mice were purchased from Jackson Laboratories, Bar Harbor, ME. Weight matched mice were housed in the laboratory animal care facility in cages (5 mice/cage) and fed a standard lab chow diet (5% fat). At 6 weeks of age, mice were divided into 2 dietary groups of 10 mice each fed semipurified AIN-93M diets containing either 10% CO or 10% FO. In the FO diet, 1% CO was added to 9% FO (Menhaden oil, ICN Biomedical, Costa Mesa, CA) to prevent essential fatty acid deficiency. The diets were supplemented with equal amounts of antioxidant supplements (vitamin E and tertiary butyl hydroxy quinone (TBHQ)), to prevent peroxidative damage during storage. The composition of the semi-purified diet is presented in Table 1. Fresh diet was provided and leftover food was removed daily to prevent rancidity. Diets were prepared weekly and stored in aliquots at -20°C . Oils used in the study were always stored under nitrogen to prevent oxidation. The fatty acid compositions of the standard chow diet, and the CO and FO are presented in Table 2. The animals were maintained on a 12 hr light/12 hr dark cycle. NIH guidelines provided in "The guide for the care and use of laboratory animals" were strictly followed and all studies were approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. Body weight and proteinuria using chemstrips (Roche Diagnostic Corporation, Indianapolis, IN) were measured in all mice every four weeks.

Collection of Spleen Tissues and Bones

At 12 months, mice were sacrificed by cervical dislocation and tissues from 5 randomly selected animals were used for further analysis. Spleens and lymph nodes were collected, instantly frozen in liquid nitrogen and stored at -80°C until the time of study. Leg joint bones were also collected from 4 mice from each group and fixed in 10% formalin for histology.

Table 1. Composition of the Experimental Diets

Ingredient ¹	Percent
Casein	14.00%
Corn starch	42.43%
Dextronized corn starch	14.50%
Sucrose	9.00%
Cellulose	5.00%
AIN-93 mineral mix	3.50%
AIN-93 vitamin mix	1.00%
L-cystine	0.18%
Choline bitartrate	0.25%
TBHQ	0.10%
Vitamin E	0.04%
Corn oil and/or Fish oil ²	10.00%

¹ All diet ingredients purchased from ICN, Irvine, CA.

² Fish oil diet supplemented with 1% corn oil (FO = 9%, CO = 1%).

Table 2. Fatty Acid Composition of Lab Chow and Oils¹

Fatty acids	Lab Chow	Corn oil	Menhaden oil
12:0	ND ²	ND	0.15
14:0	0.12	0.02	9.05
15:0	ND	ND	0.73
16:0	14.01	12.77	15.52
16:1n-7	ND	ND	15.28
17:0	0.10	ND	0.57
17:1n-9	ND	ND	1.97
18:0	2.41	0.43	2.13
18:1n-9	24.21	27.42	5.10
18:1n-7	ND	ND	6.95
18:2n-6	51.55	57.87	1.62
γ -18:3n-3	ND	ND	1.00
18:3n-3	6.23	0.81	1.79
18:4n-6	ND	ND	4.41
20:0	0.35	0.27	ND
20:1n-9	0.52	0.40	1.18
20:3n-6	ND	ND	0.10
20:4n-6	ND	ND	0.71
20:5n-3	ND	ND	14.06
22:0	0.48	ND	ND
22:5n-6	ND	ND	0.48
22:5n-3	ND	ND	1.00
22:6n-3	ND	ND	8.69

¹ Expressed as percentage of total fatty acids.

² ND = not detected.

Chemicals

Xanthine, xanthine oxidase, ferricytochrome C, β -nicotinamide adenine dinucleotide phosphate (reduced form) (β -NADPH), glutathione (reduced), hydrogen peroxide, glutathione reductase, 1-chloro-2, 4-dinitrobenzene and cumene hydroperoxide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fatty acid methyl ester standards were purchased from Nu-Chek (Elysian, MN). All other chemicals were of analytical grade.

Preparation of Spleen Homogenates

The frozen spleen tissues were rinsed in ice-cold physiological saline and minced with scissors. 10% homogenates were prepared in 0.01 M Tris-HCl buffer (pH 7.4) and centrifuged at $10,000 \times g$ and the supernatants were used for antioxidant enzyme assays. The protein content of spleen supernatants was determined by the microplate procedure of the bicinchoninic acid (BCA) protein assay as described by the supplier (Pierce Chemical Company, Rockford, IL).

Measurement of Bone Mineral Density, Bone Mineral Content, Lean Body Mass and Body Fat Mass

Bone mineral density (BMD), lean body mass (LBM) and body fat mass (BFM) were measured by dual energy x-ray absorptiometry (DEXA) at 6, 9, and 12 months using a Lunar PIXImus mouse bone densitometer (General Electric) and data

analysis was carried out manually with PIXImus software [36]. Calibration of the instrument was conducted as suggested by the manufacturer. An aluminum/lucite phantom (TBMD = 0.0700 g/cm², percentage fat = 14.0%) was placed on the specimen tray and measured 25 times without repositioning. Thereafter, the phantom was analyzed daily before animal testing for quality control purposes. Before bone scanning was performed, mice were anesthetized with intra-muscular injections of Ketamine/Rompun/NaCl (3/2/5). The densitometer was calibrated daily with a phantom supplied by the instrument manufacturer. During the measurements, the animals were lying in a prone position with posterior legs maintained in external rotation with tape. The hip, knee and ankle articulations were in 90° flexion. Upon completion of the scanning, BMD was determined in the following bone areas using the PIXImus software (version 1.46)—distal end of left femur (DLF) (knee joint) to include cancellous (trabecular) bone, proximal left tibia (PLT), central left femur (CLF), central left tibia (CLT) and lumbar spine (vertebra L3). The intrascan coefficients of variation (CVi) were 0.79%, 3.30%, 1.35%, 3.48% and 1.19% for DLF, PLT, CLF, CLT and L3, respectively. The interscan coefficients of variation (CVr) were 5.47%, 3.86%, 5.12%, 1.36% and 2.37% for DLF, PLT, CLF, CLT and L3, respectively. The CVs are in agreement with studies examining the precision and accuracy of PIXImus densitometer [47,48].

Fatty Acid Analysis of Spleen Tissues

Spleen total lipids were extracted by the method of Bligh and Dyer [49] using chloroform-methanol (1:2) and chloroform-methanol-water (1:2:0.8). Extractions were performed in an atmosphere of nitrogen and butylated hydroxy toluene (BHT) was added to prevent oxidation during processing. The organic phase, containing the total lipid extracts, was dried under a stream of nitrogen and the residue methylated according to the method of Kates [50]. Fatty acid methyl esters were separated and quantified by gas-liquid chromatography using a Hewlett-Packard 5890A series II gas chromatograph, equipped with a DB225MS capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector (FID). The injection and detector port temperatures were 225°C and 250°C, respectively. The oven temperature was maintained at 170°C for one minute and then increased to 215°C at the rate of 5°C/minute. Helium was used as the carrier gas. The running time of each sample was approximately 36 minutes. The fatty acid methyl esters were identified by comparison of retention times with fatty acid methyl ester standard (68A) from Nu Chek, Elysian, MN as an external standard. Quantification was done by an integrator (Hewlett-Packard 3396 series II) attached to the GLC machine and results are expressed as area percentages. Oil samples were treated similarly beginning at the methylation step.

Determination of Mouse Spleen Antioxidant Enzymes

Antioxidant enzyme activities were measured as described previously by us [51]. The activity of CAT was measured using its peroxidative function according to the method of Johansson and Borg [52]. SOD activity was determined by the inhibition of cytochrome C reduction by the method of Flohe and Otting [53]. GSH-P_x activity was measured by following NADPH oxidation using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide [54]. GST activity was measured by the catalytic reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with the sulfhydryl group of glutathione [55].

Bone Histology

At the end of the study knee joints collected from 4 mice in each group were fixed in 10% buffered formalin, decalcified in TBD-2, embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin (H&E) stains using standard techniques for light microscopic examination as previously described [36].

RT-PCR Analysis of LN Tissues

Total RNA from LN tissues was extracted using TRIZOL according to manufacturer's instructions and RT-PCR analysis performed as previously described [56]. Briefly, the RNA was reverse transcribed into complementary DNA (cDNA). The cDNA was subjected to enzymatic amplification in a DNA thermal cycler using specific primers. The specific primers used were as follows: RANKL: 5'-TTT GCA GGA CTC GAC TCT GGA G and 5'-TCC CTC CTT TCA TCA GGT TAT GAG; OPG: 5'-ATC ATT GAA TGG ACA ACC CAG G and 5'-TGC GTG GCT TCT CTG TTT CC; GAPDH: 5'-GAT CGT GGA AGG GCT AAT GA and 5'-GAC TTT GCC TAC AGC CTT GG. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide.

Statistical Analysis

Values are expressed as mean ± SEM. Bone mineral density results were statistically analyzed using paired t-test and $p < 0.05$ for F ratio was considered statistically significant. Antioxidant enzymes, fatty acid composition and mRNA data were analyzed by student's t-test and $p < 0.05$ for F ratio was considered statistically significant.

RESULTS

There was no difference in body weight, lean mass or fat mass between CO and FO fed mice at any given time point (data not shown).

Effect of Dietary N-3 and N-6 Fatty Acids on Femoral BMD

The effect of type of dietary fat on change in femoral BMD from 6 months to 12 months age in MRL/lpr mice is shown in Table 3. BMD increased significantly in DLF and CLF ($p < 0.05$) bone regions in FO fed mice between 6 and 9 months age which then remained steady till 12 months age. On the other hand, CO feeding significantly decreased DLF BMD between 6 to 9 months and decreased further at 12 months though insignificantly. Overall the BMD levels remained significantly ($p < 0.0001$) higher in FO fed mice compared to CO fed mice from 9 months to the end of the study period.

Effect of Dietary N-3 and N-6 Fatty Acids on Tibial BMD

BMD in the PLT bone region from FO-fed mice remained higher and uniform throughout the period of study. However, BMD of CO fed mice decreased between 6 and 9 months and decreased further with age ($p < 0.001$). In the CLT bone region, BMD increased significantly ($p < 0.05$) in FO fed mice between 6 and 9 months age. BMD decreased slightly though insignificantly with age in this bone region between 9 and 12 months age. BMD decreased significantly in CO fed mice between 6 and 9 months age and remained significantly lower until 12 months age (Table 3).

Effect of Dietary Fat on Vertebral BMD

BMD mineral density was analyzed in third lumbar vertebra at different time points. BMD in FO fed mice increased with

Table 3. Effect of CO and FO on Bone Mineral Density (Grams/Cm²) in Different Bone Areas-Distal Left Femur (DLF), Proximal Left Tibia (PLT), Central Left Femur (CLF), Central Left Tibia (CLT) and Third Lumbar Vertebra (L3) of Female MRL/Lpr Mice

Bone region	Age (months)	CO	FO
DLF	6	0.138 ± 0.004	0.149 ± 0.003 ^b
	9	0.127 ± 0.004	0.161 ± 0.003 ^b
	12	0.123 ± 0.003	0.157 ± 0.003 ^a
PLT	6	0.110 ± 0.001	0.123 ± 0.002 ^b
	9	0.100 ± 0.003	0.125 ± 0.003 ^b
	12	0.097 ± 0.002	0.123 ± 0.003 ^b
CLF	6	0.111 ± 0.004	0.122 ± 0.005
	9	0.113 ± 0.005	0.136 ± 0.005 ^d
	12	0.119 ± 0.004	0.134 ± 0.004 ^d
CLT	6	0.063 ± 0.002	0.061 ± 0.001
	9	0.055 ± 0.002	0.066 ± 0.002 ^c
	12	0.057 ± 0.002	0.064 ± 0.001 ^d
L3	6	0.105 ± 0.005	0.124 ± 0.005
	9	0.090 ± 0.003	0.113 ± 0.003 ^b
	12	0.091 ± 0.004	0.118 ± 0.004 ^d

Data expressed as mean ± SEM of 7–10 mice/group. Data was analyzed using paired t test; ^a $p < 0.0001$, ^b $p < 0.001$, ^c $p < 0.01$, ^d $p < 0.05$ vs CO. FO: Fish oil; CO: Corn oil.

age ($p < 0.05$) whereas BMD decreased in CO fed mice between 6 and 9 months which then remained steady till the end of the study period (Table 3).

Effect of Dietary N-6 and N-3 Fatty Acids on Spleen Fatty Acid Content

The effect of dietary n-3 and n-6 fatty acids on fatty acid composition of spleen total lipids is presented in Table 4. As expected, the type of dietary fat significantly affected the fatty acid composition. Spleens of CO fed mice had higher levels of n-6 fatty acids (18:2n-6 and 20:4n-6) compared to FO mice. On the other hand, FO diet fed mice had significantly higher levels of 16:1 and 22:6n-3 compared to CO fed mice. While 20:5n-3 and 22:5n-3 was present only in the FO fed groups, 22:4n-6 and 22:5n-6 was present only in the CO fed groups. ANOVA analysis for main effects showed that dietary fat significantly affected all the fatty acids except 16:0.

The effects of dietary n-3 and n-6 fatty acids on the content of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), n-6/n-3 ratio, polyunsaturated/saturated fatty acid (P/S) ratio and peroxidizability index (PI) in spleen total lipids are also presented in Table 4. While the levels of MUFAs and n-3 fatty acids were significantly higher in FO fed mice, PUFAs, n-6 fatty acids and n-6/n-3 ratio were significantly lower compared to CO fed

Table 4. Effect of CO and FO on Fatty Acid Composition of Spleen Total Lipids of Female MRL/Lpr Mice¹

Fatty acids	CO	FO
14:0	0.67 ± 0.04	1.32 ± 0.15
16:0	24.05 ± 0.07	26.87 ± 0.45
16:1	1.25 ± 0.20	3.31 ± 0.91*
18:0	22.56 ± 0.78	20.85 ± 1.86*
18:1	11.16 ± 1.57	14.99 ± 3.17*
18:1	2.29 ± 0.03	2.95 ± 0.19*
18:2	7.42 ± 0.69	3.57 ± 0.06*
20:3	0.65 ± 0.02	0.61 ± 0.05
20:4	17.13 ± 0.54	5.19 ± 0.39*
20:5	—	4.17 ± 0.13*
22:4	3.37 ± 0.05	—
22:5	2.21 ± 0.15	—
22:5	—	3.12 ± 0.08
22:6	3.75 ± 0.02	8.87 ± 0.81*
Saturates	48.15 ± 2.19	47.93 ± 1.78
MUFAs	15.25 ± 1.41	22.55 ± 3.36*
PUFAs	34.58 ± 1.40	25.53 ± 1.45*
P/S	0.68 ± 0.06	0.54 ± 0.01
n-6	30.82 ± 1.38	9.37 ± 0.43*
n-3	3.75 ± 0.02	16.16 ± 1.02*
n-6/n-3	8.21 ± 0.32	0.58 ± 0.01*
Per. Index	138.83 ± 5.65	141.93 ± 4.42

¹ Values expressed as percent of total fatty acids; values are mean ± SEM of 5 mice/group; * significantly different from corn oil controls at $p < 0.05$ by Student's t test. CO = corn oil, FO = fish oil. MUFAs = monounsaturated; PUFAs = polyunsaturated; P/S = polyunsaturated/saturated ratio; peroxidation index was calculated based on the formula: (monoenesX1) + (dienesX2) + (trienesX3) + (tetraenesX4) + (pentaenesX5) + (hexaenesX6).

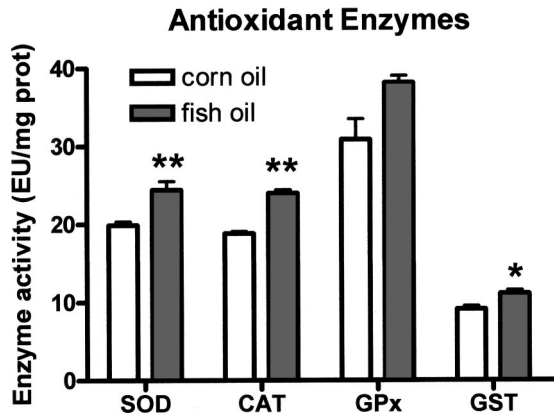


Fig. 1. Effect of corn oil and fish oil on antioxidant enzyme activities in spleen tissues of MRL/lpr mice. Values are mean \pm SEM for 5 mice/group. Enzyme units are superoxide dismutase— μ mol cytochrome C reduced/min, catalase— μ mol formaldehyde produced/min, glutathione peroxidase— μ mol NADPH oxidized/min, glutathione S-transferase— μ mol 1-chloro-2,4-dinitrobenzene-glutathione conjugate formed/min \times 100. *significantly different at $p < 0.05$ by Student's t test. **significantly different at $p < 0.01$ by Student's t test. SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GST = glutathione S-transferase.

mice. There was no difference in PI between the dietary groups. ANOVA analysis for main effects showed that the effect of dietary fat was significant for MUFAs, PUFAs, n-6/n-3 ratio, P/S ratio and PI. There was no overall effect of fat on SFAs.

Effect of Dietary N-6 and N-3 Fatty Acids on Mouse Spleen Antioxidant Enzymes

The effects of dietary fat on spleen antioxidant enzyme activities are presented in Fig. 1. Activities of SOD, CAT and

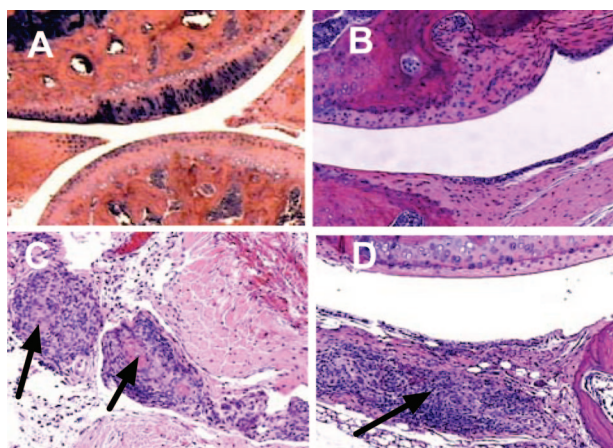


Fig. 2. Effect of corn oil and fish oil on knee joint histology. **A.** Fish oil diet fed mouse with normal knee joint, **B.** Fish oil diet fed mouse with minimal inflammatory joint changes, **C.** Corn oil diet fed mouse with vasculitis in soft tissue adjacent to the joint, **D.** Corn oil diet fed mouse with focal lymphocytic synovitis and increased thickness of synovial lining cells.

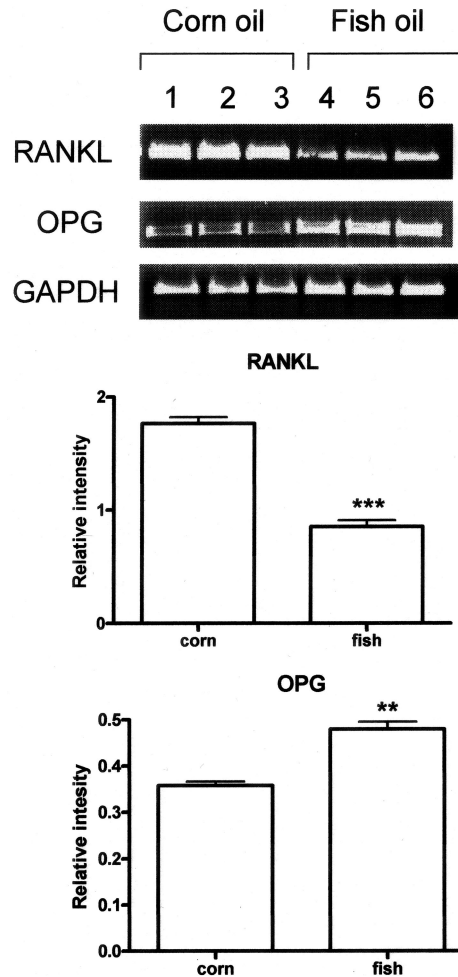


Fig. 3. RT-PCR analysis of mRNA expression for receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) in lymph nodes of mice fed corn oil or fish oil. **A** marked decrease in RANKL and significant increase in OPG was observed in fish oil-fed mice compared to corn oil-fed mice. **B.** Relative expression of RANKL and OPG is shown. The intensity of the bands was determined by densitometry using an AlphaImager 200 (Alpha Innotech Corp) imaging system and normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are mean \pm SEM for bands 1–3 and 4–6. Band intensities in fish oil-fed mice are significantly different from corn oil-fed mice at ** $p < 0.01$ and *** $p < 0.001$.

GST were significantly higher ($p < 0.01$, 0.01 , and 0.05 respectively) in FO fed mice compared to CO fed mice. GSH-Px activity in mice fed FO was close to being statistically significantly higher compared to mice fed CO.

Effect of Dietary N-6 and N-3 Fatty Acids on Knee Joint Bone Histology

Histological evaluation of the knee joints of MRL/lpr mice on the corn oil diet revealed mild synovitis with a patchy infiltrate of lymphocytes. Mild synovial hyperplasia with an increased thickness of the synovial lining cells was also

present. A vasculitis with fibrinoid necrosis and juxta-articular soft tissue inflammation was also present in some animals. These changes appeared markedly attenuated in the fish oil diet fed group (Fig. 2).

Effect of Dietary N-6 and N-3 Fatty Acids on the Expression of RANKL and OPG in LN Tissues

RNA was isolated from LN of MRL/lpr mice fed corn and fish oil for analysis of RANKL and OPG by RT-PCR (Fig. 3a). The results show a significantly decreased expression of RANKL and an enhanced expression of OPG in fish oil fed mice compared to corn oil fed mice. Fig. 3b shows the densitometric analysis of the expression of RANKL and OPG in corn oil and fish oil fed mice.

DISCUSSION

In the present study, BMD was measured *in vivo* using a DEXA scanner in cancellous and cortical bone of femur, tibia and lumbar spine, and bone histology was also performed. The results indicate for the first time that FO fed arthritis-prone mice consistently maintained a higher BMD than CO fed mice throughout the period of the study in most of the bone areas analyzed. In a previous study using a collagen induced model of arthritis, Enokida *et al.* measured time course changes in BMD in mature rats and the study showed that juxta-articular trabecular bone was vulnerable to bone loss early in the course of arthritis [57]. In our study, FO fed mice maintained higher BMD in trabecular bone compared to CO fed mice throughout the period of study. These findings are of great interest considering the increase in n-6 fatty acid consumption and corresponding decrease in n-3 fatty acid consumption over the past 30 years in the US population [58]. Increased n-6 fatty acids along with saturated fats have been linked to a rise in cardiovascular diseases and certain cancers [25]. In fact, a recent clinical study showed that increase in (n-3)/(n-6) PUFA ratio may reduce the risk of breast cancer in premenopausal women [59].

Earlier studies from this laboratory have shown that FO supplementation significantly increases the activities and mRNA expression of CAT, SOD and GSH-Px antioxidant-enzymes in hepatic and renal tissues of autoimmune-prone NZB/W mice [31,44]. Omega-3 fatty acids at hypotriglyceridemic doses are known to enhance the activities of the hepatic SOD, CAT, GSH-Px and GST [60]. In the present study, FO fed mice maintained significantly higher activities of SOD and CAT in spleen tissues, compared to CO fed mice. Nitric oxide (NO) reacts with superoxide radical to generate peroxynitrite, which under acidic conditions (as is present in sites of inflammation) yields the highly toxic hydroxyl radical, which could lead to free radical mediated bone loss. Nitric oxide synthetase

(NOS), a key enzyme involved in NO synthesis is found elevated in experimental arthritis models [13]. Moreover, pro-inflammatory cytokines such as IL-1 β and TNF- α are also involved in the formation of peroxynitrite radical by increasing the activity of NOS. MRL/lpr strain has been reported to have elevated activity of NOS and inhibition of this enzyme reduces degree of arthritis in these mice [13]. A recent study showed increased peroxynitrites and decreased catalase activity in MRL/lpr mice [61]. In our study, higher levels of SOD in FO fed mice could scavenge superoxide radicals and inhibit generation of peroxynitrite from NO by this pathway.

Numerous studies have indicated the protective role of n-3 fatty acids on autoimmune renal disease in animal models [28–31,62]. This effect was associated with decreased expression of pro-inflammatory cytokines like interleukins IL-1, IL-6 and TNF- α [27,44]. IL-1 and IL-6 are potent bone resorption-promoting factors which mediate bone breakdown in early RA. These are released from inflamed synovium and stimulate bone resorption both *in vitro* and *in vivo* [63]. These cytokines are increased in synovial fluid in RA joints suggesting that they may be key factors in the development and progression of arthritis [63]. Although the development of collagen disease involving vasculitis and arthritis is under the control of a different set of genes in MRL/lpr mice [64], down regulation of pro-inflammatory cytokines could also be one of the mechanisms by which n-3 fatty acids prevent bone loss in these mice.

Recent studies have pointed to the involvement of NF- κ B and RANKL in bone loss associated with RA [2,65]. We earlier showed that RANKL expression is increased in activated CD4⁺ T-cells of CO Ovx mice but not in FO Ovx mice [36]. In the same study, n-3 fatty acids, EPA and DHA, alone or in combination, inhibited bone marrow macrophage NF- κ B activation induced by RANKL *in vitro*, in contrast to n-6 fatty acids. The results indicated that n-3 fatty acids could prevent osteoclast activation, *in vitro* and it could be one of the mechanisms by which fish oil prevents bone loss.

RANKL is a potent activator and OPG is a potent inhibitor of osteoclastogenesis, and osteoclast activity. Thus, a reduction of RANKL expression and/or increase in OPG expression would lead to a reduction in both the formation and activity of osteoclasts [66]. Recently, Yoneda *et al.* showed that estrogen deficiency caused by ovariectomy can profoundly increase the incidence of arthritis in MRL/lpr mice whereas the administration of estrogen prevented the development of arthritis and decreased RANKL expression in synovial tissue [67]. We also found lower mRNA expression of RANKL, and higher mRNA expression of OPG in FO fed mice compared to CO fed mice. Reduced RANKL, along with increased OPG could lead to decrease in osteoclastogenesis and osteoclast activity, which results in inhibition of osteoclastic bone resorption and eventually higher bone mass and BMD in FO fed mice, compared to CO fed mice.

CONCLUSION

The results obtained in our present study as well as from our earlier studies [36] suggest that the beneficial effects of FO in maintaining higher BMD could be due to decreased RANKL/OPG ratio and higher activity of antioxidant enzymes. Thus FO could become an ideal therapeutic dietary supplement either alone or as an adjuvant to other antiresorptive drug therapy to prevent bone loss in RA or SLE. Further experiments are underway to study the effect of n-3 fatty acids in very young ovariectomized MRL/lpr mice to prevent the accelerated development of arthritis [67] before the onset of renal disease and to clarify the molecular mechanisms involved in maintenance of higher BMD and attenuation of inflammation and bone erosion by providing purified, deodorized FO to these RA disease-prone mice.

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