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Protective effect of dietary long-chain *n*-3 polyunsaturated fatty acids on bone loss in gonad-intact middle-aged male rats

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This study evaluated the effect of a fat blend containing long-chain (LC) *n*-3 PUFA on bone mineral density (BMD) and bone metabolism in gonad-intact middle-aged male rats (12 months old, *n* 28). Seven rats were killed on day 0 of dietary intervention to determine the baseline BMD. The remaining rats (seven per group) were fed a diet with one of the following dietary lipid treatments (g/kg diet): 167 g safflower oil + 33 g menhaden oil (N6 + N3 diet, control), 200 g safflower oil (N6 diet, almost devoid of LC *n*-3 PUFA), or 190 g menhaden oil + 10 g corn oil (N3 diet, rich in LC *n*-3 PUFA) for 20 weeks. After 20 weeks, all dietary treatment groups had a lower BMD compared with the baseline reference. However, rats fed the N3 diet had the highest bone mineral content and cortical + subcortical BMD compared with those fed the N6 and control N6 + N3 diet. Compared with the control (N6 + N3) group, rats fed the N3 diet had higher values for serum insulin-like growth factor-I, parathyroid hormone, 1,25-$(OH)_2$ vitamin D_3 and bone-specific alkaline phosphatase activity, but lower bone NO production and urinary Ca, whereas rats fed the N6 diet had higher bone prostaglandin E_2 production and serum pyridinoline. These findings indicate a protective action of LC *n*-3 PUFA on ageing-induced bone loss in gonad-intact middle-aged male rats through a modulation of local factors and systemic calcitrophic hormones.

Menhaden oil: *n*-3 PUFA: Bone metabolism: Gonad-intact middle-aged male rats

Although women have been the main focus for research on osteoporosis, emerging evidence indicates that ageing-induced osteoporosis is a common occurrence in men (Rowe & Kahn, 1987). The mechanism for ageing-induced bone loss in middle-aged and elderly men is not well understood (Center *et al.* 1999). Some postulate that there is an imbalance between bone formation and bone resorption apparently regulated by bone-derived local factors such as prostaglandins (Mundy, 2003), NO (Ralston *et al.* 1995) and cytokines (Mundy, 2003; Raisz & Rodan, 2003) and systemic hormones such as insulin-like growth factors (IGF), parathyroid hormone and vitamin D (Mundy 2003; Raisz & Rodan, 2003). A logical approach is therefore to investigate changes in both local factors and systemic hormones associated with the development of osteoporosis.

High-fat diets are pervasive in Western cultures (Rizek *et al.* 1983), and when this is coupled with elevated risk of chronic disease caused by ageing, it is reasonable to speculate that the source of dietary fat could be a contributory lifestyle factor associated with osteoporosis and age-related bone loss in men (Hou *et al.* 1990). When considering sources of fat, a body of scientific evidence based on results in cell cultures (Watkins *et al.* 2003), animals (Sakaguchi *et al.* 1994; Kruger *et al.* 1996; Schlemmer *et al.* 1999; Sun *et al.* 2003; Watkins *et al.* 2003, 2005) and human subjects (Kruger *et al.* 1998) indicates that long-chain (LC) *n*-3 PUFA may protect skeletal health and potentially improve conditions associated with male osteoporosis. However, all animal studies employed a moderate fat level in the experimental diets, with an oestrogen-deficiency bone loss model in female rodents. No study has evaluated the effect of a high-fat diet rich in EPA (20:5*n*-3), docosapentaenoic acid (22:5*n*-3) and DHA (22:6*n*-3) on bone metabolism and bone mass during male ageing. Therefore, in this study, we investigated the effect of menhaden oil (rich in EPA, docosapentaenoic acid and DHA) as part of a high-fat diet on bone metabolism and bone mineral density (BMD) in gonad-intact middle-aged male rats. Based on the protective effect of LC *n*-3 PUFA against bone loss in ovariectomized female rats (Sakaguchi *et al.* 1994; Kruger *et al.* 1996; Schlemmer *et al.* 1999; Watkins *et al.* 2003, 2005) and mice (Sun *et al.* 2003), we hypothesized that ageing-induced bone loss in gonad-intact male rats would be minimized with LC *n*-3 PUFA. We further hypothesized that such an effect of LC *n*-3 PUFA on bone loss would be due to the modulation of local and systemic factors that regulated bone metabolism.

Abbreviations: BALP, bone-specific alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; DEXA, dual-energy X-ray absorptiometry; IGF-I, insulin-like growth factor-I; LC, long-chain; PGE_2, prostaglandin E_2; Pi, inorganic phosphate; PTH, parathyroid hormone; PYD, pyridinoline.

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Forty per cent of the energy intake of the experimental diet was provided by the dietary fat source (20 %w/w) in this study. This design incorporates the high-fat diet that is pervasive in Western cultures and represents an important risk factor for the prevalence of chronic diseases, including osteoporosis, during male ageing (Kopelman, 2000). Similar dietary fat levels have been used in experimental rodent diets (Vaskonen *et al.* 1996; Choi *et al.* 2004; Bhattacharya *et al.* 2005). The male aged rat was selected because (1) men do not normally experience an abrupt loss of sex hormones, as occurs in women following the menopause, and (2) the middle-aged male rat has completed its bone growth and commenced bone loss (Wang *et al.* 2001). Studying the effects of LC *n*-3 PUFA on bone remodelling in middle-aged male rats will advance our understanding of their effects on skeletal biology in terms of minimizing bone loss in elderly men.

Materials and methods

Preparation of rat diets

All rats were maintained on the NIH-31 diet (Nadon, 2004) at the animal laboratory facilities before being shipped to our laboratory. Upon arrival, rats were assigned to dietary treatment groups and fed a semi-purified basal diet (modified AIN-93 diet; Dyets, Bethlehem, PA, USA) supplemented with one of the following lipid treatments (g/kg diet): 167 g safflower oil + 33 g menhaden oil (control N6 + N3 diet containing 6·1 % w/w LC *n*-3 PUFA), 200 g safflower oil (N6 diet; almost devoid of LC *n*-3 PUFA, containing only 0·3 % w/w LC *n*-3 PUFA) or 190 g menhaden oil + 10 g corn oil (N3 diet; rich in LC *n*-3 PUFA, containing 35 % w/w LC *n*-3 PUFA). The fatty acid and ingredient compositions of the diets are shown in Table 1. The *n*-6 : *n*-3 PUFA ratio in the N6, N6 + N3 and N3 diets was 242, 10·0 and 0·16, respectively. The N6 + N3 diet was the control diet because it contained adequate levels of essential PUFA and the *n*-6 : *n*-3 PUFA ratio was 10:1, as recommended for human dietary intake (Simopoulos *et al.* 1999; Watkins *et al.* 2000) and within the range found in Western diet. Compared with the control N6 + N3 diet, the N6 diet was almost devoid of LC *n*-3 PUFA, whereas the N3 diet contained a higher level of *n*-3 LC PUFA. In order to prevent essential fatty acid deficiency, 1 % corn oil was added to the N3 diet. All diets were isocaloric and isonitrogenous. Fresh diets were prepared every 14 d and kept at −20°C until fed to the rats.

Experimental design

Twenty-eight male F344 × BNF1 rats (12 months old, average weight 492 ± 15·6 g), obtained from the National Institute on Aging, Bethesda, MD, USA, were randomized into four groups

Table 1. Fatty acid and ingredient composition of the NIH-31 diet and dietary treatments

		Dietary treatment‡§		
Fatty acid	NIH-31† Mean	N6 + N3 diet Mean	N6 diet Mean	N3 diet Mean
		g/100 g total fatty acids		
14 : 0 (myristic)	0·62	1·24	0·15	6·70
16 : 0 (palmitic)	14·48	8·24	6·41	17·38
16 : 1*n*-7 (palmitoleic)	1·34	0·08	0·09	ND
18 : 0 (stearic)	3·42	2·56	2·39	3·39
18 : 1*n*-9 (oleic)	25·06	14·80	15·93	9·18
18 : 1*n*-7 (vaccenic)	1·37	1·05	0·64	3·09
18 : 2*n*-6 (linoleic)	45·80	61·23	72·61	4·27
18 : 3*n*-3 (α-linolenic)	4·27	0·38	0·16	1·48
18 : 4*n*-3 (stearidonic)	ND	0.54	ND	3.19
20 : 1*n*-9 (gondoic)	0·61	0·45	0·28	1·29
20 : 4*n*-6 (arachidonic)	0·26	0·14	ND	0·83
20 : 5*n*-3 (EPA)	ND	2·09	ND	12·57
22 : 5*n*-3 (docosapentaenoic)	0·22	0·42	ND	2·51
22 : 6*n*-3 (DHA)	0·94	2·67	0·14	15·28
SAT	19·37	12·84	9·63	28·81
Total MUFA	28·66	18·33	17·35	23·16
Total PUFA	51·94	67·60	72·91	40·83
n-6 PUFA	46·51	61·50	72·61	5·80
n-3 PUFA	5·43	6·10	0·30	35·03
n-6:*n*-3 PUFA	8·57	10·09	242	0·16

SAT, total saturated fatty acids; ND, not detected.

† All rats were maintained on NIH-31 diet at the animal laboratory facilities before being shipped to our laboratory. The NIH-31 diet contained the following (g/kg diet): protein, 184·20; fat, 44·7; fibre, 40·5; ash, 66·4; nitrogen-free extract, 559·1; moisture, 105·1.

‡ The semi-purified basal diet (modified AIN-93 diet) for experimental diets contained the following (g/kg diet): casein, 200·00; L-cystine, 3·00; sucrose, 100·00; cornstarch, 292·48; DYETROSE, 107·00; oil, 200·00; cellulose, 50·00; mineral mix, 35·00; vitamin mix, 10·00; choline bitartrate, 2·50; tert-Buthlhydroquinone, 0·02. The mineral mix contained (mg/kg diet): $CaCO_3$, 12495·00; K_2HPO_4, 6860·00; $C_6H_5O_7K_3{\cdot}H_2O$, 2477·00; NaCl, 2590·00; K_2SO_4, 1631·00; MgO, 840·00; $C_6H_5O_7Fe$ USP, 212·10; $ZnCO_3$, 57·75; $MnCO_3$, 22·05; $CuCO_3$, 10·50; KIO_3, 0·35; Na_2SeO_4, 0·369; $(NH_4)_2MoO_4{\cdot}H_2O$, 0·278; $Na_2O_3Si{\cdot}9H_2O$, 50·75; $CrK(SO_4)_2{\cdot}12H_2O$. 9·625; LiCl, 0·609; H_3BO_3, 2·853; NaF, 2·223; $NiCO_3$, 1·113; NH_4VO_3, 0·231. The vitamin mix contained (mg/kg diet): thiamine HCl, 6·00; riboflavin, 6·00; pyridoxine HCl, 7·00; niacin, 30·00; calcium pantothenate, 16·00; folic acid, 2·00; biotin, 0·20; cyanocobalamine (B-12) (0·1 %), 25·00; all-*trans*-retinyl palmitate (500, 000 IU/g), 8·00; all-*rac*-α-tocopheral acetate (500 IU/g), 25·00; cholecalciferol (400,000 IU/g), 2·50; phylloquinone, 0·75.

§ Dietary lipid contents (per kg): safflower oil 167 g + menhaden oil 33 g (N6 + N3 diet); safflower oil 200 g (N6 diet); menhaden oil 190 g + corn oil 10 g (N3 diet).

of seven rats each. The rats were assigned to the baseline and three dietary treatment (N6 + N3, N6, N3) groups. Rats in the baseline group were killed on day 0 of dietary intervention, and serum and bone samples were collected for later analysis. Values from the baseline group were compared with the results obtained at the end of the 20-week feeding period to determine any age effect on the parameters being studied. Rats in each dietary treatment group were fed their respective diets for 20 weeks. Rats were housed individually under a controlled temperature of 21 ± 2°C with a 12 h light–dark cycle. Feed and distilled water were provided *at libitum*. Rats were weighed every other week and examined daily. All procedures were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

Prior to the rats being killed, 72 h urine samples were collected from each animal and stored at − 20°C until analysed. All animals were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Blood samples were drawn from the heart into Vacutainer tubes (BD, Franklin Lakes, NJ, USA), serum being isolated and immediately stored at − 80°C for biochemical analysis. Final body weights were recorded, and the femora, tibias and humeri were harvested and cleaned of adhering soft tissue. The left tibial samples were kept on ice at the time of collection and then frozen at − 80°C for fatty acid analysis. The right humeri were processed for determination of *ex vivo* prostaglandin E_2 (PGE_2) and NO production (see later). The left femur samples were preserved in 70 % v/v ethanol for determination of BMD.

Analysis of fatty acid composition

Lipids in the diets and bone samples were extracted as described previously (Watkins *et al.* 1996). Fatty acid concentrations in the diets and bones were measured using a gas chromatograph, and data were expressed as the area percentage of fatty acid methyl esters in the lipids, as previously described (Watkins *et al.* 2000).

Ex vivo *production of prostaglandin E_2 and NO*

Ex vivo PGE_2 production in bone organ cultures was performed as described by Watkins *et al.* (2000). PGE_2 and NO concentrations in the bone organ culture medium were measured by RIA and Griess assay, respectively, as described by Shen *et al.* (2004). PGE_2 and NO values were expressed as nanograms and nanomoles per gram of dry bone weight, respectively.

Serum insulin-like growth factor-I, parathyroid hormone, 25-OH vitamin D_2 and 1,25-$(OH)_2$ vitamin D_3 levels

Serum IGF-I concentration was measured using the IGF-I (Direct) RIA kit (ALPCO Diagnostics, Windham, NH, USA). Serum parathyroid hormone (PTH) concentration was measured using the rat bioactive intact PTH ELISA kit (ALPCO Diagnostics). The intra- and interassay CV for PTH were 9·4 and 4·3 %, respectively. Concentrations of 25-OH vitamin D_2 and 1,25-$(OH)_2$ vitamin D_3 in serum were measured using a radioreceptor-binding ELISA (DiaSorin Inc. Stillwater, MN, USA). The intra- and interassay CV for 25-OH vitamin D_2 were 7·0 and 4·1 %, and for 1,25-$(OH)_2$ vitamin D_3 were 17·3 and 8·5 %, respectively.

Serum bone-specific alkaline phosphatase, pyridinoline, Ca and phosphate levels

Serum bone-specific alkaline phosphatase (BALP) activity was measured using a semi-automated quantitative assay as previously described (Hoffmann *et al.* 1994). The concentration of serum pyridinoline (PYD), a product of the breakdown of bone and cartilage collagen, was measured using the Metra Serum PYD ELISA (Quidel, San Diego, CA, USA). The intra- and interassay CV for PYD were 8·3 and 8·7 %, respectively. Serum and urinary Ca, inorganic phosphate (Pi) and creatinine concentrations were measured by an automated clinical chemistry analyser (Model RxL; Dade Behring, Deerfield, IL, USA). Data for urinary Ca and Pi concentrations were expressed as mg/mg creatinine.

Bone mineral density

The total bone area, bone mineral content (BMC) and BMD of the whole left femur of each rat were determined by dual-energy X-ray absorptiometry (DEXA; HOLOGIC QDR-2000 plus DEXA; Hologic Inc., Waltham, MA, USA). The machine was set at an ultra-high-resolution mode with a line spacing of 0·0254 cm, a resolution of 0·0127 cm and a collimator diameter of 0·9 cm. The total, trabecular, cortical, and subcortical regions of the excised metaphyseal distal femurs of rats were also scanned by a peripheral quantitative computerized tomography X-ray machine (STRATEC XCT-960, Norland Medical Systems, Fort Atkinson, WI, USA).

Statistical analysis

Data are presented as means with their standard errors. All data were analysed with SigmaStat software (version 2.03; Systat Software Inc., Richmond, CA, USA). Differences between baseline and each dietary treatment group were analysed by student's *t* test to determine age effect ($\alpha = 0{\cdot}05$). The differences between the three dietary treatment groups (N6 + N3, N6, N3) were analysed by one-way ANOVA followed by Tukey's test to determine the effect of fat type ($\alpha = 0{\cdot}05$).

Results

Diet and bone fatty acid analysis

Body weight and food consumption were not affected by the dietary treatments. The *n*-6:*n*-3 PUFA ratio was calculated from the analysed fatty acid values of the formulated dietary treatments (Table 1). The *n*-6:*n*-3 PUFA ratio ranged from 0·16 to 242. The N6 diet had the highest ratio (242) and contained 72·61 g of 18:2*n*-6 (linoleic acid) per 100 g total fatty acids. The N3 diet had the lowest ratio (0·16) and contained 12·6 g EPA, 2·51 g docosapentaenoic acid and 15·3 g DHA per 100 g total fatty acids.

The fatty acid composition of the cortical bone of middle-aged male rats was significantly influenced by the dietary PUFA treatment compared with the baseline group (Table 2). Significant differences in fatty acid profiles were also observed between the treatment groups. Relative to the control N6 + N3 group, rats fed the N6 diet (almost devoid of LC *n*-3 PUFA) had higher values for 18 : 2*n*-6, 20 : 4*n*-6, total PUFA and total *n*-6 PUFA, but lower values for 14 : 0, 16 : 0, 18 : 1*n*-7, 20 : 5*n*-3, 22 : 5*n*-3, 22 : 6*n*-3, total saturated fatty acids and total *n*-3 PUFA in cortical bone. In contrast, rats fed the N3 diet (high in LC *n*-3 PUFA) had

Table 2. Fatty acid composition in cortical bone of gonad-intact middle-aged male rats
(Mean values with their standard errors, *n* 7)

			Dietary treatment†					
	Baseline		N6 + N3		N6		N3	
Fatty acid	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
			g/100 g total fatty acids					
14 : 0 (myristic)	1·04	0·003	1·40	0·02[b*]	0·70	0·01[c*]	3·67	0·09†[a*]
16 : 0 (palmitic)	19·7	0·002	16·90	0·16[b*]	14·2	0·27[c*]	23·03	0·50[a*]
16 : 1*n*-7 (palmitoleic)	1·72	0·003	1·93	0·27[b]	1·08	0·07[b]	6·12	0·39[a*]
18 : 0 (stearic)	5·86	0·01	4·88	0·15*	4·78	0·10*	5·48	0·29
18 : 1*n*-9 (oleic)	22·3	0·15	17·7	0·32*	18·3	0·16*	18·21	0·44*
18 : 1*n*-7 (vaccenic)	0·77	0·21	3·12	0·06[b*]	2·57	0·07[c*]	4·86	0·07[a]
18 : 2*n*-6 (linoleic)	32·77	0·06	41·98	0·77[b*]	49·89	0·46[a*]	15·43	0·59[c*]
20 : 4*n*-6 (arachidonic)	2·64	0·01	1·54	0·04[b*]	2·44	0·40[a]	1·57	0·09†[b*]
20 : 5*n*-3 (eicosapentaenoic)	0·14	0·07	0·52	0·02[b*]	0·02	0·01[c]	2·32	0·16[a*]
22 : 5*n*-3 (docosapentaenoic)	1·20	0·03	1·28	0·04[b]	0·20	0·02[c*]	2·47	0·07[a*]
22 : 6*n*-3 (docosahexaenoic)	1·20	0·07	2·76	0·12[b*]	0·42	0·04[c*]	8·58	0·23[a*]
SAT	27·52	0·38	23·88	0·30[b*]	20·38	0·38[c*]	33·26	0·84[a*]
Total MUFA	29·61	0·34	23·52	0·50[b*]	22·60	0·24[b*]	30·11	0·81[a]
Total PUFA	41·30	0·20	49·85	0·56[b*]	56·05	0·27[a*]	32·49	0·31[c*]
n-6 PUFA	37·72	0·20	45·06	0·58[b*]	55·25	0·30[a*]	18·25	0·58[c*]
n-3 PUFA	3·58	0·10	4·87	0·16[b*]	0·80	0·07†[c]	14·28	0·44[a*]
n-6:*n*-3 PUFA	10·58	0·28	9·31	0·34[b]	72·48	7·84[a*]	1·29	0·08[c*]

N6 + N3, safflower oil + menhaden oil diet; N6, safflower oil diet; N3, menhaden oil diet; SAT, total saturated fatty acids.
[a,b,c] Mean values within a row with unlike superscript letters differ significantly between three dietary treatments by one-way ANOVA followed by Tukey's test ($P < 0·05$).
* Mean values within a row indicated the difference between baseline group and dietary treatment groups by Student's *t* test ($P < 0·05$).
† For details of diets, see Table 1.

higher values for 14:0, 16:0, 16:1n-7, 18:1*n*-7, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3, total saturated fatty acids, total MUFA and total *n*-3 PUFA, but lower values for 18:2*n*-6 and total *n*-6 PUFA in cortical bone compared with those fed the control diet (N6 + N3 diet).

Local factors and systemic calcitrophic hormones

Compared with the baseline group: the N6 + N3 group had a lower value for PGE_2 production but no difference in NO production and serum IGF-I concentration; the N6 group showed no difference in PGE_2, NO or IGF-I; the N3 group had lower levels of PGE_2 and NO production, but a higher value for serum IGF-I concentration (Table 3). Compared with the rats fed the control N6 + N3 diet, rats fed the N6 diet had a significantly higher PGE_2 level, whereas those fed the N3 diet had lower NO production in bone culture but higher IGF-I concentration in serum.

Compared with the baseline group, all dietary treatment groups had significantly higher concentrations of calcitrophic hormones, including PTH, 25-OH vitamin D_2 and 1,25-$(OH)_2$ vitamin D_3. The N3 group had significantly higher concentrations of serum systemic calcitrophic hormones than the N6 + N3 control group (Table 3).

Serum bone biomarkers

There were no differences in serum bone turnover biomarkers (BALP, PYD) between the N6 + N3 and baseline group (Table 3). The rats fed the N6 diet had a higher serum PYD concentration but the same serum BALP activity compared with those in the baseline group. Rats fed the N3 diet showed significantly higher BALP activity but no difference in serum PYD compared with baseline. When compared with the N6 + N3 control group, the N6 group had a higher PYD concentration, whereas the N3 group had higher BALP activity.

Serum and urinary Ca and phosphate

Compared to the baseline group: all dietary treatment groups had higher urinary Pi values; the N6 and N3 groups had a higher serum Pi; the N6 + N3 and N6 groups had a higher urinary Ca (Table 3). Compared with the N6 + N3 control group, the N6 and N3 groups had a significantly higher level of serum Pi. The N3 group had higher serum Pi but lower urinary Ca and Pi compared with the N6 + N3 control group.

Bone mineral density

There were no differences in the DEXA total area of the femoral bone region between the baseline group and the dietary treatment groups (Table 4). Both the dietary treatment groups, however, had significantly lower values for whole-femur BMD (as measured by DEXA), trabecular and cortical + subcortical bone densities (as measured by peripheral quantitative computerized tomography), compared with the baseline group. There was no difference in BMC between the baseline and the N3 groups, however; both the N6 + N3 control and N6 groups had a lower BMC compared with baseline. Moreover, rats fed the N3 diet had significantly higher BMC and cortical + subcortical bone density values than those fed the control N6 + N3 diet. There was no difference in any aspect of BMD between the N6 + N3 control group and the N6 group.

Table 3. Effect of different lipid treatments on local factors, systemic hormones and bone turnover biomarkers in gonad-intact middle-aged male rats

(Mean values with their standard errors, *n* 7)

			Dietary treatment†					
	Baseline		N6 + N3		N6		N3	
Experimental groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Local factor (bone organ culture)								
PGE_2 (ng/g bone dry weight)	41·7	5·4	8·1	1·7^{*b}	27·9	5·6^{a}	7·3	3·4^{*b}
NO (nmol/g bone dry weight)	30·0	4·8	24·8	3·7^{a}	23·2	4·1^{a}	13·2	2·6^{*b}
Systemic hormones (serum)								
IGF-I (ng/ml)	736	39	658	40^{b*}	727	47^{b*}	938	69^{a*}
PTH (pg/ml)	279	49	1024	55^{b*}	848	85^{b*}	1334	45^{a*}
25-(OH) vitamin D_2 (pg/ml)	27·8	0·6	19·2	0·2^{b*}	18·7	0·4^{b*}	31·5	0·9^{a*}
1,25-$(OH)_2$ vitamin D_3 (pg/ml)	28·8	2·3	127·9	9·3^{b*}	134·8	8·9^{a,b*}	176·0	19·5^{a*}
Serum bone turnover biomarker								
BALP (units/l)	67·1	3·4	69·8	5·4^{b}	74·2	6·1^{b}	122·8	6·2^{a*}
PYD (nmol/l)	4·6	0·2	5·1	0·7^{b}	8·3	1·1^{a*}	5·1	0·5^{b}
Others								
Serum Ca (mg/dl)	8·5	0·03	9·0	0·07*	9·1	0·05*	9·1	0·07*
Serum Pi (mg/dl)	4·7	0·3	5·3	0·1^{b}	6·5	0·3^{a*}	7·3	0·2^{a*}
Urinary Ca (mg/mg creatinine)	0·08	0·003	0·13	0·013^{a*}	0·13	0·014^{a*}	0·07	0·005^{b}
Urinary Pi (mg/mg creatinine)	0·06	0·01	0·84	0·03^{a*}	0·84	0·03^{a*}	0·36	0·02^{b*}

N6 + N3, safflower oil + menhaden oil diet; N6, safflower oil diet; N3, menhaden oil diet; PGE_2, prostaglandin E_2; IGF-I, insulin-like growth factor-I; PTH, parathyroid hormone; BALP, bone-specific alkaline phosphatase; PYD, pyridinoline; Pi, inorganic phosphate.

[a,b] Mean values within a row with unlike superscript letters differ significantly among three dietary treatments by one-way ANOVA followed by Tukey's test ($P < 0·05$).

* Mean values within a row indicated the difference between baseline group and dietary treatment groups by Student's *t* test ($P < 0.05$).

† For details of diets, see Table 1.

Discussion

In the present investigation, a model of gonad-intact, middle-aged male rats was successfully used to study the relationships between dietary PUFA treatment and bone metabolism associated with ageing-induced bone loss. Compared with the 12-month-old baseline group, all dietary treatment groups fed the high-fat diets for 20 weeks had lower values for femur BMC (15·3, 13·6 and 5·7 % for the N6, control N6 + N3 and N3 groups, respectively). This decrease could be due to ageing and/or the high fat content of the diets. The ageing effect on BMC has been reported by Wang *et al.* (2001): gonad-intact 17-month-old Sprague-Dawley male rats had a lower value for femur neck BMC (5 %) than 12-month-old rats.

For the current study, all rats in the baseline and dietary treatment groups were purchased from the same vendor at the same time. After 20 weeks, all rats were of the same age but had been fed diets with different fats. Our results showed that: (1) bone fatty acid composition in the different groups reflected the effects of dietary treatments, as reported in previous studies (Li *et al.* 1999; Watkins *et al.* 2000, 2003), with results significantly different from the baseline; (2) there was no difference in the amount of food consumption between the three dietary groups; (3) there were significant differences in bone parameters in

Table 4. Effect of different lipid treatments on bone mineral density in gonad-intact, middle-aged male rats

(Mean values with their standard errors, *n* 7)

			Dietary treatment†					
	Baseline		N6 + N3		N6		N3	
Experimental groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
DEXA								
BMC (g)	0·59	0·01	0·51	0·01^{b*}	0·51	0·01^{b*}	0·56	0·02^{a}
Total area (cm^2)	2·30	0·03	2·47	0·06	2·32	0·05	2·36	0·04
BMD (g/cm^2)	0·259	0·003	0·219	0·003*	0·218	0·003*	0·227	0·004*
pQCT								
Total density (mg/cm^3)	776	13	610	13*	621	21*	653	5*
Trabecular bone density (mg/cm^3)	487	18	241	9*	259	16*	259	10*
C + S density (mg/cm^3)	1019	11	919	16^{b*}	920	23^{b*}	968	12^{a*}

N6 + N3, safflower oil + menhaden oil diet; N6, safflower oil diet; N3, menhaden oil diet; DEXA, dual-energy X-ray absorptiometry; BMC, bone mineral content; BMD, bone mineral density; pQCT, peripheral quantitative computerized tomography; C + S, cortical + subcortical;

[a,b] Mean values within a row with unlike superscript letters differed significantly between the three dietary treatments by one-way ANOVA followed by Tukey's test ($P < 0.05$).

* Mean values within a row indicated the difference between the baseline group and dietary treatment groups by Student's *t* test ($P < 0.05$).

† For details of diets, see Table 1.

those fed with N6 or N3 diets compared with the control N6 + N3 diet. Therefore, the relative difference in bone loss should be mainly due to the variation in the dietary fat source. The fatty acid and methyl ester analysis of bone shown in Table 2 indicates that rats fed the N3 diet had significantly higher (threefold) amounts of *n*-3 PUFA and a reduction in *n*-6 PFUA of 50 % (in linoleate of 64 %) compared with those fed the N6 + N3 diet.

Our data showed that BMC in the N3 group was not significantly different from that in the baseline group, but was higher than that in the control N6 + N3 and N6 groups, suggesting that diets rich in LC *n*-3 PUFA resulted in less bone loss in middle-aged male rats during bone remodelling. Such a bone-mass conservation by LC *n*-3 PUFA agrees with that reported for ovariectomy-induced osteopenia in female rats (Watkins *et al.* 2003, 2005) and mice (Sun *et al.* 2003).

It was noted that the safflower oil used in this study contained a small amount of cholecalciferol (vitamin D_3; 5 μg vitamin D_3 per 100 g oil), whereas menhaden oil contained 10 μg vitamin D_3 per 100 g oil (Staffas & Nyman, 2003). In addition to the vitamin D_3 present in the menhaden oil, 25 μg cholecalciferol per kilogram of diet was added as a supplement to all dietary treatments. As a result, the amounts of cholecalciferol in the N3, N6 + N3 and N6 diets were 44·5, 36·5 and 35 μg/kg diet, respectively. Because the amount of food consumption was the same in each group, the total intake of cholecalciferol in the N3 group was 27 % higher than that in the N6 group.

Higher values for PTH (57 %), 25-OH vitamin D_2 (68 %) and 1,25-$(OH)_2$ vitamin D_3 (31 %) in the N3 group could be due to a different dietary fat and/or vitamin D_3 level in the menhaden oil. Such a small increase in vitamin D_3 in the N3 diet might, at least partially, have favoured bone remodelling during ageing. Treatments with vitamin D_3 and vitamin D analogues have been demonstrated to reduce bone loss in ovariectomized female rats (Shiraishi *et al.* 1999) and aged male rats (Li *et al.* 2004). Compared with the rats fed safflower oil in the N6 diet (almost devoid of LC *n*-3 PUFA), the N3 diet (rich in LC *n*-3 PUFA) favoured bone conservation in rats through a reduction in local catabolic factors such as PGE_2 (Spencer *et al.* 1991, Li *et al.* 1999; Watkins *et al.* 2000) and NO (MacIntyre *et al.* 1991; Ralston *et al.* 1995; van't Hof *et al.* 2000; Gyurko *et al.* 2005), and increased circulating anabolic factors such as IGF-I (Spencer *et al.* 1991; Ammann *et al.* 1996) and BALP (Watkins *et al.* 2000), as evidenced by favourable changes in terms of lowered serum PYD (Kelly *et al.* 2003; Watkins *et al.* 2003) and urinary Ca (Buck *et al.* 1991; Claassen *et al.* 1995) levels. In addition, a recent study reported by Weatherill *et al.* (2005) provided some evidence that dietary LC *n*-3 and *n*-6 PUFA may also modulate bone formation and resorption via regulating T-cell function and the production of cytokines such as IL-1, IL-6 and transforming necrosis factor α. Future studies are needed to evaluate such possible mechanisms.

The dietary *n*-6:*n*-3 PUFA ratio plays an important role in bone metabolism (Watkins *et al.* 2000; Weiler & Kruger, 2004; Weiss *et al.* 2005). Although our results showed no difference in the BALP and BMD data between the N6 + N3 and N6 groups, in the present study supplementation of the control N6 + N3 diet with LC *n*-3 PUFA significantly decreased the bone *ex vivo* PGE_2 production and serum PYD concentration of rats compared with those in the N6 diet (almost devoid of LC *n*-3 PUFA), indicating a lower resorption rate in rats fed the N6 + N3 diet.

Despite the differences between gonad-intact, middle-aged male rats and middle-aged men in terms of the impact of fish-oil supplementation on bone metabolism, we believe that our study presents a critical first step towards assessing the effects of high fish oil consumption on skeletal metabolism in middle-aged men. Our data have demonstrated that a diet rich in LC *n*-3 PUFA, antagonistic to arachidonic acid in terms of prostanoid action, mitigated ageing-induced bone loss in intact, middle-aged male rats during skeletal remodelling. This suggests that dietary supplementation rich in LC *n*-3 PUFA might contribute to the maintenance of a healthy skeletal system in middle-aged men. Future investigations should test the potential for a protective effect of a high LC *n*-3 PUFA diet on bone structure and mechanical properties to further our understanding of the role of lipid nutrition in skeletal health and the prevention of pathological bone loss (osteoporosis) during male ageing.

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