The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats

Owen Kelly¹, Siobhan Cusack¹, Christopher Jewell¹ and Kevin D. Cashman¹,²*

¹Department of Food and Nutritional Sciences and
²Department of Medicine, University College, Cork, Republic of Ireland

(Received 14 October 2002 – Revised 13 May 2003 – Accepted 2 June 2003)

The effect of polyunsaturated fatty acids (PUFAs), in particular conjugated linoleic acid (CLA), on Ca and bone metabolism is unclear. In a 2 x 2 factorial design study, forty male 4-week-old rats were fed a control diet containing 70 g added fat (soyabean oil (SBO; n-6 PUFA-rich diet) or menhaden oil–safflower oil (MSO; n-3 PUFA-rich diet))/kg diet with 0 or 10 g CLA/kg for 8 weeks. Ex vivo prostaglandin E₂ biosynthesis by bone organ culture was significantly higher (P<0.001) in rats consuming SBO compared with MSO, irrespective of CLA. Addition of the CLA treatment to either diet further lowered (P<0.05) ex vivo prostaglandin E₂ production. Neither PUFA type nor CLA altered circulating or femoral mRNA levels of osteocalcin (a marker of bone formation) or insulin-like growth factor-I (a mediator of bone metabolism). While urinary pyridinium crosslinks levels (markers of bone resorption) were unaffected by CLA irrespective of PUFA type, they were significantly higher (P<0.05) in rats consuming SBO compared with MSO irrespective of CLA. Net fractional (%) and absolute (mg) Ca absorption were significantly (P<0.01 and P<0.05 respectively) higher in CLA-supplemented than unsupplemented animals fed on the n-3 PUFA-rich diet, whereas CLA had no effect in animals fed the n-6 PUFA-rich diet. There was no effect of CLA supplementation on bone mineral mass. In conclusion, CLA supplementation over 8 weeks appeared to enhance Ca absorption in young growing rats fed an n-3 PUFA-rich diet, but had no measurable effect on bone metabolism or bone mass over this time frame.

Calcium absorption: Bone metabolism: Conjugated linoleic acid: Polyunsaturated fatty acid

While dietary factors, such as Ca, Mg, Na, Cu, non-digestible oligosaccharides and vitamins D and K, amongst others, have attracted considerable attention (Cashman, 2002), the influence of dietary lipids on Ca metabolism and bone health has received much less research emphasis (Kruger & Horrobin, 1997). There are some animal data to suggest that supplementation of the diet with γ-linoleic acid (18 : 3n-6)–eicosapentaenoic acid (20 : 5n-3) (3:1, w/w) may promote intestinal Ca absorption, Ca balance and bone Ca content as well as reducing urinary pyridinium excretion (marker of bone resorption) in experimental rats, relative to control animals supplemented with linoleic acid (18 : 2n-6)–α-linolenic acid (18 : 3n-3) (3:1, w/w) (Claassen et al. 1995a,b). These benefits to Ca absorption and bone were not evident in the lower γ-linoleic acid:eicosapentaenoic acid ratio groups (Claassen et al. 1995b), suggesting the n-6:n-3 fatty acid ratio may be important. These observations have been supported by the results of a controlled pilot study in elderly women (mean age 79·5 years) with senile osteoporosis: these suggest that γ-linoleic acid and eicosapentaenoic acid (together with Ca) have beneficial effects on bone turnover as well as on the bone mineral density of the lumbar spine and femur (Kruger et al. 1998). On the other hand, van Dokkum et al. (1983) showed that increasing the linoleic acid level in the diet of young men participating in a mineral balance study significantly reduced faecal Ca, indicating stimulation of Ca absorption by n-6 essential fatty acids. Therefore, the effects of different bioactive fatty acids on Ca and bone metabolism, as well as their mechanisms of action, are unclear.

While much of the attention over the last 5 years has focused on the possible beneficial affects of conjugated linoleic acid (CLA), another potentially bioactive polyunsaturated fatty acid (PUFA), on body composition, lipoprotein metabolism, inflammation and carcinogenesis (for reviews, see Kritchevsky, 2000; Roche et al. 2001a), recently attention has focused on a possible beneficial effect of CLA on Ca absorption and bone health. Park et al. (1997) have reported that dietary supplementation

Abbreviations: CLA, conjugated linoleic acid; GAPDH, glyceraldehyde phosphate dehydrogenase; IGF, insulin-like growth factor; MSO, menhaden oil–safflower oil; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SBO, soyabean oil.

* Corresponding author: Professor Kevin D. Cashman, fax +353 21 4270244, email k.cashman@ucc.ie
with CLA in experimental mice led to a reduction in whole-body fat and an increase in body protein, water and ash; the increase in whole-body ash content suggests that CLA may enhance bone mineralization and protect against bone loss. This contention was supported by the finding that bone ash was higher in the tibia of CLA-fed chicks compared with control animals (Cook et al. 1997). The mechanism by which CLA may increase bone ash is unclear. Watkins et al. (1997) found that a dietary source of anhydrous butterfat (a rich natural source of CLA) stimulated the rate of bone formation in young growing chicks by modulating prostaglandin (PG) E2 production in bone. PGE2 plays an important role in the local regulation of bone formation and bone resorption (Marks & Miller, 1993). However, Li et al. (1999) recently reported that dietary CLA (10 g/kg diet for 8 weeks) actually led to lowered ex vivo PGE2 production in bone organ culture from young growing rats fed a diet high in either n-3 or n-6 PUFA. This decreased PGE2 biosynthesis may have led to the reduced rates of mineral deposition and bone formation in CLA-supplemented rats, even though bone mass and mineral content were unaffected by CLA supplementation. n-3 PUFA also lowered ex vivo PGE2 production compared with n-6 PUFA, an effect independent of CLA (Li et al. 1999). There may also be situations where CLA and n-3 and/or n-6 PUFA interact. For example, Li et al. (1999) found that CLA supplementation increased serum levels of a particular binding protein for insulin-like growth factor (IGF)-I (a mediator of bone metabolism), namely IGF-binding protein-3, in rats given a diet high in n-6 PUFA, but decreased it in rats fed a diet high in n-3 PUFA.

Recent research using the Caco-2 cell model (a useful in vitro model for predicting Ca absorption in human subjects; Fleet & Wood, 1999) also suggests that chronic (2–3-week) exposure of these cells to specific isomers of CLA can stimulate paracellular Ca transport (Jewell & Cashman, 2003a,b). In this way, therefore, CLA may indirectly influence bone mass by making more Ca available for calcification. However, to date, there has been no study of the effect of CLA on Ca absorption in vivo.

Therefore, the objective of the present study was to investigate the effect of PUFA type (n-3 v. n-6), CLA, and their possible interactions, on Ca absorption, bone metabolism (i.e. bone formation and bone resorption) and bone composition in young growing rats.

Materials and methods

Preparation of rat diets

The basal diet (AIN-93G (Reeves et al. 1993) without fat) contained one of the following lipid treatments: soyabean oil (a diet rich in n-6 PUFA; SBO) or menhaden oil–safflower oil (56:44, w/w), a diet rich in n-3 PUFA; MSO) at 70 g/kg diet with or without added CLA (Table 1). For diets containing CLA (SBO+CLA and MSO+CLA), 10 g dietary SBO or MSO/kg respectively were replaced with CLA (generously provided by Loders Croklaan B.V., Wormerveer, Holland). The SBO diet (AIN-93G) also served as a control diet since it contained all known nutrients for the growing rat as recommended by the American Institute of Nutrition (Reeves et al. 1993). A dietary level of 10 g CLA/kg diet was used in the present study, because this dietary level of CLA was shown to modulate bone formation in a previous study of young growing rats (Li et al. 1999). All diets were isoenergetic and isonitrogenous. Fresh diets were prepared every 14 d and kept at −20°C until fed.

Experimental design

Forty male weanling rats, 28-d-old, Wistar strain (average weight 47.3 g), obtained from the Biological Services Unit, University College, Cork, Republic of Ireland, were randomized by weight into four groups of ten rats each. The rats were assigned to four dietary groups (SBO, MSO, SBO+CLA and MSO+CLA) following a 2x2 factorial design. Rats were housed individually, feed was provided ad libitum at 17.00 hours each day and all animals were given distilled water ad libitum for the duration of the study. Rats were weighed weekly and examined daily for general condition.

During the last week of the study, all rats were placed in individual metabolism cages with a grid-floor and a facility for separate collection of faeces and urine. To acclimatize the animals to the new environment, rats were placed in these cages 2 d before the beginning of a 4 d metabolic
period for determination of net dietary Ca, Mg and P absorption and urinary pyridinium crosslink excretion. *Ad libitum* intake of diets was measured during a 2 d period to determine average food intake per group. To assure complete and equivalent consumption of all food offered during the 4 d metabolic period, an equalized feeding paradigm was used. In the present study, the amount of food offered to all rats during the 4 d balance period was limited to 90 % of the *ad libitum* food intake of the group that ate the least amount of food during the previous 2 d. Feed was provided at 09.00 hours each day over the period and any remaining feed at 09.00 hours the following day was weighed. Quantitative collections of faeces were made over the 4 d period and these were pooled for each rat and stored at −20°C until required for analysis.

During the metabolic period, urine samples (24 h) were collected for each animal in vessels that were covered with Al foil to prevent degradation of the pyridinium crosslinks by light. The urine samples for each animal were pooled and the volumes recorded. Portions of the pooled urine samples were acidified with 12 m-m-HCl (225 μl/100 ml urine) and stored at −20°C until required for analysis.

Net Ca, Mg and P absorption were calculated as the difference between mineral intake from the diet and mineral recovered in the faeces during the 4 d period.

After 56 d on their respective diets, all animals were anaesthetized with diethyl ether and blood was drawn from the heart into vacutainer tubes, processed to serum and immediately stored at −80°C until required. Final body weights were recorded, and femora and tibias were harvested and cleaned of adhering soft tissue. The distal epiphyses were removed from the left femora, and the metaphyseal–diaphyseal bone shafts were freed of bone marrow and blood. These were immediately placed in Al foil, then immersed in 20 ml Hank’s balanced salt solution (Sigma Chemical Co. Ltd) and incubated with shaking at 37°C for 2 h. After incubation, the bone culture medium was collected and PGE2 concentrations measured in duplicate using a recently developed ELISA (Amersham Pharmacia Biotech UK Limited, Amersham, Bucks., UK). The intra- and inter-assay CV were 4·2 and 5·2 % respectively. The accuracy of mineral analysis was assured in each analytical run by appropriate recovery of mineral in dry-ashed samples of National Institute of Standards and Technology-certified bone meal (standard reference material no. 1486; Laboratory of the Government Chemist, London, UK).

**Femoral mass**, **length**, **volume and density**. The length of each right femur was measured with a vernier caliper. Bone volume and density were measured by Archimedes’ principle as described by Doyle & Cashman (2003).

**Serum osteocalcin and insulin-like growth factor-I** levels. Serum osteocalcin concentrations were measured in duplicate using the Rat-Mid osteocalcin ELISA (Osteometer Biotech A/S, Osteopark, Herlev, Denmark). The intra- and inter-assay CV were 4·0 and 6·2 % respectively. Serum IGF-I concentrations were measured in duplicate using a recently developed ELISA (Biomedical Technologies Inc., Stoughton, MA, USA). The intra- and inter-assay CV were 3·2 and 5·2 % respectively.

**Ex vivo production of prostaglandin E2 by bone organ cultures**. *Ex vivo* PGE2 production in bone organ cultures was performed as described by Watkins et al. (1996, 1997). In brief, shafts from the right tibia, once removed, were carefully flushed with a 0·15 M-NaCl solution to remove marrow cells. A weighed section of bone shaft was immersed in 20 ml Hank’s balanced salt solution (Sigma Chemical Co. Ltd) and incubated with shaking for 2 h at 37°C. After incubation, the bone culture medium was collected and PGE2 concentrations measured in duplicate using a recently developed ELISA (Amersham Pharmacia Biotech UK Limited, Amersham, Bucks., UK). The intra- and inter-assay CV were 3·2 and 5·2 % respectively. The protein concentration of the bone culture medium was determined by using the method of Lowry et al. (1951) and the PGE2 values were expressed as ng/mg protein.

**Reverse transcription and quantitative polymerase chain reaction analysis for femoral insulin-like growth factor-I and osteocalcin mRNA**. RNA was isolated and analysed in the left femora from each rat within a group (n = 8) as described by Fleet & Hock (1994). Total RNA (1 μg) from each rat was made into cDNA by the reverse transcription reaction described by Fleet & Hock (1994). Primers sets for osteocalcin, IGF-I and glyceraldehyde phosphate dehydrogenase (GAPDH) were derived from previously published sequences (Fleet & Hock, 1994). Quantitative polymerase chain reaction analysis of the samples was performed using the LightCycler system and software package for the analysis of fluorescent data (Roche-Diagnostics, Mannheim, Germany), as described by Witter et al. (1997). The dilution series of normal
control cDNA served to provide a standard curve and allowed the quantification of the polymerase chain reaction-produced yield for all samples. Polymerase chain reaction mixtures contained 4 mM-MgCl$_2$ and 0·5 μM (IGF-I) or 1·0 μM (osteocalcin, GAPDH) of each primer in 20 μl volumes. Forty amplification cycles were performed, consisting of a denaturation step at 95°C, annealing at 50°C (GAPDH), 58°C (osteocalcin) or 64°C (IGF-I), and an elongation step at 72°C for 8 (GAPDH), 12 (osteocalcin) or 9 (IGF-I) s. A single fluorescence measurement was taken at the end of each elongation step. Identification of amplicons was performed by generation of a melting curve. The characteristic melting temperature for each amplicon was initially confirmed by agarose (2%) gel–ethidium bromide electrophoresis with visualization on a u.v. transilluminator. All data for osteocalcin and IGF-I gene expression were normalized to the expression of GAPDH, a ubiquitously expressed housekeeping gene, in each animal.

Statistical methods

Data for all variables were normally distributed (as determined by the method of Kolmogorov and Smirnov) and were of equal variances (as determined by Bartlett’s test) and thus allowed for parametric tests of significance. Results are presented as mean values with their pooled standard errors. Data were subjected to two-way ANOVA, with variation attributed to PUFA type and CLA (Snedecor & Cochran, 1967). To follow up ANOVA, all pairs of mean values were compared by the method of least significant difference (Snedecor & Cochran, 1967). The effects of CLA supplementation and dietary PUFA type on physical properties and macromineral content of femora are shown in Table 2. Femoral length, ash weight, proportion of ash and femoral density were unaffected by either dietary PUFA type or CLA supplementation. Femur dry weight and bone mineral mass tended to be lower ($P=0·075$ and $P=0·058$ respectively) in rats fed SBO (n-6 PUFA-rich diet) compared with those fed MSO (n-3 PUFA-rich diet), irrespective of CLA. There was no effect of dietary PUFA type or CLA supplementation on the concentration or content of femoral Ca, Mg and P (Table 2).

Results

Mean body-weight gain did not differ among groups (results not shown). The effects of CLA supplementation and dietary PUFA type on physical properties and macromineral content of femora are shown in Table 2. Femoral

<table>
<thead>
<tr>
<th>PUFA type...</th>
<th>CLA supplementation...</th>
<th>SBO</th>
<th>- CLA</th>
<th>+ CLA</th>
<th>MSO</th>
<th>- CLA</th>
<th>+ CLA</th>
<th>Pooled SEM</th>
<th>Statistical significance of variance ratio (P), effects of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (mm)</td>
<td></td>
<td>28·13</td>
<td>28·23</td>
<td>28·21</td>
<td>27·99</td>
<td>0·02</td>
<td>0·593</td>
<td>0·727</td>
<td>0·456</td>
</tr>
<tr>
<td>Dry wt (mg)</td>
<td></td>
<td>363</td>
<td>366</td>
<td>375</td>
<td>378</td>
<td>8</td>
<td>0·075</td>
<td>0·705</td>
<td>0·949</td>
</tr>
<tr>
<td>Ash wt (mg)</td>
<td></td>
<td>0·24</td>
<td>0·24</td>
<td>0·25</td>
<td>0·25</td>
<td>0·01</td>
<td>0·134</td>
<td>0·414</td>
<td>0·835</td>
</tr>
<tr>
<td>Ash (g/kg dry wt)</td>
<td></td>
<td>66·1</td>
<td>66·1</td>
<td>66·0</td>
<td>67·1</td>
<td>5·4</td>
<td>0·743</td>
<td>0·445</td>
<td>0·534</td>
</tr>
<tr>
<td>Density (g/mm$^3$)</td>
<td></td>
<td>0·00143</td>
<td>0·00140</td>
<td>0·00143</td>
<td>0·00139</td>
<td>0·00002</td>
<td>0·620</td>
<td>0·174</td>
<td>0·872</td>
</tr>
<tr>
<td>Bone mineral mass (mg/mm)</td>
<td></td>
<td>8·57</td>
<td>8·62</td>
<td>8·61</td>
<td>9·05</td>
<td>0·17</td>
<td>0·058</td>
<td>0·422</td>
<td>0·581</td>
</tr>
<tr>
<td>Ca (mg/g dry wt)</td>
<td></td>
<td>27·4</td>
<td>28·3</td>
<td>294</td>
<td>281</td>
<td>9</td>
<td>0·391</td>
<td>0·848</td>
<td>0·118</td>
</tr>
<tr>
<td>Mg (mg/g dry wt)</td>
<td></td>
<td>4·23</td>
<td>4·39</td>
<td>4·20</td>
<td>4·29</td>
<td>0·15</td>
<td>0·652</td>
<td>0·413</td>
<td>0·792</td>
</tr>
<tr>
<td>P (mg/g dry wt)</td>
<td></td>
<td>114</td>
<td>113</td>
<td>114</td>
<td>113</td>
<td>1</td>
<td>0·938</td>
<td>0·439</td>
<td>0·754</td>
</tr>
<tr>
<td>Ca (mg/bone)</td>
<td></td>
<td>99</td>
<td>103</td>
<td>108</td>
<td>106</td>
<td>3</td>
<td>0·112</td>
<td>0·728</td>
<td>0·359</td>
</tr>
<tr>
<td>Mg (mg/bone)</td>
<td></td>
<td>1·53</td>
<td>1·61</td>
<td>1·58</td>
<td>1·62</td>
<td>0·06</td>
<td>0·611</td>
<td>0·299</td>
<td>0·763</td>
</tr>
<tr>
<td>P (mg/bone)</td>
<td></td>
<td>41·4</td>
<td>41·3</td>
<td>42·8</td>
<td>42·8</td>
<td>0·8</td>
<td>0·086</td>
<td>0·957</td>
<td>0·897</td>
</tr>
</tbody>
</table>

PUFA, polyunsaturated fatty acid; SBO, soyabean oil; MSO, menhaden oil–safflower oil; CLA, conjugated linoleic acid.

* For details of diets and procedures, see Table 1 and p. 744.
unaffected by PUFA type or CLA supplementation. Similarly, net absolute P absorption was unaffected by PUFA type or CLA supplementation.

**Discussion**

The findings of the present study suggest that while CLA had no effect on net absolute Mg and P absorption, it may enhance Ca absorption in rats fed a diet rich in n-3 PUFA. This stimulatory effect of CLA on Ca absorption was not observed in rats fed a diet rich in n-6 PUFA. This is the first study, to our knowledge, that has investigated the effect of CLA on Ca absorption in *vivo*. This stimulatory effect of CLA on Ca absorption in *vivo*, however, supports the findings from recent *in vitro* studies that showed that specific isomers of CLA significantly increased paracellular Ca transport in Caco-2 cells (Jewell & Cashman, 2003a,b). Paracellular Ca transport is generally thought to be the predominant route of intestinal Ca absorption *in vivo* when Ca intake is adequate to high (Bronner, 1998). The mechanism by which CLA enhanced Ca absorption is unclear. Roche et al. (2001b) suggested that the effect of CLA on paracellular epithelial permeability might arise due to an alteration in the cellular distribution of occludin (an integral structural protein component of the tight junction between neighbouring intestinal cells). It is also unclear as to why CLA only promoted Ca absorption in a diet rich in n-3 and not n-6 PUFA. IGF-I, an important paracrine and autocrine regulatory polypeptide of many cells, is believed to play a role in intestinal Ca absorption (Fleet et al. 1994; Fatayerji et al. 2000). While circulating IGF-I levels were unaffected by either PUFA or CLA in the present study, IGF-binding protein-3 (although not measured) may have been differentially modulated by an interaction between CLA and n-3 and/or n-6 PUFA. For example, Li et al. (1999) found that CLA supplementation increased serum IGF-binding protein-3 levels in rats given a diet high in n-6 PUFA, but decreased it in rats fed a diet high in n-3 PUFA. A reduction in IGF-binding protein-3 in the n-3 PUFA+CLA-supplemented rats may have increased the biological activity of IGF-I and in that way may have increased Ca absorption.

The additional Ca absorbed by CLA-supplemented rats fed on an n-3 PUFA-rich diet would be of little value unless it was retained by these animals. Unfortunately, the retention of Ca could not be estimated in the present study, because of contamination of urine samples with spill food. However, fish oil, rich in n-3 fatty acids, has been shown to decrease urinary Ca excretion in animals by a down-regulation of PGE$_2$ (Buck et al. 1991; Claassen et al. 1995a). Therefore, it is unlikely that the additional Ca absorbed by rats fed the diet rich in n-3 fatty acids and supplemented with CLA was excreted in urine. Rather, Ca retention may have been improved by the CLA-induced down-regulation of PGE$_2$ levels in these MSO+CLA-fed rats, relative to those receiving the unsupplemented MSO. However, this would need to be confirmed.

### Table 3. The effect of different lipid treatments on urinary pyridinoline (Pyr) and deoxypyridinoline (Dpyr) concentrations and serum osteocalcin and insulin-like growth factor (IGF)-I in young male rats

<table>
<thead>
<tr>
<th>PUFA type and CLA supplementation</th>
<th>SBO</th>
<th>MSO</th>
<th>Statistical significance of variance ratio ($P$), effects of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− CLA</td>
<td>+ CLA</td>
<td>− CLA</td>
</tr>
<tr>
<td>Urine:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyr (nmol/mmol Cr)</td>
<td>110</td>
<td>95</td>
<td>82</td>
</tr>
<tr>
<td>Dpyr (nmol/mmol Cr)</td>
<td>194</td>
<td>176</td>
<td>155</td>
</tr>
<tr>
<td>Serum:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>42·6</td>
<td>45·4</td>
<td>41·2</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>336</td>
<td>350</td>
<td>358</td>
</tr>
</tbody>
</table>

PUFA, polyunsaturated fatty acid; SBO, soyabean oil; MSO, menhaden oil–safflower oil; CLA, conjugated linoleic acid; Cr, creatinine.

* For details of diets and procedures, see Table 1 and p. 744.
Table 4. The effect of different lipid treatments on food intake and apparent absorption of calcium, phosphorus and magnesium in young male rats

<table>
<thead>
<tr>
<th>PUFA type…</th>
<th>SBO</th>
<th>MSO</th>
<th>Statistical significance of variance ratio (P), effects of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA supplementation…</td>
<td>− CLA</td>
<td>+ CLA</td>
<td></td>
</tr>
<tr>
<td>Food intake (g/4 d)†</td>
<td>73·4</td>
<td>69·7</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>38·9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39·9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>133&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Net Mg absorption:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>54·1</td>
<td>57·3</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>14·9</td>
<td>14·8</td>
<td></td>
</tr>
<tr>
<td>Net P absorption:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>84·6</td>
<td>84·6</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>2062</td>
<td>2030</td>
<td></td>
</tr>
</tbody>
</table>

PUFA, polyunsaturated fatty acid; SBO, soyabean oil; MSO menhaden oil–safflower oil; CLA, conjugated linoleic acid.

<sup>a,b</sup>Mean values within a row with unlike superscript letters were significantly different (ANOVA followed by least significant difference test; P<0·05).

† Mean food consumption over the 4 d balance period (see p. 745).

especially as the increased Ca absorption in these rats was not translated into differences in bone mass or mineral content. Bone mass or mineral content were also unaffected by CLA supplementation of rats fed a diet high in n-6 PUFA. Therefore, while CLA supplementation did not appear to influence the physical properties or mineral content of bone over 8 weeks, Sinha et al. (1988a,b) has suggested that several months may be required to induce measurable changes in bone by dietary alteration.

There was a tendency for femoral dry weight and bone mineral mass to be lower in rats fed a diet high in n-6 fatty acids (SBO) compared with those fed a diet high in n-3 fatty acid (MSO). These findings are in agreement with those of Li et al. (1999), who reported similar findings in young growing male rats. The findings of both studies in young growing rats are also consistent with the results of a previous study that showed that chicks fed an n-6 PUFA-rich diet (SBO) for 21 d had reduced total bone and cortical bone areas in cross-sections of tibiae compared with chicks given menhaden oil (rich in n-3 PUFA) (Watkins et al. 1996). The reduced femoral dry weight and bone mineral mass in rats fed the n-6 PUFA-rich diet in the present study is consistent with the increased rate of bone resorption (as determined by the urinary pyridinium link, which is sensitive and specific markers; Black et al. 1989; Egger et al. 1994) in these animals, relative to the animals fed a diet rich in n-3 PUFA. In contrast, Claassen et al. (1995b) have shown that increasing the n-6 PUFA:n-3 PUFA (3:1, w/w) reduced urinary pyridinium crosslink excretion in young growing male rats. The n-6 and n-3 PUFA used by Claassen et al. (1995b), however, were from earlier primrose (Oenothera biennis) oil and fish oil respectively, whereas the n-6 and n-3 PUFA in the present study were from SBO and MSO respectively. Therefore, the particular bioactive fatty acids per se in these oils may be as important to bone health as the overall n-6 PUFA:n-3 PUFA ratio in the diet.

The mechanism by which the n-3 PUFA lowered the rate of bone resorption in the present study is unclear. However, rats receiving a diet high in n-3 fatty acids (MSO) had significantly lower ex vivo PGE<sub>2</sub> production in bone (tibia) organ culture compared with that in rats receiving a diet high in n-6 fatty acids (SBO). This finding is in close agreement with that of Li et al. (1999), who reported a similar response of ex vivo PGE<sub>2</sub> production in rat tibia and femur to PUFA type. PGE<sub>2</sub> is an important factor in the regulation of local bone metabolism, including bone resorption as well as bone formation (Raisz & Fall, 1990; Marks & Miller, 1993). Therefore, the n-3 PUFA may have lowered the rate of bone resorption as a consequence, at least in part, of their suppressive effect on PGE<sub>2</sub> biosynthesis.

Addition of the CLA treatment to either diet further lowered ex vivo PGE<sub>2</sub> production. Because CLA is incorporated into membrane phospholipids, it may compete with other PUFA in the formation of arachidonic acid (the precursor of PGE<sub>2</sub>) to inhibit PGE<sub>2</sub> biosynthesis (Li & Watkins, 1998). However, while CLA further reduced the biosynthesis of PGE<sub>2</sub>, particularly in the n-6 PUFA-rich group, it had no effect on the rate of bone resorption in either dietary PUFA group.

As already mentioned, PGE<sub>2</sub> is also an important regulatory factor in the rate of bone formation (Raisz & Fall, 1990). For example, reduced production of PGE<sub>2</sub> in chicks fed a diet high in n-3 PUFA was associated with an increased rate of bone formation (Xu et al. 1994; Watkins et al. 1996). However, in the present study as well as the study by Li et al. (1999), n-3 PUFA had no effect on the rate of bone formation in young growing rats, as assessed by serum osteocalcin (a biochemical marker of bone formation in rats (Creedon et al. 1999) used in both studies) or by histomorphometry (used by Li et al. 1999). Furthermore, while CLA reduced PGE<sub>2</sub> biosynthesis in bone, it had no effect on serum osteocalcin in either study. Of note, Li et al. (1999) reported a reduced rate of mineral apposition and bone formation (measured by histomorphometry) in tibia of CLA-supplemented animals when compared with unsupplemented animals.
irrespective of dietary PUFA type; the authors associated this with the inhibitory effect of CLA on ex vivo PGE2 production in bone of these rats. These findings might suggest that serum osteocalcin may lack sensitivity as a technique for detecting subtle dietary-induced changes in the rate of bone formation. However, femoral osteocalcin mRNA levels were also unaffected by CLA supplementation in the present study, suggesting no effect of CLA on osteocalcin synthesis at the transcriptional level.

PGE2 is known to be a potent regulator of IGF-I levels, at least in bone (McCarthy et al. 1991; Schmid et al. 1992). IGF-I is the most abundant growth factor in bone and is believed to function as both a systemic and local growth factor for bone tissue (Delany et al. 1994). In the present study, CLA had no effect on serum IGF-I levels in young growing rats, irrespective of PUFA type. This was despite the fact that CLA supplementation of these rats led to a reduction in PGE2 biosynthesis in bone. This is in contrast to the findings of Li et al. (1999), who showed that CLA reduced the circulating levels of IGF-I in growing rats. However, a reduction in PGE2 does not necessarily lead to a reduction in IGF-I levels. For example, in both the present study and that of Li et al. (1999), a diet high in n-3 PUFA had no effect on serum IGF-I in growing rats, despite markedly reducing ex vivo PGE2 production in bone, relative to that in rats fed a diet high in n-6 PUFA. There has also been some uncertainty as to whether changes in circulating concentrations of the IGF-I reflect local (bone tissue) concentrations, and the amount in bone tissue may be more important for bone formation (Rodan & Rodan, 1995). However, in the present study, IGF-I mRNA levels in femora were unaffected by CLA supplementation; this is consistent with the serum findings.

In conclusion, there have been significant changes over the past few decades in the fat composition of the food supply, in agricultural methods of food preparation and in the eating habits of industrialized societies (Watkins et al. 2001). The present dietary n-6:n-3 fatty acids ratio may be far from the optimal recommended intake of n-3 fatty acids to protect against chronic disease risk (Simopoulos et al. 1999). The findings of the present study in young growing rats suggest that a diet high in n-3 fatty acids may be beneficial for bone metabolism and mass. Furthermore, n-3 fatty acids appeared to interact with CLA in promoting intestinal Ca absorption, which, if sustained, would also benefit bone in the longer term. The mechanisms of action of these effects by bioactive fatty acids require further investigation.

The level of CLA used in the present study (10 g/kg diet), although higher than that found in conventional diets without supplementation, compares favourably with the range used in other animal studies (5–15 g/kg) that examined anti-inflammatory and anti-carcinogenic properties of CLA (Ip et al. 1991, 1996; Chin et al. 1994) as well as effects on bone (Li et al. 1999). However, further research is needed to evaluate more typical dietary levels (including levels achieved with consumption of CLA supplements, a practice that is gaining in popularity) on Ca and bone metabolism, preferably in studies with human subjects.

Acknowledgement

This work was supported by funding made available by the Irish Government under the National Development Plan 2000–2006.

References


Watkins BA, Shen CL, Allen KG & Seifert MF (1996) Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J Bone Miner Res 11, 1321–1332.


