

The effect of calcium intake on bone composition and bone resorption in the young growing rat

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A low Ca intake by both rats and man increases bone resorption, decreases bone mass and increases the risk of osteoporosis. The skeletal effect of high Ca intakes is less clear, particularly during periods of bone mineral accrual. Twenty-four female 5-week-old rats, Wistar strain, were randomized by weight into three groups of eight rats each and fed *ad libitum* a semi-purified diet containing 2 (Ca-restricted), 5 (normal) or 20 (Ca-supplemented) g Ca/kg for 3 weeks. When compared with the normal Ca diet, urinary Ca excretion was unaffected by the dietary restriction of Ca for 3 weeks, but was greater ($P<0.001$) in Ca-supplemented rats. Urinary pyridinoline (Pyr) and deoxypyridinoline (Dpyr) levels were significantly greater during weeks 2 (Pyr $P<0.05$, Dpyr $P<0.001$) and 3 (Pyr $P<0.01$, Dpyr, $P<0.001$) of dietary Ca restriction, but were unaffected by Ca supplementation. Femoral dry weight and the concentration of Mg and P in femora were unaffected by dietary Ca concentration. Femoral Ca concentration was reduced ($P<0.05$) in the Ca-restricted group compared with the other two groups. In conclusion, these results suggest that increasing dietary Ca intake, well above the recommended level, had no effect on bone mineral composition or bone resorption (as assessed with urinary pyridinium crosslinks) in young growing female rats. In addition, these results confirm the findings of previous studies which have shown that bone Ca content in young growing rats was reduced by dietary Ca restriction and that this reduction results, at least in part, from an increased rate of bone resorption.

Calcium: Pyridinium crosslinks: Bone: Rats

There is evidence that bone mass in early adult life (peak bone mass) is an important factor influencing bone mass and fracture susceptibility in later life (Hui *et al.* 1989; Melton *et al.* 1990; Hansen *et al.* 1991). Thus, achievement of optimal peak bone mass is critical for delaying or preventing osteoporosis (Matkovic *et al.* 1979; Jackman *et al.* 1997).

The importance of Ca intake for the development and maintenance of peak bone mass is well established. A low Ca intake by both rats and man increases bone resorption (Egger *et al.* 1994; Shapses *et al.* 1995; Ginty *et al.* 1998; Talbott *et al.* 1998), decreases bone mass (Matkovic *et al.* 1990; Persson *et al.* 1993; Talbott *et al.* 1998) and increases the risk of osteoporosis (Heaney, 1996).

The skeletal effect of high Ca intakes is less clear, particularly during periods of bone mineral accrual. There is some concern that very high Ca intakes during the period of growth could potentially interfere with the bone remodelling process. On the other hand, a number of studies have

shown that increasing Ca intake (by 300–1000 mg/d, from foods or supplements) above the usual dietary intakes (typically in the range, 300–900 mg Ca/d) enhances the rate of increase in bone mineral content and bone mineral density in children and adolescents (Johnston *et al.* 1992; Lloyd *et al.* 1993; Lee *et al.* 1994; Chan *et al.* 1995; Cadogan *et al.* 1997; Slemenda *et al.* 1997; Dibba *et al.* 1998). The Ca levels used in some of these studies were designed to raise levels to the recommended concentration from a baseline of low Ca, whereas in others they raised levels well above the recommended concentration. The higher Ca intake, if sustained, may lead to a higher peak bone mass at maturity (Institute of Medicine, 1997). The mechanism by which high Ca intake benefits peak bone mass is thought to be suppression of the remodelling rate which leads to a measurable increase in bone mass over time (Heaney, 1994). However, there have been few studies on the effect of increasing dietary Ca intake above the usual

Abbreviations: Dpyr, deoxypyridinoline; Pyr, pyridinoline.

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dietary intake on bone turnover, or remodelling, in children and adolescents. Two studies have reported that Ca supplementation significantly reduced serum levels of osteocalcin, a marker of bone formation, in children and that the reductions in serum osteocalcin with Ca supplementation were accompanied by greater increases in bone mineral density (Johnston *et al.* 1992; Dibba *et al.* 1998, 1999). The effect of Ca supplementation on the rate of bone resorption is less clear. Johnston *et al.* (1992) found that Ca supplementation (1000 mg/d) for 3 years had no effect on serum levels of tartrate-resistant acid phosphatase, a marker of bone resorption, in prepubertal children with a mean usual Ca intake of 900 mg/d. Cadogan *et al.* (1997) did not observe any change in a number of indicators of bone resorption and bone formation (including serum osteocalcin) in adolescent girls whose mean usual Ca intake of 746 mg/d was supplemented daily with an additional 568 ml milk (approximately 660 mg Ca/d) for 18 months, although bone mineral content and density were reported to increase. In the adolescent rat Peterson *et al.* (1995) found that Ca supplementation over 8 weeks reduced the rate of bone resorption, as assessed by bone histomorphometry at the tibia, even though tibial bone mineral density was unchanged. There has been no study of the effect of very high Ca intakes (achievable by diet plus supplemental Ca) on bone resorption or bone remodelling in the young growing rat, despite evidence of possible adverse effects on bone development (Persson *et al.* 1993).

Pyridinium crosslinks, pyridinoline (Pyr) and deoxypyridinoline (Dpyr), are products of the post-translational modification of collagen, and their urinary excretion has been used as a specific and sensitive index of bone resorption in man (Eyre, 1992; Robins & New, 1997) and rats (Black *et al.* 1989; Egger *et al.* 1994). Low Ca intakes by both rats (Egger *et al.* 1994; Talbott *et al.* 1999) and man (Shapses *et al.* 1995; Ginty *et al.* 1998) have been shown to

increase the level of excretion of these pyridinium crosslinks in urine.

The aim of the present study was to investigate the effect of increasing Ca intake to a high level on bone mineral composition and on pyridinium crosslinks of collagen, using the young growing female rat as a model. In addition, the effect of moderate restriction of dietary Ca on these variables was also re-evaluated. The juvenile rat is an acceptable model for skeletal studies as it shows the same biological mechanisms for bone growth, bone modelling and remodelling as in children and adolescents (Kalu, 1991; Frost & Jee, 1992; Peterson *et al.* 1995).

Materials and methods

Preparation of rat diets

The AIN-76 purified diet (American Institute of Nutrition, 1977) was used in the present study (Table 1).

Experimental design

Twenty-four female Wistar rats, 5 weeks old (average weight 102 g), obtained from the Biological Services Unit, University College, Cork, Republic of Ireland were randomized by weight into three groups of eight rats each. The three groups were fed *ad libitum* on semi-purified diets (AIN-76) containing 2.0 (Ca-restricted), 5.0 (normal), or 20.0 (Ca-supplemented) g Ca/kg for 21 d. Rats were housed individually in metabolism cages with a grid-floor and a facility for separate collection of faeces and urine. Feed was provided 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. Urine samples (24 h) were collected for each animal during the last 3 d of each week of the study in vessels 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-HCl (225 μ l/100 ml urine) and stored at -20°C until required for analysis.

After 21 d on the respective diets, all animals were killed by over-exposure to diethyl ether, and final body weights were recorded. The right femora were harvested and cleaned of adhering soft tissue. The femora were dried overnight at 110°C , weighed and stored in sealed containers until required for mineral analysis.

Experimental techniques

Urinary pyridinoline and deoxypyridinoline. Pooled urine samples were analysed in duplicate using a three-step procedure. Aliquots (250 μ l) of pooled urine samples were hydrolysed with an equal volume of 12 M-HCl at 110°C for 18 h. The crosslinks from the urine hydrolysates were then extracted by CF1 cellulose chromatography with the use of an internal standard (acetylated pyridinoline; MetraBiosystems Ltd, Wheatley, Oxon., UK) and were measured using a reversed-phase HPLC method with fluorescence detection (Colwell *et al.* 1993). The acetylated pyridinoline was used in accordance with the method described by Calabresi *et al.*

Table 1. Composition of the modified AIN-76 diet (American Institute of Nutrition, 1977)

Ingredient	Content (g/kg)
Casein	200.0
DL-Methionine	3.0
Maize starch	150.0
Sucrose	495.0, 487.5, 450.0*
Fibre	50.0
Maize oil	50.0
AIN mineral mix†	35.0
AIN vitamin mix‡	10.0
Calcium carbonate	5.0, 12.5, 50.0*
Choline bitartrate	2.0

* Representing diets containing (/kg) 2.0 (Ca-restricted), 5.0 (normal) or 20.0 (Ca-supplemented) g Ca respectively.

† Containing (g/kg): potassium dihydrogen phosphate 376, dipotassium hydrogen phosphate 160, sodium chloride 74, magnesium oxide 24, manganous carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite 0.01, chromium potassium sulfate 0.55, sucrose 354.

‡ Containing (/kg): nicotinic acid 3 g, calcium pantothenate 1.6 g, riboflavin 600 mg, thiamin hydrochloride 600 mg, pyridoxine hydrochloride 700 mg, pteroylmonoglutamic acid 200 mg, biotin 20 mg, cyanocobalamin 1 mg, cholecalciferol 2.5 mg, menaquinone 5.0 mg, retinyl palmitate 120 mg, DL- α -tocopheryl acetate 5000 mg.

Table 2. Effect of dietary calcium concentration on urinary calcium, pyridinolone (Pyr) and deoxypyridinolone (Dpyr) concentrations in young growing female rats*
(Mean values with their standard errors for eight rats per group)

Group	Dietary Ca (g/kg)	Urine																
		Ca (mg/d)			Pyr (nmol/d)			Dpyr (nmol/d)										
		Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	SE	SE						
Ca-restricted	2.0	0.8 ^a	0.2	0.1	1.0 ^a	0.1	8.7 ^a	0.6	14.7 ^a	1.3	16.3 ^a	1.0	11.5 ^a	0.6	15.3 ^a	1.0	16.6 ^a	1.2
Normal Ca	5.0	1.0 ^a	0.2	0.2	1.2 ^a	0.1	9.5 ^a	0.6	10.2 ^b	0.6	11.4 ^b	1.0	10.6 ^a	0.7	10.8 ^b	0.9	11.3 ^b	1.0
Ca-supplemented	20.0	3.7 ^b	0.3	0.5	4.0 ^b	0.4	9.2 ^a	0.3	10.7 ^b	0.9	12.0 ^b	0.8	11.3 ^a	0.5	12.2 ^b	1.3	12.0 ^b	0.8
ANOVA (one-way): P		<0.001		<0.001	<0.001		0.518		0.011		0.007		0.537		<0.001		<0.001	

^{a,b}Mean values within a column with different superscript letters were significantly different (ANOVA followed by least significant difference test; $P < 0.05$).
* For details of diets and procedures, see Table 1 and pp. 454–455.

(1994) and Robins *et al.* (1994). The crosslink contents of urine samples were quantified by external standardization using a commercially-available Pyr–Dpyr HPLC calibrator (MetraBiosystems Ltd). The intra-assay CV for Pyr and Dpyr measured as the variation between ten chromatograms obtained between column regenerations as described by Colwell *et al.* (1993) were 6 and 9% respectively. The interassay CV for Pyr and Dpyr were 7 and 8% respectively.

Femoral calcium, phosphorus and magnesium and urinary calcium levels. Weighed femora (dried) were digested in 10 ml 16 M-HNO₃–12 M-HClO₄ (2:1, v/v) on a hot plate (S & J Juniper & Co., Harlow, Essex, UK) until the sample colour resembled that of the reagent blank. Ca and Mg were analysed in duplicate in femoral digests by atomic absorption spectrophotometry (Pye-Unicam Atomic Absorption Spectrophotometer, Model SP9; Pye Unicam, Cambridge, Cambs., UK) after appropriate dilution with LaCl₃ solution (5 g/l; BDH Ltd, Poole, Dorset, UK). Ca was analysed in duplicate in urine by atomic absorption spectrophotometry after appropriate dilution with the LaCl₃ solution. A range of Ca and Mg standards was used to obtain Ca and Mg calibration curves. The intra- and interassay CV for Ca were 2.8 and 7.8%, and for Mg were 3.2 and 8.8% respectively. P was determined in the femoral digests by the method of Weissman & Pileggi (1974). The intra- and interassay CV for P were 4.2 and 6.1% respectively.

Statistical methods

Data are presented as means with their standard errors. All data were subjected to one-way ANOVA, with variation attributed to dietary Ca (Snedecor & Cochran, 1967). To follow up the ANOVA, all pairs of means were compared by the method of least significant difference (Snedecor & Cochran, 1967).

Results

Mean body-weight gain did not differ among groups (Fig. 1). The influence of dietary Ca concentration on urinary Ca, Pyr and Dpyr levels is shown in Table 2. Urinary Ca excretion was unaffected by the dietary restriction of Ca to 2.0 g/kg diet for 3 weeks. Urinary Ca excretion was greater in the Ca-supplemented group compared with the normal and Ca-restricted groups. While unaffected during week 1, urinary Pyr and Dpyr levels were significantly greater during weeks 2 ($P < 0.05$, $P < 0.001$ respectively) and 3 ($P < 0.01$, $P < 0.001$ respectively) of Ca restriction. Urinary Pyr and Dpyr were unaffected by Ca supplementation for 3 weeks.

The influence of dietary Ca concentration on femoral dry weight and macromineral (Ca, Mg and P) concentrations is shown in Table 3. Femoral dry weight and the concentration of Mg and P in femora were unaffected by dietary Ca concentration. Femoral Ca concentration was reduced in the Ca-restricted group compared with the normal and Ca-supplemented groups, with no significant difference in femoral Ca concentration between the last two groups.

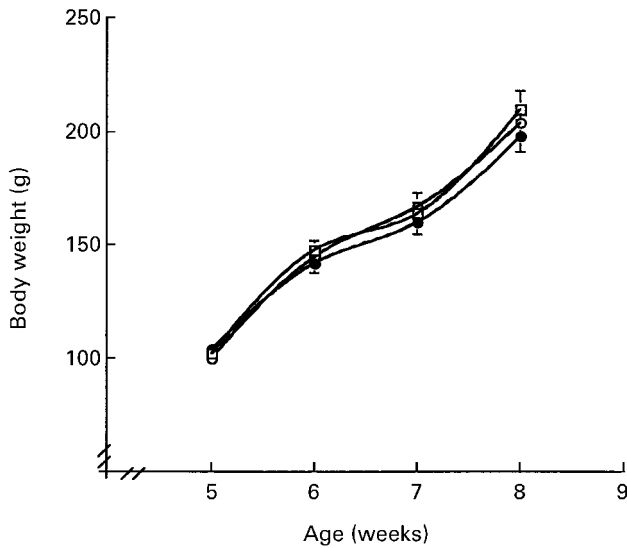


Fig. 1. Body weights over time in rats fed on diets containing different levels of calcium. Groups were: (□), normal calcium diet; (●), Ca-restricted diet; (○), Ca-supplemented diet. Values are means with their standard errors represented by vertical bars for eight rats. No significant differences were found between groups at any of the time points (by ANOVA, $P < 0.05$). For details of diets, see Table 1.

Discussion

In the present study, mean body-weight gain by young growing rats was unaffected by dietary Ca concentration. This finding is in agreement with those of other studies (Thomas *et al.* 1988, 1991; Takeda *et al.* 1993; Peterson *et al.* 1995; Talbott *et al.* 1999), but not all (Persson *et al.* 1993), which have examined the effect of dietary Ca on weight gain in the rat. Increasing dietary Ca concentration to a high level (20 g/kg diet) significantly ($P < 0.05$) increased urinary Ca excretion in rats in the present study. On the other hand, restricting dietary Ca intake to a low level (2.0 g/kg diet) had no effect on urinary Ca excretion. These findings are consistent with the results of previous studies (Makynen *et al.* 1995; Cashman & Flynn, 1996). The elevated urinary Ca levels in rats on the high-Ca diet in the present study probably reflected a greatly increased absolute absorption of Ca by these rats. For example, Cashman & Flynn (1996) showed that absolute Ca absorption from a

10 g meal in rats fed habitually on diets containing 20, 5 and 2 g Ca/kg diet was 36.9, 24.2 and 17.8 mg Ca respectively. A similar response of urinary Ca to a high dietary Ca intake has been noted in man (Ginty *et al.* 1998).

In the present study moderate dietary restriction of Ca reduced femur Ca concentration, but had no effect on femoral dry weight or on the Mg or P content of the femur. A reduced femoral Ca level, but not Mg or P levels, arising from an increased rate of bone resorption has also been noted in a recent study in which young growing rats were fed a high-salt diet (Creedon & Cashman, 2000). It may be that, as suggested by Goulding & Gold (1988), the duration of the present study was too short to elicit significant osteopenia. Several studies have shown that bone Ca content (Thomas *et al.* 1988, 1991 Persson *et al.* 1993; Takeda *et al.* 1993; Peterson *et al.* 1995), bone strength (Thomas *et al.* 1988, 1991) and bone density (Persson *et al.* 1993; Talbott *et al.* 1998) in young growing rats were reduced by dietary Ca restriction (i.e. in the range 0.3–2.5 g Ca/kg diet). Such skeletal alterations may have arisen because of an increased rate of bone resorption or a reduced rate of bone formation and mineralization, or both, secondary to Ca restriction.

In the present study, moderate dietary restriction of Ca increased the rate of bone resorption, as measured by the urinary excretion of pyridinium crosslinks of collagen which are regarded as specific markers of bone resorption in rats (Black *et al.* 1989; Egger *et al.* 1994). This finding is in agreement with the findings of other studies that reported increased excretion of urinary pyridinium crosslinks (Egger *et al.* 1994; Talbott *et al.* 1999) and [³H]tetracycline (Egger *et al.* 1994; Talbott *et al.* 1998, 1999), another marker of bone resorption, in Ca-restricted young rats. Recently, Talbott *et al.* (1999) found that 20-week-old female rats fed a diet containing 1 g Ca/kg diet had significantly ($P < 0.05$) higher mean urinary Pyr and Dpyr levels (over 9 weeks) compared with rats fed a diet containing 5 g Ca/kg diet. As a marker of bone formation, such as serum osteocalcin or bone-specific alkaline phosphatase, was not included in the present study, it is not clear whether the increased rate of bone resorption reflected an increased rate of bone turnover (i.e. bone resorption and formation). An increased rate of bone turnover has been associated with a reduction in bone mass (Hansen *et al.* 1991) and disruption of the trabecular network (Parfitt, 1984).

Table 3. Effect of dietary calcium concentration on femur dry weight and concentrations of calcium, magnesium and phosphorus in young growing female rats*

(Mean values with their standard errors for eight rats per group)

Group	Dietary Ca (g/kg)	Dry wt (mg)		Ca (mg/g dry wt)		Mg (mg/g dry wt)		P (mg/g dry wt)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ca-restricted	2.0	274 ^a	10	266 ^a	4	3.84 ^a	0.33	104.9 ^a	2.4
Normal Ca	5.0	277 ^a	13	286 ^b	5	3.76 ^a	0.27	103.4 ^a	2.2
Ca-supplemented	20.0	279 ^a	18	287 ^b	3	3.80 ^a	0.23	105.6 ^a	2.8
ANOVA (one-way), P value		0.417		0.029		0.983		0.813	

^{a,b}Mean values within a column with different superscript letters were significantly different (ANOVA followed by least significant difference test; $P < 0.05$).

* For details of diets and procedures, see Table 1 and pp. 454–455.

An increased rate of bone resorption, as assessed by bone histomorphometry, in young rats fed a Ca-restricted diet has also been reported by Peterson *et al.* (1995). In their study, young (4-week-old) female rats fed a diet containing 2.5 g Ca/kg diet for 8 weeks had higher rates of bone resorption compared with rats fed diets containing 5 and 10 g Ca/kg diet. Furthermore, in addition to the increased rate of bone resorption, dietary restriction of Ca led to reduced rates of bone formation, mineral apposition and longitudinal growth, and to reduced trabecular number and bone volume (Peterson *et al.* 1995). If low Ca intakes during adolescence in man leads to elevated rates of bone resorption, as it appears to do in rats, then this increase may have a deleterious effect on peak bone mass.

In the present study increasing the dietary Ca content to fourfold the requirement had no effect on femoral dry weight or on femoral Ca, Mg or P concentrations. This finding is in agreement with those of Peterson *et al.* (1995) which showed that increasing the dietary Ca content to twofold the requirement had no effect on bone mass and Ca content in young growing rats. Persson *et al.* (1993), on the other hand, reported that increasing the dietary Ca content to tenfold the requirement impaired bone development (i.e. bone wet weight, ash weight and bone density) in young growing rats. In the present study the rate of bone resorption, as assessed by the urinary pyridinium crosslinks, was unaffected by Ca supplementation for 3 weeks. In contrast, Peterson *et al.* (1995) reported that the rate of bone resorption, as assessed by bone histomorphometry, was reduced by a doubling of the recommended Ca intake of young growing female rats over 8 weeks. However, despite the reduction in the rate of bone resorption, there was no effect of Ca supplementation on the rate of bone formation or mineral apposition, or on bone volume (Peterson *et al.* 1995). The reasons for the discordant findings of the present study and that of Peterson *et al.* (1995) are unclear. Peterson *et al.* (1995) fed rats a high-Ca diet for a longer period than that in the present study. It is also possible that the urinary pyridinium crosslink assay for determining the rate of bone resorption is a less-sensitive technique compared with bone histomorphometry.

While increasing the Ca intake to high levels (as CaCO₃) had no effect on bone resorption in the present study, it may lead to a reduction in the intestinal absorption of Mg (Greger *et al.* 1981) and P (Institute of Medicine, 1997), which may have adverse consequences for bone metabolism. However, interactions between high levels of Ca and Mg or P have not shown evidence of depletion of the affected nutrient (Shils, 1994). Furthermore, no depletion of either femoral Mg or femoral P was evident in rats fed the high-Ca diet in the present study.

The current criterion of the National Research Council (1978) for determining the Ca requirement of the rat is the level of Ca needed for maximum mineralization of bone during growth. The method typically employed in this determination is the measurement of bone weight, bone ash and total bone Ca (Bernhart *et al.* 1969; National Research Council, 1978). Urinary pyridinium crosslinks were utilized in the present study in order to provide better interpretation of the bone mineral composition data, but also because biochemical markers of bone turnover, such as the

pyridinium crosslinks, have been suggested as being of potential use in predicting the long-term effects of Ca on bone turnover and bone mass and, thus, in defining optimal Ca intakes (Cashman & Flynn, 1999). Interestingly, the pyridinium crosslink data from the present study support the National Research Council (1978) Ca requirement for the rat (5 g Ca/kg diet). In addition, the findings of the present study lend further support to the hypothesis that Ca is a threshold nutrient (i.e. at suboptimal intakes the ability of the organism to store Ca as bone tissue is limited by the intake of Ca, but increasing Ca intake above that required as optimal for genetic or mechanical purposes does not lead to increased storage of Ca).

In conclusion, the findings of the present study confirm the findings of previous studies that have shown that bone Ca content in young growing rats was reduced by dietary Ca restriction, and that this reduction results, at least in part, from an increased rate of bone resorption. In addition, increasing dietary Ca intake above the recommended level had no effect on bone mineral composition or on bone resorption, as assessed with urinary pyridinium crosslinks of collagen, in young growing female rats. These findings would suggest that high Ca intake levels, well in excess of requirements, may not have detrimental effects on the bone remodelling process, at least in the short term. The utilization of the pyridinium crosslinks of collagen (as markers of bone resorption), as well as inclusion of a marker of bone formation (such as osteocalcin or bone-specific alkaline phosphatase) in future Ca supplementation trials in children and adolescents would greatly extend our knowledge of the effect on bone turnover of increasing dietary Ca intakes above usual levels.

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