Calcium Requirements of Growing Rats Based on Bone Mass, Structure, or Biomechanical Strength Are Similar\textsuperscript{1,2}

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Abstract

Although calcium (Ca) supplementation increases bone density, the increase is small and the effect on bone strength and fracture risk is uncertain. To investigate if bone mass, morphology, and biomechanical properties are affected by deficient to copious dietary Ca concentrations, the long bones (tibia and femur) of growing female Sprague-Dawley rats (8/group) were assessed after 13 wk of consuming 1, 2, 3, 4, 5, 6, or 7 g Ca/kg of a modified AIN-93G diet. Dietary phosphorous (P) and vitamin D remained constant at recommended concentrations. The assessment included mineralization, density, biomechanical properties of breaking by a 3-point flexure test, and morphological properties by microcomputed topography scanning of trabecular bone of the proximal tibia metaphysis. Dietary treatment did not affect food intake, weight gain, renal and muscle Ca concentrations, and bone hydroxyproline. All bone parameters measured were significantly impaired by Ca deficiency in rats fed the diet containing 1 g Ca/kg. Modest impairments occurred with some parameters (bone density, biomechanical bending moment, modulus of elasticity, and stress) in rats fed 2 g Ca/kg, but all parameters stabilized between 2 and 3 g/kg diet, with no differences between 3 and 7 g/kg. The results suggest that a threshold response in bone Ca retention or bone mass at ~2.5 g Ca/kg diet is associated with similar threshold responses in bone breaking strength and related biomechanics as well as trabecular structural properties. There was no evidence of a relative P deficiency or of improved or impaired bone strength and structure as Ca intakes increased beyond those needed to maximize bone density. J. Nutr. 138: 1462-1468, 2008.

Introduction

Increased calcium (Ca) ingestion is commonly recommended for bone health and prevention of osteoporosis and fractures (1,2). Although Ca supplementation significantly increases bone density, the increase is small, whether in children (3) or adults (4), and the impact on bone strength and fracture risk is uncertain. Bone density may not be a reliable indicator of bone strength (5). For example, in a prospective study of 202 postmenopausal women, fluoride treatment significantly increased bone mineral density of the lumbar spine and femoral neck and trochanter but also increased the number of nonvertebral fractures (6). Contrary to expectations, a recent meta-analysis of randomized clinical trials found that Ca supplementation (without additional vitamin D) increased hip fracture risk in men and women (7). Although animal models can be used to directly test the effects of diet on bone strength, most animal studies have tended to focus on determining the Ca requirement to improve either bone mass or density (8). In addition, such studies have tended to use a constant Ca:phosphorus (P) molar ratio, whereas Ca supplementation of human diets commonly does not include P. The objective of the present investigation was to test the influence of increasing dietary Ca concentrations (without increasing dietary P), from nutritionally inadequate to copious levels, on bone mineral density, structure, and breaking strength biomechanics.

Methods

Animals and diets. The experimental protocol was approved by the local USDA-Agricultural Research Service animal care committee and all experimental procedures complied with National Research Council guidelines for animal research (9). Female Sprague-Dawley rats obtained from Sasco were housed individually in wire-bottomed stainless steel cages, with a 12-h-light/-dark cycle (lights on at 0600). These growing rats were ~4 wk old; initial body weights were (mean ± SD) 59 ± 4 g. The rats were assigned randomly, with blocking for body weight, to 7 dietary treatment groups containing 8 rats each. The experimental diets were modified from the AIN-93G diet (10) to obtain Ca concentrations of 1, 2, 3, 4, 5, 6, and 7 g/kg diet, with Ca lactate as the Ca source, substituted for equal weights of dietary cornstarch. The resulting analyzed Ca concentrations of the diets were 1.09, 2.12, 3.25, 4.26, 5.22, 6.27, and 7.28 g/kg, respectively (Table 1).
TABLE 1 Dietary intake, body weight, and bone density of weanling rats fed incremental Ca concentrations for 13 wk\(^1\)

<table>
<thead>
<tr>
<th>Ca group</th>
<th>Diet Ca</th>
<th>Food intake</th>
<th>Body weight</th>
<th>Body weight gain</th>
<th>Tibia fresh weight</th>
<th>Tibia density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td>g/d</td>
<td>g</td>
<td>g/13 wk</td>
<td>g</td>
<td>g/cm(^2)</td>
</tr>
<tr>
<td>1</td>
<td>1.09 ± 0.04</td>
<td>14.5 ± 1.9(^{a,b})</td>
<td>229 ± 14</td>
<td>170 ± 15</td>
<td>0.52 ± 0.04(^{a})</td>
<td>1.30 ± 0.02(^{c})</td>
</tr>
<tr>
<td>2</td>
<td>2.12 ± 0.04</td>
<td>15.8 ± 1.5(^{b})</td>
<td>262 ± 31</td>
<td>203 ± 32</td>
<td>0.66 ± 0.05(^{a})</td>
<td>1.41 ± 0.02(^{b})</td>
</tr>
<tr>
<td>3</td>
<td>3.25 ± 0.08</td>
<td>14.7 ± 1.1(^{b})</td>
<td>244 ± 16</td>
<td>185 ± 13</td>
<td>0.64 ± 0.06(^{b})</td>
<td>1.44 ± 0.02(^{ab})</td>
</tr>
<tr>
<td>4</td>
<td>4.26 ± 0.02</td>
<td>14.2 ± 1.8(^{a})</td>
<td>237 ± 21</td>
<td>178 ± 19</td>
<td>0.64 ± 0.05(^{a})</td>
<td>1.46 ± 0.03(^{a})</td>
</tr>
<tr>
<td>5</td>
<td>5.22 ± 0.02</td>
<td>16.2 ± 1.8(^{a})</td>
<td>261 ± 31</td>
<td>203 ± 28</td>
<td>0.70 ± 0.06(^{a})</td>
<td>1.46 ± 0.03(^{a})</td>
</tr>
<tr>
<td>6</td>
<td>6.27 ± 0.02</td>
<td>13.6 ± 1.2(^{b})</td>
<td>229 ± 13</td>
<td>170 ± 10</td>
<td>0.63 ± 0.05(^{a})</td>
<td>1.45 ± 0.01(^{a})</td>
</tr>
<tr>
<td>7</td>
<td>7.28 ± 0.02</td>
<td>14.3 ± 1.8(^{a})</td>
<td>252 ± 28</td>
<td>192 ± 23</td>
<td>0.64 ± 0.07(^{a})</td>
<td>1.47 ± 0.02(^{a})</td>
</tr>
</tbody>
</table>

\(^1\) Data are means ± SD, n = 3 (diet) or 8 (rat measures). For rat data, means in a column with superscripts without a common letter differ, P < 0.05.

All other dietary components (e.g., P) were kept constant. The AIN-93G diet (10) provided 25 μg cholecalciferol and 3 g P/kg, the latter from a combination of potassium phosphate and casein (10). Thus, Ca:P molar ratios were -0.25, 0.50, 0.75, 1.0, 1.3, 1.5, and 1.8 for the present study. Dietary intake was monitored intermittently (daily for 4 d/week) by weighing consumption without adjustment for spillage.

Rats were fed the experimental diets for 13 wk and after 1 night without food were killed by anesthetizing with ether followed by exsanguination from the descending aorta. The rats' hind legs were dissected. To prevent desiccation of the bone samples, the legs were frozen intact, with thawing and removal of adhering tissue delayed until we conducted the density and biomechanical measurements.

Physical and biomechanical testing. Volumetric bone density of the left tibia was determined by Archimedes' principle. After deagging the bone in deionized water, the bone was weighed while submerged and again after removal from the water (11).

Biomechanical assessment of the breaking of the left femur was conducted by a 3-point bending test using a custom-made apparatus. Interfaced with a computer, this apparatus consists of 2 supports (fulcrums) and a movable crosshead driven by a step motor. The increase in force corresponding to each downward movement of the crosshead (0.0169 mm) was measured with an electronic load cell until fracture. The resulting force-displacement data were used together with an electronic digital caliper (0.01 mm resolution; Fred V. Fowler) and microscopic (40X magnification) measurements of bone geometry to determine breaking force, bending moment, stress, and modulus of elasticity with formulas described by Crenshaw et al. (12).

Bone structural analysis. The right proximal tibia was prepared for scanning electron microscopy and structural analysis. The bone was cut longitudinally in a plane that passed perpendicular to a line segment extending from the crest of the tibia to the fibula attachment and through the lateral edge of the cruciate ligament insertion. The cut bone sample was fixed in 10% buffered formalin prior to incubation with 5% H2O2, and then rinsed twice and averaged to obtain an ROI comprised of 418 serial slices. Following acquisition of the spatial data, structural analysis was accomplished with a dedicated computer program (microCT 40 software; version 4.04; Scanco USA) (14). A volume of interest (VOI) that contained only trabecular primary and secondary spongiosa was defined within the ROI (Fig. 1A). The VOI consisted of 100 consecutive image sagittal slices, each 10 μm in width, and was bounded medially as the first slice located from the midperistomial tibial surface at a distance equal to 25% of width of the proximal tibial growth plate (GP) measured in the sagittal plane at the widest point; laterally as slice 100 of 100 slices; proximally by the interface between the GP and the primary spongiosa; distally by the transverse plane that intersected the secondary spongiosum at a distance measured from the center of the GP equal in length to the width of the GP; and anteriorly and posteriorly by the respective peripheral trabecular-cortical bone interfaces.

Boundary contours were drawn for reference slices 1, 25, 50, 75, and 100 and then boundaries of all intervening slices were morphed between the reference slices. Segmentation values (for detection of bone material within the VOI) were kept constant for all evaluations: lower threshold, 350; upper threshold, 1000; Gauss sigma, 0.8; Gauss support, 1.0. The resulting 3-dimensional image analysis provides an assessment of the bone volume fraction [bone volume (BV)/total volume (TV)] as well as trabecular number, thickness, separation, and structural model index (SMI) as described by Hildebrand et al. (15) and connectivity density (a measure of the degree to which a structure is multiply connected) as described by Odgaard and Gundersen (16).

Chemical analyses of diet and tissues. Following biomechanical assessment, the left femur was demarrowed with cold 0.25 mol/L sucrose, pulverized in liquid nitrogen, bophilized, and diluted in 6 mol/L HCl (1:5 v/v), then analyzed colorimetrically for hydroxyproline using Ehrlich's reagent (17).

Serum alkaline phosphatase was measured colorimetrically (18). Diet and tissue samples were analyzed for Ca concentrations by inductively coupled argon plasma emission spectrophotometry after nitric acid and hydrogen peroxide digestion. Analytical accuracy was monitored by assaying a locally prepared typical diet standard, yielding results that were (mean ± SD) 102 ± 8% of established acceptable values.

Statistics. The effects of dietary treatment were determined by using ANOVA, with Tukey's pairwise contrasts to compare between treatment groups (SAS version 9.1.3) (19). A change point (broken line) model (20) was used to analyze the relationship between dietary Ca concentrations and different bone parameters, assuming an asymptote with a slope of 0 and defining the Ca requirement as the change point. The NLIN procedure in SAS was used to estimate the change point and appropriate 95% CI. Results were considered significant at P < 0.05.

Results

Food intake was independent of dietary Ca concentration, with a significant difference only between the most extreme means,
which did not represent the extremes in Ca concentration (Table 1). Consistent with this, neither body weight nor final weight gain were affected by the dietary Ca concentration (Table 1). Tibia weight was significantly reduced only at the lowest Ca intake (Table 1), and reached a threshold weight in rats fed between 2 and 7 g Ca/kg diet. Tibia density was lower than in all other groups in rats fed 1 g Ca/kg diet, was lower than in most other groups in those fed 2 g/kg, and reached a threshold that did not differ among the rats fed 3–7 g Ca/kg (Table 1).

The serum alkaline phosphatase concentration was significantly greater than in all other groups, which did not differ from one another, in the rats fed the lowest level of dietary Ca, 1 g/kg (Table 2). Femur hydroxyproline concentrations were not affected by dietary Ca (Table 2). Tibia Ca concentrations increased in a stepwise fashion between 1 and 3 g Ca/kg diet, reaching a threshold concentration that remained unchanged between 3 and 7 g/kg (Table 2). Data were similar when expressed on a per tibia basis, although without a significant difference between rats fed 2 and 3 g Ca/kg diet. The tibia P concentration also was significantly lower in rats fed the diet lowest in Ca than in all other groups, but it did not differ among those fed between 3 and 7 g/kg (Table 2). The Ca:P molar ratio of the tibia did not differ among the groups (data not shown). There was no evidence of soft tissue calcification, based on the lack of significant difference among the groups in the Ca concentration of the kidney or gastrocnemious muscle (Table 2).

Most indices of bone strength generally increased to threshold levels as dietary Ca increased (Table 3). The maximum breaking force was reached in rats fed 2 g Ca/kg; bending moment, stress, and modulus of elasticity maximized at or above a dietary concentration of 3 g/kg (Table 3).

Consistent with these results, Ca deficiency impaired trabecular bone structure in the proximal tibia (as assessed by micro-CT), with significant differences from other groups occurring only in rats fed the lowest Ca diet for most of the parameters (Table 4). Compared with dietary Ca concentrations between 2 and 7 g/kg, the lowest dietary Ca concentration of 1 g/kg resulted in a lower bone volume density (BV/TV), a lower connectivity density (a measure of trabecular elements proposed to be more closely related to strength and stiffness than bone volume density), a lower mean trabecular number, and more trabecular separation. The trabecular thickness, although significantly lower in rats fed 1 g Ca/kg than in those fed 7 g/kg (Table 4), also appeared to follow this general response pattern of an early increase to a stable maximum threshold when data for individual rats were examined (Fig. 2). The trabecular number and related separation were more substantially affected by Ca deficiency than the trabecular thickness (Fig. 2). The differences in the SMI indicate that Ca deficiency resulted in trabeculae with a more cylindrical or rod-like shape, in contrast to a more flattened, plate-like shape with adequate dietary Ca (sphere SMI = 4; cylinder SMI = 3; and plate SMI = 0). The 3-dimensional micro-CT and scanning electron microscopy images of the primary and secondary spongiosa in the proximal tibia metaphysis further demonstrated the effects of Ca deficiency (1–2 g Ca/kg diet) on bone structure (Fig. 1). The scanning elec-

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**FIGURE 1** Structural changes in metaphyseal trabecular bone in the rat proximal tibia in response to graded levels of dietary calcium. (A) The VOI within the ROI was immediately distal of the GP and contained only trabecular primary and secondary spongiosa as detected by micro computed topography. The scanning electron micrographs (cut face up) and corresponding VOI (cut face down) were from the bones with BV/TV ratios closest to the mean ratio of each dietary group: 1 (B), 2 (C), 3 (D), 4 (E), 5 (F), 6 (G), 7 (H) g Ca/kg diet. Calcium deficiency induced severe reduction of the trabecular bone mass in the metaphysis. However, there was robust conservation of the metaphyseal trabeculae that were situated in the long axis of the lines of force generated by mechanical loads on the articulating condyles proximally and passed on to the diaphyseal shaft.

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**TABLE 2** Chemical composition of tissues of weanling rats fed incremental Ca concentrations for 13 wk

<table>
<thead>
<tr>
<th>Ca group</th>
<th>Serum alkaline phosphatase</th>
<th>Femur hydroxyproline</th>
<th>Tibia Ca</th>
<th>Tibia P</th>
<th>Kidney Ca</th>
<th>Muscle Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μkat/L</td>
<td>μmol/g dw</td>
<td>mmol/g dw</td>
<td>mmol/tibia</td>
<td>mmol/g dw</td>
<td>mmol/tibia</td>
</tr>
<tr>
<td>1</td>
<td>1.6 ± 0.3ab</td>
<td>84 ± 6</td>
<td>4.4 ± 0.2</td>
<td>1.4 ± 0.1bc</td>
<td>2.7 ± 0.1bc</td>
<td>0.8 ± 0.0bc</td>
</tr>
<tr>
<td>2</td>
<td>1.0 ± 0.1ab</td>
<td>71 ± 12</td>
<td>5.1 ± 0.3</td>
<td>2.1 ± 0.1bc</td>
<td>3.1 ± 0.1bc</td>
<td>1.3 ± 0.1bc</td>
</tr>
<tr>
<td>3</td>
<td>0.9 ± 0.2ab</td>
<td>68 ± 8</td>
<td>5.4 ± 0.2</td>
<td>2.2 ± 0.2bc</td>
<td>3.2 ± 0.1ab</td>
<td>1.3 ± 0.1bc</td>
</tr>
<tr>
<td>4</td>
<td>1.0 ± 0.2ab</td>
<td>65 ± 16</td>
<td>5.3 ± 0.2</td>
<td>2.2 ± 0.2bc</td>
<td>3.2 ± 0.1ab</td>
<td>1.4 ± 0.1bc</td>
</tr>
<tr>
<td>5</td>
<td>1.1 ± 0.3ab</td>
<td>71 ± 17</td>
<td>5.4 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>3.3 ± 0.1bc</td>
<td>1.5 ± 0.1bc</td>
</tr>
<tr>
<td>6</td>
<td>1.1 ± 0.3ab</td>
<td>66 ± 14</td>
<td>5.4 ± 0.2</td>
<td>2.2 ± 0.2bc</td>
<td>3.3 ± 0.1bc</td>
<td>1.4 ± 0.1bc</td>
</tr>
<tr>
<td>7</td>
<td>1.1 ± 0.3ab</td>
<td>81 ± 20</td>
<td>5.5 ± 0.2</td>
<td>2.3 ± 0.3bc</td>
<td>3.3 ± 0.1bc</td>
<td>1.4 ± 0.1bc</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n = 8. Means in a column with superscripts without a common letter differ, P < 0.05.
tron microscopy images clearly illustrate severe reduction of the trabecular bone mass in the metaphysis of the tibia induced by calcium deficiency. However, there was robust conservation of the metaphyseal trabeculae that were situated in the long axis of the lines of force generated by mechanical loads on the articulating condyles proximally and passed on to the diaphyseal shaft.

To further compare calcium requirements derived using different bone measurement criteria, key parameters (Tables 1–4) were analyzed using a change point or broken line model. The resulting requirements, with 95% CI, demonstrate that similar dietary Ca concentrations (~2.5 g/kg diet) were sufficient to reach a threshold response in most of the measured parameters of bone density, biomechanical strength, and structure (Fig. 3). An exception to this was a greater Ca requirement (3.9 g/kg diet) to maximize biomechanical stress. The results using tibia weight or trabecular thickness as dependent variables did not fit this model well and were not used to estimate the Ca requirement.

Discussion

By testing graded increases in dietary Ca, this study clearly shows similar Ca requirements to meet threshold responses for most of the measurements of bone mass, strength, and structure. That is, the size, density, mineral retention, breaking force, flexibility (e.g., modulus of elasticity), and trabecular number and spacing of bone reached a metabolic maximum if Ca intakes met or exceeded ~2.5 g/kg diet, when dietary P and vitamin D requirements were met and kept constant.

The greater Ca requirement estimated using the criterion of biomechanical stress (Fig. 3) in contrast with the other dependent bone-related variables was unexpected. The present finding that the Ca requirement to reach maximum bone stress was greater than that to reach maximum bending moment, is contrary to observations by Miller et al. (21), as discussed by Crenshaw et al. (12). Stress is a measure of the amount of force per unit area (12), which adjusts the bending moment to take into account the area and the geometrical shape of the bone. For these tibia measurements, the cross-section of bone where force is applied is assumed to be elliptical, an assumption that is obviously not strictly met with these biological samples. Given that the other biomechanical measurements, including bending moment, suggest a Ca requirement of ~2.5 g/kg diet, the greater Ca requirement derived with the stress measurement should be viewed with caution. A further limitation of the present broken line modeling was the small number of groups consuming less than the requirement. Although the stress parameter maximized at 3.9 g/kg diet by this modeling (Fig. 3), the mean stress measurement was not different in rats fed between 3 and 7 g Ca/kg diet (Table 3).

The present data, showing effects of Ca deficiency that were corrected in rats fed 2–3 g Ca/kg diet without differences among those fed between 3 and 7 g Ca/kg, are generally consistent with more limited published observations relating dietary Ca to the biomechanical properties of bone. Breitman et al. (22) found no difference in the bone biomechanical strength, measured by 3-point flexure testing of the femurs (as in the present study) of 90-d-old ovariectomized rats fed either 2 or 25 g Ca/kg diet. Won et al. (23), also used the 3-point flexure method with femurs of rats and found that the breaking force was significantly reduced in rats fed 0.2 g Ca/kg diet but reached a maximum threshold that did not differ among those fed 3, 6, 10,

### TABLE 3
Biomechanical traits of the left femur of weanling rats fed incremental Ca concentrations for 13 wk

<table>
<thead>
<tr>
<th>Ca group</th>
<th>Breaking force kg</th>
<th>Bending moment kg/cm</th>
<th>Stress kg/cm²</th>
<th>Modulus of elasticity kg/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.2 ± 0.56</td>
<td>1.73 ± 0.18</td>
<td>880 ± 96</td>
<td>49,398 ± 2438</td>
</tr>
<tr>
<td>2</td>
<td>12.0 ± 1.24</td>
<td>2.88 ± 0.14</td>
<td>1001 ± 99</td>
<td>66,041 ± 5918</td>
</tr>
<tr>
<td>3</td>
<td>12.3 ± 1.35</td>
<td>3.31 ± 0.40</td>
<td>1214 ± 110</td>
<td>72,914 ± 5075</td>
</tr>
<tr>
<td>4</td>
<td>12.6 ± 1.79</td>
<td>3.50 ± 0.37</td>
<td>1368 ± 135</td>
<td>78,784 ± 12,543</td>
</tr>
<tr>
<td>5</td>
<td>13.8 ± 1.35</td>
<td>3.67 ± 0.28</td>
<td>1235 ± 118</td>
<td>69,057 ± 5149</td>
</tr>
<tr>
<td>6</td>
<td>12.6 ± 1.42</td>
<td>3.36 ± 0.32</td>
<td>1262 ± 67</td>
<td>79,942 ± 10,380</td>
</tr>
<tr>
<td>7</td>
<td>12.8 ± 1.59</td>
<td>3.47 ± 0.28</td>
<td>1320 ± 168</td>
<td>78,367 ± 4582</td>
</tr>
</tbody>
</table>

1 Data are mean ± SD, n = 8. Means in a column with superscripts without a common letter differ, P < 0.05.

### TABLE 4
Structural analysis of the metaphyseal primary and secondary spongiosa within the rat proximal tibia (see Fig. 1) by micro-CT of weanling rats fed incremental Ca concentrations for 13 wk

<table>
<thead>
<tr>
<th>Ca group</th>
<th>Bone volume density (BV/TV) 1/mm³</th>
<th>Connectivity density 1/mm²</th>
<th>Trabecular no.</th>
<th>Trabecular separation mm</th>
<th>Trabecular thickness mm</th>
<th>SMI²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.033 ± 0.014b</td>
<td>27 ± 28b</td>
<td>2.48 ± 0.38b</td>
<td>0.420 ± 0.069b</td>
<td>0.048 ± 0.005b</td>
<td>77.9</td>
</tr>
<tr>
<td>2</td>
<td>0.106 ± 0.033b</td>
<td>141 ± 51b</td>
<td>3.44 ± 0.36b</td>
<td>0.303 ± 0.028b</td>
<td>0.052 ± 0.003b</td>
<td>80.6</td>
</tr>
<tr>
<td>3</td>
<td>0.124 ± 0.041b</td>
<td>197 ± 80b</td>
<td>3.61 ± 0.68b</td>
<td>0.296 ± 0.057b</td>
<td>0.050 ± 0.003b</td>
<td>78.9</td>
</tr>
<tr>
<td>4</td>
<td>0.134 ± 0.056b</td>
<td>187 ± 74b</td>
<td>3.81 ± 0.66b</td>
<td>0.290 ± 0.053b</td>
<td>0.052 ± 0.004b</td>
<td>78.3</td>
</tr>
<tr>
<td>5</td>
<td>0.150 ± 0.030b</td>
<td>209 ± 45b</td>
<td>3.80 ± 0.34b</td>
<td>0.270 ± 0.027b</td>
<td>0.053 ± 0.003b</td>
<td>79.6</td>
</tr>
<tr>
<td>6</td>
<td>0.136 ± 0.043b</td>
<td>190 ± 58b</td>
<td>3.49 ± 0.50b</td>
<td>0.296 ± 0.038b</td>
<td>0.054 ± 0.003b</td>
<td>78.0</td>
</tr>
<tr>
<td>7</td>
<td>0.165 ± 0.054b</td>
<td>209 ± 72b</td>
<td>4.04 ± 0.79b</td>
<td>0.264 ± 0.054b</td>
<td>0.056 ± 0.005b</td>
<td>78.2</td>
</tr>
</tbody>
</table>

1 Data are mean ± SD, n = 8. Means in a column with superscripts without a common letter differ, P < 0.05.

2 For the structure model index, 0 indicates parallel plates, 3 cylindrical rods, and 4 spheres.
treated from 3 to 51 wk. Zhang et al. (26), studying 12-mo-old rats from 3 to 19 wk old; Ca deficiency did not affect older rats comparing 1.25 to 5.0 g Ca/kg diet, although only in rats treated with vertebrae, the Ca-deficient diet resulted in lower relative femur density (measured by dual X-ray absorptiometry) and trabecular number, separation, thickness, or SMI, indicating a more rod-like rather than plate-like structure (24), all of which were fully consistent with the present micro-CT observations with the tibia (Table 4). Turner et al. (25) found similar differences when comparing 1.25 to 5.0 g Ca/kg diet, although only in rats treated from 3 to 19 wk old; Ca deficiency did not affect older rats treated from 3 to 51 wk. Zhang et al. (26), studying 12-mo-old rats, found that Ca supplementation [56 mg Ca/(kg body weight-d)] of a diet containing 9 g Ca/kg diet did not influence micro-CT measurements (BV/TV, connectivity density, trabecular number, separation, thickness, or SMI) of the femur greater trochanter region. Together, these observations indicate that bone biomechanics and morphology are substantially influenced by dietary Ca deficiency but otherwise reach a response threshold, and that older rats are less susceptible to deficiency than younger rats. Bone mass depends on dietary P as well as Ca (8,27,28) and a relative P deficiency has been proposed as a possible explanation for increased fracture risk with a high Ca intake (7). Shapiro and Heaney (27) demonstrated that the femur strength of growing rats was impaired with inadequate dietary P (0.75 g/kg diet) and that increases in dietary Ca (1.25, 2.50, or 3.75 g/kg diet) did not effectively improve femur strength unless the Ca source also provided P. An excess of P (6, 12, or 18 g P/kg diet compared with 3 g P/kg diet) increased the Ca loss from the 45Ca-labeled skeleton of rats 8–14 mo of age (28) and the 2 highest P intakes significantly reduced bone weight, Ca, and P. However, the present results do not support concerns that an increase in dietary Ca without an accompanying increase in dietary P may adversely affect bone biomechanics and fracture risk. With a constant and adequate P concentration of 3 g/kg diet, increases in dietary Ca within the range of 3–7 g Ca/kg diet did not influence femur strength or flexibility.

The present study resulted in somewhat lower estimates of Ca requirements than previous investigations testing the effect of dietary Ca concentration on bone Ca retention. Bernhart et al. (8) reported that 3.4 g Ca/kg diet resulted in maximal mineralization of bone in 24- to 28-d-old rats studied for 3 wk. Similarly, Kaup et al. (29) reported that the tibia Ca concentration maximized in weanling rats fed ≥3.6 g Ca/kg for 4 wk and Forbes et al. (30) reported maximum femur Ca in young rats fed ~4 g Ca/kg diet for 4 wk. The Ca requirements for laboratory rats set by the NRC (31) at 5 g Ca/kg diet were based on 3- to 4-wk studies with rapidly growing rats such as those cited above.

![FIGURE 2](image_url)

**FIGURE 2** Individual (< >) and mean (—) results for trabecular number (A), separation (B), and thickness (C) within the proximal tibia (see Fig. 1A) of weanling rats fed different dietary Ca concentrations for 13 wk. The trabecular number and separation were more substantially affected by calcium deficiency than the trabecular thickness. Means (n = 8) without a common letter differ, P < 0.05.

and 20 g Ca/kg (between 12 and 16 wk of age), with a constant dietary P (5.5 g/kg) nearly twice that of the dietary P used in the present study.

The present findings on a response threshold for the morphological properties of bone are also consistent with more limited published observations. Medeiros et al. (24) found lower femur density (measured by dual X-ray absorptiometry) and reduced strength (by 3-point flexure testing) in rats fed 1 vs. 5.2 g Ca/kg diet for 5 wk. In their reported micro-CT measurements with vertebrae, the Ca-deficient diet resulted in lower relative bone volume (BV/TV), trabecular number and thickness, and greater trabecular separation and SMI, indicating a more rod-like rather than plate-like structure (24), all of which were fully consistent with the present micro-CT observations with the tibia (Table 4). Turner et al. (25) found similar differences when comparing 1.25 to 5.0 g Ca/kg diet, although only in rats treated from 3 to 19 wk old; Ca deficiency did not affect older rats treated from 3 to 51 wk. Zhang et al. (26), studying 12-mo-old rats, found that Ca supplementation [56 mg Ca/(kg body weight-d)] of a diet containing 9 g Ca/kg diet did not influence micro-CT measurements (BV/TV, connectivity density, trabecular number, separation, thickness, or SMI) of the femur greater trochanter region. Together, these observations indicate that bone biomechanics and morphology are substantially influenced by dietary Ca deficiency but otherwise reach a response threshold, and that older rats are less susceptible to deficiency than younger rats. Bone mass depends on dietary P as well as Ca (8,27,28) and a relative P deficiency has been proposed as a possible explanation for increased fracture risk with a high Ca intake (7). Shapiro and Heaney (27) demonstrated that the femur strength of growing rats was impaired with inadequate dietary P (0.75 g/kg diet) and that increases in dietary Ca (1.25, 2.50, or 3.75 g/kg diet) did not effectively improve femur strength unless the Ca source also provided P. An excess of P (6, 12, or 18 g P/kg diet compared with 3 g P/kg diet) increased the Ca loss from the 45Ca-labeled skeleton of rats 8–14 mo of age (28) and the 2 highest P intakes significantly reduced bone weight, Ca, and P. However, the present results do not support concerns that an increase in dietary Ca without an accompanying increase in dietary P may adversely affect bone biomechanics and fracture risk. With a constant and adequate P concentration of 3 g/kg diet, increases in dietary Ca within the range of 3–7 g Ca/kg diet did not influence femur strength or flexibility.

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![FIGURE 3](image_url)

**FIGURE 3** Dietary Ca requirements with 95% CI, estimated using broken line modeling and multiple bone measurement criteria in rats consuming diets for 13 wk. For each parameter presented, the broken-line model was significant, P < 0.001, with R2 from 0.39 to 0.84 (tibia weight and trabecular thickness results did not fit the model well and are not reported). The dietary Ca requirement for reaching a threshold response was similar for most of the measured parameters of bone density, biomechanical strength, and structure, with the exception of biomechanical stress.
[The NRC requirements also considered the concerns about the influence of Ca:P ratios on development of nephrocalcinosis, which can be influenced by other dietary factors such as potassium and magnesium (10)]. The generally lower requirement of ~2.5 g Ca/kg diet for maximal bone mineralization in the present study is likely because the longer (13 wk) duration reduced the overall rate of bone growth and development. Whereas maximal bone mineralization required ≥3 g Ca/kg diet in the 3- to 4-wk studies cited above, there was no evidence of bone impairment by any parameter in rats fed ≥3 g Ca/kg diet for 13 wk in the present study. This suggests that any possible short-term impairments in bone mineralization in an earlier, more rapid period of growth may be compensated for with additional “catch-up” time consuming 3 g Ca/kg diet.

Of course, the interpretation of this research is limited by the use of laboratory rats. As indicated above, the rats’ young age likely increased their Ca requirements and responsiveness to dietary differences compared with older rats. The rats in this and most other studies were also relatively sedentary, without substantial load-bearing exercise that may influence the effect of dietary Ca on bone morphology (32).

In addition, there is uncertainty in comparing the mineral concentration of rat diets to the content of human diets. The use of body surface area is recommended as the basis for conversions of the dose of experimental drugs between species (33). The rats in the present study, with a mean body weight of ~150 g, consumed ~15 g diet/d (Table 1), so the 1 g Ca/kg diet is equivalent to 0.1 g Ca/kg body weight for the rats, or using a conversion based on body surface area, 0.016 g Ca/kg body weight for humans. Thus, the dietary Ca concentrations in the present study would be equivalent to ~1 to 7 g/d for a 60-kg human, a range extending from near to well above the amounts recommended for Ca intake (2). Therefore, the present research suggests a threshold response in bone mass, morphology, and biomechanical properties that is stable across a wide range of dietary Ca intakes that meet or exceed Ca requirements. Additional Ca consumption without additional P neither enhanced nor impaired these bone properties.

Although the lowest dietary Ca concentration in the present study was inadequate for bone development in these weanling rats, it is less likely to be inadequate in fully grown rats (25) or in adult humans. The differences between rats fed the 1 and 2 g Ca/kg diets in the present study, such as the 8% increase in bone density, 16% increase in tibia Ca, 160% increase in bending moment, and 320% increase in bone volume density in rats fed the latter diet substantially exceed the small differences observed in children’s total bone mineral content or upper limb mineral density (3), as well as the ~1–2% differences observed in adult bone density (4) in human Ca supplementation trials. These responses in human trials, without a clear reduction in the incidence of fractures (4), suggest that the initial Ca status of the subjects in these trials may be close to meeting or exceeding Ca requirements for maximizing bone mass and strength.

The Ca intake required for Ca sufficiency has been difficult to define. Limited Ca balance data have been used to estimate Ca intakes associated with threshold responses in both adolescents (34,35) and adults (2), but there was no suggestion of a threshold response in Ca balance with more extensive balance data in adults (36). In the Women’s Health Initiative clinical trial (37), supplementation of 36,282 postmenopausal women with 1000 mg Ca and 400 IU (10 μg) of vitamin D for 1 y increased hip bone density by 1.06% (and the risk of renal calculi), without significantly increasing spine or whole body bone density or significantly reducing the risk of hip, spine, or total fractures [although hip fractures were reduced with Ca plus vitamin D supplementation in a subgroup identified as more adherent (37)]. In that study, baseline Ca intakes were 1150 mg/d and one-third of subjects consumed <800 mg/d, but corrections for baseline Ca intakes did not modify the nonsignificant effect of Ca plus vitamin D supplementation on fracture risk. The results from the present study with young rats cannot help resolve the problem of estimating dietary requirements for human Ca sufficiency but do suggest that once such requirements have been met, additional Ca is unlikely to improve bone mass, structure, or strength.

In conclusion, Ca deficiency clearly impaired bone mass, morphology, and biomechanical properties in growing rats, but these parameters stabilized with Ca intakes of ~2.5 g/kg diet and were not further improved or impaired with additional dietary Ca. Dietary P remained constant at recommended levels and there was no indication of a relative P deficiency adversely affecting bone with increasing Ca intake. It is important to define sufficient Ca intakes for humans, as further increases in dietary Ca are unlikely to be beneficial.

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Literature Cited


Diet calcium, bone mass, morphology, and biomechanics 1467


