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Diet Calcium Level but Not Calcium Supplement Particle Size Affects Bone Density and Mechanical Properties in Ovariectomized Rats[1,2]

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Abstract

Calcium (Ca) supplements, especially Ca carbonate ($CaCO_3$), are the main alternative sources of dietary Ca and an important part of a treatment regimen for osteoporosis, the most common metabolic bone disorder of aging and menopause. In a female ovariectomized (OVX) rat model for studying postmenopausal osteoporosis, we tested the hypothesis that a small compared with a large particle size of $CaCO_3$ (13.0- vs. 18.5-μm geometric diameter) would result in increased Ca balance and subsequently bone mass and that this would be affected by dietary Ca level. We used 6-mo-old rats that were OVX either at 6 or 3 mo of age as models of early or stable menopausal status, respectively. The rats received semipurified diets that contained either 0.4 or 0.2% dietary Ca provided from $CaCO_3$ of 2 particle sizes. A group of Sham-operated rats with intact ovaries served as control and were fed 0.4% dietary Ca from large particles. Estrogen deficiency as a result of ovariectomy had an adverse effect on bone density, mineral content, and bone mechanical properties ($P < 0.001$). Reducing dietary Ca from 0.4 to 0.2% resulted in significant adverse effects on bone density and mechanical properties ($P < 0.001$). The particle size of $CaCO_3$ did not affect total Ca balance, bone dual energy X-ray absorptiometry and peripheral quantitative computed tomography indices, bone ash and Ca content, or the mechanical determinants of bone strength. We conclude that a decrease in particle size of $CaCO_3$ to 70% of that typically found in Ca supplements does not provide a benefit to overall Ca metabolism or bone characteristics and that the amount of Ca consumed is of greater influence in enhancing Ca nutrition and skeletal strength. J. Nutr. 139: 1308–1314, 2009.

Introduction

Calcium (Ca)[5] supplements are important alternative sources of Ca that have positive effects in minimizing bone loss during aging in some (1–4), but not all (5), studies. The advantages of Ca supplements to bone are realized when vitamin D status is adequate (6). Ca and vitamin D supplementation are recommended as a first line of defense against osteoporosis (7), especially for those who do not consume the levels of dairy products recommended by the 2005 Dietary Guidelines for Americans (8). The bioavailability of Ca varies in Ca supplements and can be affected by such factors as disintegration, solubility, chelate formation, and food-drug interactions (9–12). Although solubility of a Ca source at neutral pH has been reported to have little effect on absorbability (13), the amount of Ca available during absorption is dependent in part on its dissolution by the influence of gastric acidity (14). Except for some cases of intact absorption of chelates (e.g. Co in vitamin B-12), it is usually assumed that the most soluble and therefore most absorbable form of any element is its simple ionic state, i.e. Ca^{2+} ion in this case. To release the maximum amount of ion from a salt, disintegration of the salt (15) and subsequent solubility and dissociation of the ion can be enhanced by reducing the particle size and increasing the amount of surface area. No studies to our knowledge have investigated the effects of $CaCO_3$ particle size on Ca retention and bone parameters in human or animal models. Guinotte et al. (16) investigated the difference between coarse and fine $CaCO_3$ particle size on Ca solubility in poultry. The ratio of soluble:insoluble Ca in the gizzard was 11-fold higher in chicks ingesting fine Ca compared with those fed coarse Ca in their diet. However, the particle sizes studied in poultry were much larger than the typical size found in human dietary supplements.

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[5] Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; DXA, dual energy X-ray absorptiometry; OVX, ovariectomized; pQCT, peripheral quantitative computed tomography.
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We tested the hypothesis that a smaller particle size of $CaCO_3$ would result in increased Ca balance and a consequent increase in bone mass in rats. Because human studies have shown that the efficacy of Ca supplementation can depend on both the stage of menopause (17) and amount of supplementation (18,19), we used ovariectomized (OVX)[5] rats as a model to evaluate the effect of particle size under the conditions of early and stable menopause and adequate and inadequate Ca intake.

Materials and Methods

Rats and study design. Rats were maintained and treated under protocols approved by the Purdue University Animal Care and Use Committee. A total of 265 female Sprague Dawley rats (from Harlan International) were used in 2 experiments (**Table 1**). Rats in Expt. 1 and 2 were OVX at 6 and 3 mo of age, early and stable OVX, respectively, and started the experimental diets at 6 mo of age. Dietary $CaCO_3$ supplements were provided by Delavau. Particle sizes of the Ca supplements were assessed using laser diffraction with a Beckman Coulter LS 1320. The larger size is typically used in commercially available supplements and had a particle geometric diameter (mean, median, SD) of 18.55, 17.04, and 13.56 μm. Further grinding of the large-size product produced the smaller particle size with a diameter of 13.39 μm, 11.52, and 10.4 (mean, median, SD). Semipurified diets (AIN 93M) (20) were prepared from the 2 particle sizes of $CaCO_3$. Diets contained 0.4 or 0.2% Ca, levels previously shown to provide adequate or marginal Ca to rats, respectively (21).

Both experiments included additional similar rats that underwent a Sham OVX procedure and served as control groups. These rats were fed a 0.4% Ca diet from the standard 18.5-μm particle size. Rats in Expt. 1 and 2 were killed using CO_2 gas at 8 and 9 mo of age, respectively.

Data and specimen collection. Food intake and body weight were monitored weekly throughout the experiments. During the last week of each experiment, a whole-body scan of rats from selected groups was obtained by dual energy X-ray absorptiometry (DXA) using a Prodigy scanner (GE Lunar) to examine the whole-body bone mineral density (BMD), bone mineral content (BMC), and fat content. All OVX rats were then placed in individual metabolic cages to measure a complete Ca balance, which consisted of dietary intake and urinary and fecal excretion, over 2 consecutive days. At termination, L4 vertebrae, femora, and tibiae were collected from each rat. Various measurements were conducted on the bone samples. The vertebrae were fixed in 10% neutral buffered formalin for 24 h and then stored in 70% ethanol at 4°C for imaging by peripheral quantitative computed tomography (pQCT) (XCT Research SA; Stratec Medizintechnic). Each scan was acquired with a 0.12-mm voxel size and the scan line was adjusted using the scout view of the software (Stratec, version 5.50d; Stratec Medizintechnic). A constant threshold of 364 mg/cm^3 was used to segment the bone area from the marrow regions. Total, trabecular, and cortical volumetric BMD and the total trabecular area were measured for each sample. One tibia and a femur from each rat were cleaned of soft tissues and measured by pixi DXA (GE Lunar) for BMD and BMC. Volumetric density of the bones was also estimated by the weight change in water method (22) to calculate bone mass per unit volume. Bones were then ashed in a muffle furnace at 500°C for 18 h and weighed to measure BMC. Bone ash concentration, which has been shown to be very sensitive to different levels of Ca intake (23), was determined by the ratio of ash weight:bone volume. Ash then was dissolved in 70% nitric acid (JT Baker) overnight. The acid ash mixture was diluted with distilled water and further with 0.1% lanthanum chloride for Ca measurements. The Ca content in all samples, including that collected from balance studies, was assayed by atomic absorption spectrometry (AAnalyst 300; Perkin Elmer) using standard procedures.

Contralateral limb bones dissected for mechanical testing were wrapped in saline-soaked gauze and stored at −20°C until testing, when they were thawed at room temperature and kept wet using 0.9% saline solution. Three-point bending tests were performed at the mid-diaphysis of all femurs and tibias using a servohydraulic machine (MTS-810, MTS). Bones were consistently oriented for testing with the anterior cortex in tension. Mechanical properties including bone ultimate load (breaking force), structural toughness (energy to failure), and stiffness (rigidity) were determined from the force deformation curves (24).

Statistical analysis. Results are presented as means ± SE. In each experiment, a comparison was made using 1-way ANOVA between Sham and OVX groups receiving the same diet. Two-way factorial ANOVA was used to determine the main effects and possible interactions of dietary Ca levels and particle size on Ca balance and bone parameters in OVX groups. An effect was considered significant at $P \leq 0.05$. Whole-body DXA indices were also compared in response to particle size of $CaCO_3$ and the stage of ovariectomy (Table 6) with adjustment for multiple comparisons using Bonferroni test to maintain an overall type 1 error rate of 0.05. For all parameters with significant *P*-values, intergroup differences were determined with the Tukey pairwise test. Statistical analyses were performed using Minitab 15 statistical software.

TABLE 1 Description of the experiments for studying $CaCO_3$ of different particle size at different Ca levels in 6-mo-old female rats

Dietary Ca, %	Mean geometric particle size of $CaCO_3$, μm	Rats, *n*
Expt. 1: OVX or Sham-OVX at 6 mo; killed at 8 mo		
OVX		
0.4	13	29
	18.5	29
0.2	13	29
	18.5	29
Sham		
0.4	18.5	29
Expt. 2: OVX or Sham-OVX at 3 mo; killed at 9 mo		
OVX		
0.4	13	24
	18.5	24
0.2	13	24
	18.5	24
Sham		
0.4	18.5	24

Results

In both experiments, ovariectomizing rats resulted in an increase in body weight within 1 wk of surgery, which was maintained throughout the experiment. OVX rats were 15% heavier than those in the Sham group by wk 2 after surgery. Estrogen deficiency was associated with increased food intake, which has been previously reported in rats and humans (25–27). Feed intake, feed efficiency (data not shown), and body weight (**Tables 2** and **3**) of the rats were not affected by dietary level of Ca or $CaCO_3$ particle size.

Expt. 1 (early OVX rats). Estrogen deficiency in OVX rats resulted in a lower femoral BMD, BMC, and Ca content ($P < 0.001$) compared with the Sham group (Table 2). The Ca content of the tibia was also 9% lower ($P < 0.001$) in OVX rats, which was reflected in a 4% lower tibial ash weight/dry bone weight ($P < 0.001$).

Rats receiving 0.2% compared with 0.4% dietary Ca had a lower femoral BMC ($P = 0.05$) that was associated with adverse

TABLE 2 Body weight and bone characteristics of 8-mo-old OVX rats and their Sham-operated controls fed 0.4% dietary Ca from large $CaCo_3$ particles for 2 mo (Expt. 1)[1,2]

	Sham	OVX
Body weight, *g*	310.6 ± 5.0	365.4 ± 4.0*
Femur		
pixi BMD, *g/cm²*	0.219 ± 0.001	0.195 ± 0.001*
pixi BMC, *g*	0.476 ± 0.005	0.451 ± 0.005*
Ca, *% dry weight*	24.3 ± 0.003	22.1 ± 0.002*
Tibia		
Ash, *g*	0.310 ± 0.004	0.307 ± 0.003
Ash:dry weight, *g*	0.602 ± 0.003	0.576 ± 0.003*
Ca, *% dry weight*	23.8 ± 0.002	21.8 ± 0.002*
Femoral mechanical properties		
Ultimate load, *N*	150.5 ± 3.0	148.2 ± 2.7
Toughness, *Nmm*	50.9 ± 2.4	50.4 ± 2.0
Stiffness, *Nmm*	436.4 ± 12.1	432.2 ± 14.8
Tibial mechanical properties		
Ultimate load, *N*	105.0 ± 2.3	107.2 ± 2.1
Toughness, *Nmm*	33.8 ± 1.7	35.5 ± 1.6
Stiffness, *Nmm*	245.1 ± 7.1	239.2 ± 8.1

[1] Values are means ± SE, $n = 29$. *Different from Sham, $P < 0.001$.
[2] Rats were OVX or Sham OVX at 6 mo of age.

effects on mechanical properties of bone (**Table 4**). The femoral ultimate load and toughness were 7 and 16% lower in rats receiving low Ca in their diet, respectively ($P < 0.001$). Balance data showed that reducing dietary Ca by one-half resulted in a decrease in fecal Ca by 50% ($P < 0.001$) and in urine by 15% ($P = 0.01$). However, overall Ca balance did not differ between the groups (Table 4).

The size of the $CaCo_3$ particle in the diet did not affect Ca balance, BMD, or bone strength. There was also no interaction between dietary Ca and the supplement particle size for these variables (Table 4).

TABLE 3 Body weight and bone characteristics of 9-mo-old OVX rats and their Sham-operated controls fed 0.4% dietary Ca from large $CaCo_3$ particles for 3 mo (Expt. 2)[1,2]

	Sham	OVX
Body weight, *g*	308.4 ± 5.9	372.8 ± 5.9*
Femur		
pixi BMD, *g/cm²*	0.218 ± 0.001	0.192 ± 0.001*
pixi BMC, *g*	0.480 ± 0.006	0.430 ± 0.004*
Volumetric density, *g/cm³*	1.540 ± 0.002	1.470 ± 0.002*
Ash, *g*	0.420 ± 0.005	0.382 ± 0.005*
Ash concentration, *%*	63.6 ± 0.4	56.6 ± 0.4*
Ca, *mg/g ash*	420.4 ± 4.6	415.1 ± 4.6
Tibia		
Volumetric density, *g/cm³*	1.558 ± 0.004	1.528 ± 0.003*
Ash weight, *g*	0.309 ± 0.003	0.305 ± 0.003
Ash concentration, *%*	67.0 ± 0.5	64.0 ± 0.4*
Femoral mechanical properties		
Ultimate load, *N*	158.8 ± 2.8	145.8 ± 2.4*
Toughness, *Nmm*	47.4 ± 1.5	42.6 ± 1.7*
Stiffness, *Nmm*	427.4 ± 14.0	401.6 ± 10.6*
Tibial mechanical properties		
Ultimate load, *N*	97.8 ± 1.4	100.2 ± 1.0
Toughness, *Nmm*	30.0 ± 0.9	31.4 ± 1.3
Stiffness, *Nmm*	238.9 ± 7.0	246.8 ± 6.5
Vertebral pQCT		
Trabecular area, *mm²*	60.6 ± 0.6	69.6 ± 0.4*
Trabecular density, *mg/cm³*	345.1 ± 5.1	252.8 ± 2.6*
Cortical density, *mg/cm³*	1216.0 ± 3.1	1176.8 ± 2.1*
Total density, *mg/cm³*	581.6 ± 7.4	441.9 ± 3.1*

[1] Values are means ± SE, $n = 24$. *Different from Sham, $P < 0.001$.
[2] Rats were OVX or Sham OVX at 3 mo of age.

Expt. 2 (stable OVX rats). Similar to Expt. 1, ovariectomizing rats resulted in continued adverse effects on bone parameters. The OVX rats had lower BMD, BMC, and bone ash compared with the Sham group (Table 3) ($P < 0.001$). Unlike Expt. 1 in which femoral strength and toughness were not affected by estrogen deficiency, the mechanical properties of femurs were adversely affected in this experiment, perhaps due to longer duration of estrogen deficiency. This was demonstrated by lower femoral ultimate load (8%), toughness (11%), and bone stiffness (6%) in OVX rats compared with the Sham group ($P < 0.001$) (Table 3). Tibial mechanical measurements were not affected by ovariectomizing rats. The pQCT scanning of L4 vertebrae showed that the trabecular bone area was 15% higher ($P < 0.001$) in OVX rats, reflecting a higher bone remodeling rate compared with the Sham group. The vertebral bone loss as a result of estrogen deficiency was 24% ($P < 0.001$) as measured by pQCT. This was accounted for by bone loss in both trabecular (27%; $P < 0.001$) and cortical bone (3%; $P < 0.001$) compared with Sham-operated rats.

Feeding rats 0.2% Ca compared with 0.4% Ca had adverse effects on bone characteristics (**Table 5**). Rats receiving the inadequate Ca diet had a lower femoral bone density ($P < 0.001$), ash concentration ($P = 0.002$), and bone stiffness ($P = 0.03$). The tibial bone density, ultimate breaking load, and stiffness were also lower in rats fed the lower Ca diet ($P < 0.05$). The pQCT data of the vertebral bone also showed a lower density of trabecular, but not of cortical bone, in the 0.2% Ca-fed rats ($P = 0.003$) (Table 5). They also had a 50% lower Ca excretion in feces and 30% less Ca in urine than rats fed 0.4% Ca ($P < 0.001$), but overall Ca balance was not affected by dietary Ca concentration.

Bone characteristics or Ca balance did not differ as a result of small or large $CaCO_3$ particle size in diets. The vertebrae scanned by pQCT also showed no affect of particle size. Dietary Ca level and the particle size of the Ca supplement did not interact to affect the variables measured.

Whole-body DXA. In both experiments, whole-body BMD was lower in the OVX rats than in the Sham group ($P < 0.001$) and the body fat content was at least 3 times that of Sham group in early OVX rats and more than 2 times that of the Sham group in stable OVX rats ($P < 0.001$) (**Table 6**). The content of body fat was less in the stable OVX rats than in early OVX rats. The stable OVX rats had greater whole-body BMC than early OVX rats ($P < 0.001$) due to being 1 mo older at the time of termination, but whole-body BMD did not change. Providing dietary Ca to OVX rats from large compared with small particle sizes of the supplement did not differentially affect whole-body BMC, BMD, or fat content.

Discussion

We hypothesized that a smaller particle size of $CaCO_3$ in rat diets would improve bone mass via increased Ca balance.

TABLE 4 Body weight, Ca retention, and bone characteristics of 8-mo-old OVX rats fed 2 levels of dietary Ca from large or small $CaCO_3$ particles for 2 mo (Expt. 1)[1,2]

	0.4% Ca		0.2% Ca		2-Way ANOVA *P*-values		
	Large particle	Small particle	Large particle	Small particle	Ca	Size	Ca × size
Body weight, *g*	365.4 ± 4.0	361.7 ± 4.8	371.6 ± 5.0	356.2 ± 6.0	NS	NS	NS
Ca balance, *mg/2 d*							
Intake	114.1 ± 2.2	118.0 ± 2.8	60.9 ± 1.6	57.1 ± 1.5	<0.001	NS	NS
Fecal excretion	106.4 ± 3.4	102.4 ± 4.3	56.4 ± 2.6	51.8 ± 1.9	<0.001	NS	NS
Urinary excretion	4.4 ± 0.2	4.2 ± 0.2	4.0 ± 0.3	3.3 ± 0.2	0.01	NS	NS
Total balance	3.3 ± 2.7	11.4 ± 3.9	0.5 ± 1.7	2.0 ± 1.7	NS	NS	NS
Net C absorption	7.7 ± 2.7	15.6 ± 3.9	4.5 ± 1.9	5.3 ± 1.8	NS	NS	NS
Femur							
pixi BMD, g/cm^2	0.195 ± 0.001	0.199 ± 0.001	0.197 ± 0.001	0.192 ± 0.001	NS	NS	NS
pixi BMC, *g*	0.451 ± 0.005	0.448 ± 0.007	0.447 ± 0.007	0.425 ± 0.007	0.05	NS	NS
Ca, *% dry weight*	22.1 ± 0.002	21.7 ± 0.002	23.0 ± 0.002	22.3 ± 0.003	NS	NS	NS
Tibia							
Ash, *g*	0.307 ± 0.003	0.306 ± 0.004	0.302 ± 0.004	0.293 ± 0.004	NS	NS	NS
Ash:dry weight, *g*	0.576 ± 0.003	0.589 ± 0.002	0.579 ± 0.004	0.580 ± 0.004	NS	NS	NS
Ca, *% dry weight*	21.8 ± 0.002	21.8 ± 0.003	21.8 ± 0.002	21.8 ± 0.002	NS	NS	NS
Femoral mechanical properties							
Ultimate load, *N*	148.2 ± 2.7	149.5 ± 2.2	139.9 ± 2.7	141.3 ± 2.2	<0.001	NS	NS
Toughness, *Nmm*	50.4 ± 2.0	50.6 ± 1.9	42.5 ± 1.3	43.5 ± 2.4	<0.001	NS	NS
Stiffness, *Nmm*	432.2 ± 14.8	439.1 ± 7.3	422.2 ± 11.5	430.6 ± 13.1	NS	NS	NS
Tibial mechanical properties							
Ultimate load, *N*	107.2 ± 2.1	104.0 ± 2.5	102.5 ± 1.8	102.7 ± 2.3	NS	NS	NS
Toughness, *Nmm*	35.5 ± 1.6	33.0 ± 1.6	32.9 ± 0.8	34.2 ± 1.9	NS	NS	NS
Stiffness, *Nmm*	239.2 ± 8.1	243.0 ± 6.7	231.8 ± 7.5	238.3 ± 7.5	NS	NS	NS

[1] Values are means ± SE, $n = 24$.
[2] Rats were OVX or Sham OVX at 6 mo of age.

Particle size of $CaCO_3$ did not affect Ca balance or endpoint bone parameters in rats. Particle size of Ca sources do influence Ca nutrition in chickens and laying hens (28,29), but this is unlikely to be related to Ca absorption. Because eggshell is usually calcified during the night when no Ca is available from the feed, the advantage of using coarser and less soluble particles is the constant release of Ca, with consequent lower mobilization of Ca from bone (30,31). Therefore, Ca salts with larger particle sizes have an indirect sparing effect on bone and no influence on the mechanism of Ca absorption. In this experiment, the range of particle size, expressed as mean geometric diameter, was broad within each product and the distribution about the mean was not normal. We did not observe a difference in Ca retention due to particle size. Power calculations showed that with the SD and sample size in our study, we had an 80% chance to detect a significant difference in Ca retention of 19.2 mg/2 d at $P < 0.05$. The small size of the particle is unlikely to affect Ca uptake at the enterocyte level, because Ca is absorbed in its ionic form, with an ionic radius scale of picometer (1×10^{-12} m) compared with the finest supplemental Ca of micrometer (1×10^{-6} m) scales. Particle size, however, may influence overall Ca absorption outside the range used in our study. For example, nanotechnology has been reported to improve the absorption rate of drugs or nutrients due to an increase in surface area and resulting solubility of the particles (32–34). The process involves milling micrometer- to nanometer-sized drug crystals. Nanometer- compared with micrometer-sized pearl powder has shown a higher bioavailability of Ca in humans as assessed by serum and urinary Ca and serum parathyroid hormone concentrations (35). In rats, supplementing the diet with nano-Ca-enriched milk was reported to increase urinary excretion of Ca (36). The study was limited in sample size. The authors also reported a lower feed efficiency in supplemented rats but did not explain their finding. Nevertheless, there is no report to our knowledge on the effect of supplementation with nanometer-scale Ca particles on the endpoint bone parameters. We studied a practical range from a commercial production standpoint.

On the other hand, the amount of Ca is a major determinant of Ca absorption. Although Ca absorption efficiency decreases with increasing Ca load, the total amount absorbed still increases (37). The Ca intake is particularly important in acquiring peak bone mass during puberty to protect later in life against bone fracture risks (38,39). The ex vivo bone measurements in rats fed marginal dietary Ca in these experiments showed a decrease in bone density and consequent mechanical measures. We targeted 0.2% as a suboptimal Ca intake rather than a more overt Ca-deficient diet, such as 0.02% Ca as studied by others (40). In contrast to our data that reducing dietary Ca from 0.4 to 0.2% in rats adversely affected the bone mechanical properties, Breitman et al. (41) found no difference in femoral or vertebral BMD and peak load between OVX rats fed 0.2 or 2.5% Ca diets. They also reported that femoral toughness, which was 15% lower in rats fed the lower level of Ca was not different between the 2 groups. It appears that their study did not have enough power to detect significant differences due to the small sample size ($n = 10$/group). Our data suggest that strategies focusing on enhancing the Ca intake can be more effective in improving overall Ca nutrition than those modifying products for greater Ca absorbability.

Reducing dietary Ca from 0.4 to 0.2% resulted in a decrease in fecal Ca loss by 50% and urine Ca loss by 15–30% ($P <$

TABLE 5 Body weight, Ca retention, and bone characteristics of 9-mo-old OVX rats fed 2 levels of dietary Ca from large or small $CaCO_3$ particles for 3 mo (Expt. 2)[1,2]

	0.4% Ca		0.2% Ca		ANOVA		
	Large particle	Small particle	Large particle	Small particle	Ca	Size	Ca × size
Body weight, *g*	372.8 ± 5.9	375.5 ± 10.2	381.6 ± 9.2	371.2 ± 10.0	NS	NS	NS
Ca balance, *mg/2 d*							
Intake	107.5 ± 2.7	105.6 ± 2.6	53.3 ± 1.3	54.5 ± 1.5	<0.001	NS	NS
Fecal excretion	104.5 ± 6.0	104.0 ± 5.7	50.7 ± 2.2	54.0 ± 1.9	<0.001	NS	NS
Urinary excretion	4.7 ± 0.2	4.5 ± 0.3	3.5 ± 0.3	3.2 ± 0.3	<0.001	NS	NS
Total balance	−1.7 ± 4.2	−2.9 ± 4.1	−0.9 ± 1.8	−2.7 ± 2.3	NS	NS	NS
Net Ca absorption	3.0 ± 4	1.6 ± 6.2	2.6 ± 3.0	0.5 ± 3.3	NS	NS	NS
Femur							
pixi BMD, *g/cm²*	0.192 ± 0.001	0.192 ± 0.001	0.187 ± 0.001	0.188 ± 0.001	0.003	NS	NS
pixi BMC, *g*	0.430 ± 0.004	0.432 ± 0.006	0.425 ± 0.006	0.422 ± 0.007	NS	NS	NS
Volumetric density, *g/cm³*	1.470 ± 0.002	1.476 ± 0.003	1.463 ± 0.002	1.460 ± 0.002	<0.001	NS	NS
Ash, *g*	0.382 ± 0.005	0.386 ± 0.005	0.376 ± 0.005	0.372 ± 0.007	NS	NS	NS
Ash concentration, *%*	56.6 ± 0.4	57.4 ± 0.3	55.8 ± 0.3	55.8 ± 0.3	0.002	NS	NS
Ca, *mg/g ash*	415.1 ± 4.6	422.4 ± 2.3	421.0 ± 4.1	423.7 ± 2.9	NS		
Tibia							
Volumetric density, *g/cm³*	1.528 ± 0.003	1.528 ± 0.003	1.520 ± 0.003	1.515 ± 0.004	0.01	NS	NS
Ash weight, *g*	0.305 ± 0.003	0.306 ± 0.003	0.299 ± 0.003	0.299 ± 0.004	NS	NS	NS
Ash concentration, *%*	64.0 ± 0.4	63.8 ± 0.5	63.2 ± 0.4	63.0 ± 0.5	NS	NS	NS
Femoral mechanical properties							
Ultimate load, *N*	145.8 ± 2.4	147.4 ± 2.0	143.2 ± 2.4	142 ± 3.1	NS	NS	NS
Toughness, *Nmm*	42.6 ± 1.7	43.3 ± 1.3	41.3 ± 1.9	39.8 ± 1.8	NS	NS	NS
Stiffness, *Nmm*	401.6 ± 10.6	392.6 ± 12.3	380.7 ± 10.9	384.5 ± 11.5	0.03	NS	NS
Tibial mechanical properties							
Ultimate load, *N*	100.2 ± 1.0	99.8 ± 1.4	95.0 ± 1.5	96.0 ± 1.8	0.03	NS	NS
Toughness, *Nmm*	31.4 ± 0.9	29.2 ± 0.7	28.9 ± 0.7	29.5 ± 1.0	NS	NS	NS
Stiffness, *Nmm*	246.8 ± 6.5	245.6 ± 6.9	234.2 ± 5.8	230.2 ± 6.0	0.03	NS	NS
Vertebral pQCT							
Trabecular area, *mm²*	69.6 ± 0.4	68.5 ± 0.4	70.1 ± 0.5	67.9 ± 1.4	NS	NS	NS
Trabecular density, *mg/cm³*	252.8 ± 2.6	252.1 ± 3.8	241.6 ± 3.1	238.5 ± 4.1	0.003	NS	NS
Cortical density, *mg/cm³*	1176.8 ± 2.1	1178.0 ± 2.6	1170.8 ± 2.1	1173.1 ± 1.9	NS	NS	NS
Total density, *mg/cm³*	441.9 ± 3.1	441.8 ± 5.0	425.4 ± 4.1	422.8 ± 6.4	<0.001	NS	NS

[1] Values are means ± SE, $n = 24$.
[2] Rats were OVX or Sham OVX at 3 mo of age.

0.001) with no difference in total Ca balance. Therefore, it was somewhat surprising that bone strength was adversely affected. The apparent discrepancy is likely due to the lower sensitivity of balance assays relative to a number of bone measures such as BMD, BMC, and mechanical properties. Therefore, whereas the Ca balance was adaptively maintained in rats fed the low-Ca diet, bone strength was negatively affected, suggesting that the body's mechanisms to excrete less Ca at marginal intakes are not sufficient to support the structural integrity of bone.

Bone parameters were also largely diminished in response to ovariectomy. Comparatively, hormone deficiency exceeded the effects of low dietary Ca on bone, regardless of whether the rats were recently or were stable OVX. Consistent with other studies (42–44), ovariectomy led to a reduction in bone density at a number of skeletal sites. The mechanism by which estrogen deficiency causes bone loss is still under investigation (45,46). The bone antiresorptive activity of estrogen is a result of multiple genomic and nongenomic effects of the hormone on

TABLE 6 Effects of ovariectomy and dietary $CaCO_3$ particle size in the diet on DXA indices in rats receiving 0.4% dietary Ca for 2 (Expt. 1) or 3 mo (Expt. 2)[1]

	Expt. 1			Expt. 2		
		Early OVX			Stable OVX	
Whole-body DXA	Sham	Large	Small	Sham	Large	Small
BMD, *g/cm²*	0.313 ± 0.001[a]	0.297 ± 0.001[b]	0.298 ± 0.001[b]	0.311 ± 0.001[a]	0.302 ± 0.001[b,c]	0.305 ± 0.002[c]
BMC, *g*	8.86 ± 0.19[a]	8.17 ± 0.25[b]	7.93 ± 0.27[b]	9.70 ± 0.30[c]	9.40 ± 0.20[c]	9.80 ± 0.40[c]
Fat, *g*	23.5 ± 1.7[a]	80.8 ± 4.6[b]	88.3 ± 6.1[b]	19.3 ± 3.5[a]	52.6 ± 7.5[c]	34.9 ± 6.7[c]

[1] Values are means ± SE; $n = 16$. Means in a row without a common letter differ, $P < 0.001$.

bone marrow and bone cells, which decrease osteoclast differentiation and capacity of bone resorption and increase their apoptosis via modulation of immune system cytokines (47).

Our study included a large sample size to examine various Ca and bone parameters in response to the particle size of the Ca supplement and to early and stable hormone deficiency. Bone parameters were unaffected by particle size of the Ca supplement in rats that underwent oviarectomy either immediately or 3 mo before the feeding trial. In contrast, bone parameters were largely diminished in response to inadequate dietary Ca and to estrogen deficiency. We conclude that hormonal status and an adequate intake of Ca are major factors in determining Ca and bone status and particle size of the Ca supplement has no effect.

Literature Cited

1. Dawson-Hughes B, Dallal GE, Krall EA, Sadowski L, Sahyoun N, Tannenbaum S. A controlled trial of the effect of calcium supplementation on bone density in postmenopausal women. N Engl J Med. 1990;323:878–83.
2. Reid IR, Ames RW, Evans MC, Gamble GD, Sharpe SJ. Effect of calcium supplementation on bone loss in postmenopausal women. N Engl J Med. 1993;328:460–4.
3. Aloia JF, Vaswani A, Yeh JK, Ross PL, Flaster E, Dilmanian FA. Calcium supplementation with and without hormone replacement therapy to prevent postmenopausal bone loss. Ann Intern Med. 1994;120:97–103.
4. Cumming RG, Nevitt MC. Calcium for prevention of osteoporotic fractures in postmenopausal women. J Bone Miner Res. 1997;12: 1321–9.
5. Bischoff-Ferrari HA, Dawson-Hughes B, Baron JA, Burckhardt P, Li R, Spiegelman D, Specker B, Orav JE, Wong JB, et al. Calcium intake and hip fracture risk in men and women: a meta-analysis of prospective cohort studies and randomized controlled trials. Am J Clin Nutr. 2007;86:1780–90.
6. Boonen S, Lips P, Bouillon R, Bischoff-Ferrari HA, Vanderschueren D, Haentjens P. Need for additional calcium to reduce the risk of hip fracture with vitamin d supplementation: evidence from a comparative metaanalysis of randomized controlled trials. J Clin Endocrinol Metab. 2007;92:1415–23.
7. Department of Health and Human Services and the Surgeon General Office. The Surgeon General's Report on Bone Health and Osteoporosis. Rockville (MD): Public Health Service Office of the Surgeon General; 2004. p. 1–10.
8. Department of Health and Human Services and USDA. Dietary guidelines for Americans. Executive summary. Washington (DC): U.S. Government Printing Office; 2005. p. v–ix, App. B4–B5, p. 59–60.
9. Heaney RP, Weaver CM. Oxalate: effect on calcium absorbability. Am J Clin Nutr. 1989;50:830–2.
10. Pak CY, Poindexter J, Finlayson B. A model system for assessing physicochemical factors affecting calcium absorbability from the intestinal tract. J Bone Miner Res. 1989;4:119–27.
11. Brennan MJ, Duncan WE, Wartofsky L, Butler VM, Wray HL. In vitro dissolution of calcium carbonate preparations. Calcif Tissue Int. 1991;49:308–12.
12. Whiting SJ, Pluhator MM. Comparison of in vitro and in vivo tests for determination of availability of calcium from calcium carbonate tablets. J Am Coll Nutr. 1992;11:553–60.
13. Heaney RP, Recker RR, Weaver CM. Absorbability of calcium sources: the limited role of solubility. Calcif Tissue Int. 1990;46:300–4.
14. Greger JL, Krashoc CL. Effects of a variety of calcium sources on mineral metabolism in anemic rats. Drug Nutr Interact. 1988;5:387–94.
15. Carr CJ, Shangraw RF. Nutritional and pharmaceutical aspects of calcium supplementation. Am Pharm. 1987;NS27:49–50, 4–7.
16. Guinotte F, Gautron J, Nys Y, Soumarmon A. Calcium solubilization and retention in the gastrointestinal tract in chicks (Gallus domesticus) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. Br J Nutr. 1995;73:125–39.
17. Elders PJ, Lips P, Netelenbos JC, van Ginkel FC, Khoe E, van der Vijgh WJ, van der Stelt PF. Long-term effect of calcium supplementation on bone loss in perimenopausal women. J Bone Miner Res. 1994;9: 963–70.
18. Boonen S, Vanderschueren D, Haentjens P, Lips P. Calcium and vitamin D in the prevention and treatment of osteoporosis: a clinical update. J Intern Med. 2006;259:539–52.
19. Tang BM, Eslick GD, Nowson C, Smith C, Bensoussan A. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis. Lancet. 2007;370:657–66.
20. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993;123:1939–51.
21. Forbes RM, Weingartner KE, Parker HM, Bell RR, Erdman JW Jr. Bioavailability to rats of zinc, magnesium and calcium in casein-, egg- and soy protein-containing diets. J Nutr. 1979;109:1652–60.
22. Zhang B, Coon CN. The relationship of various tibia bone measurements in hens. Poult Sci. 1997;76:1698–701.
23. Cheng TK, Coon CN. Sensitivity of various bone parameters of laying hens to different daily calcium intakes. Poult Sci. 1990;69:2209–13.
24. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993;14:595–608.
25. Parker GC, McKee ME, Bishop C, Coscina DV. Whole-body metabolism varies across the estrous cycle in Sprague-Dawley rats. Physiol Behav. 2001;74:399–403.
26. Geary N. Estradiol, CCK and satiation. Peptides. 2001;22:1251–63.
27. Lyons PM, Truswell AS, Mira M, Vizzard J, Abraham SF. Reduction of food intake in the ovulatory phase of the menstrual cycle. Am J Clin Nutr. 1989;49:1164–8.
28. Rao KS, Roland DA Sr. Influence of dietary calcium level and particle size of calcium source on in vivo calcium solubilization by commercial Leghorns. Poult Sci. 1989;68:1499–505.
29. Rao KS, Roland DA Sr. In vivo limestone solubilization in commercial Leghorns: role of dietary calcium level, limestone particle size, in vitro limestone solubility rate, and the calcium status of the hen. Poult Sci. 1990;69:2170–6.
30. Zhang B, Coon CN. The relationship of calcium intake, source, size, solubility in vitro and in vivo, and gizzard limestone retention in laying hens. Poult Sci. 1997;76:1702–6.
31. Lichovnikova M. The effect of dietary calcium source, concentration and particle size on calcium retention, eggshell quality and overall calcium requirement in laying hens. Br Poult Sci. 2007;48:71–5.
32. Merisko-Liversidge EM, Liversidge GG. Drug nanoparticles: formulating poorly water-soluble compounds. Toxicol Pathol. 2008;36: 43–8.
33. Douroumis D, Fahr A. Nano- and micro-particulate formulations of poorly water-soluble drugs by using a novel optimized technique. Eur J Pharm Biopharm. 2006;63:173–5.
34. Jinno J, Kamada N, Miyake M, Yamada K, Mukai T, Odomi M, Toguchi H, Liversidge GG, Higaki K, et al. Effect of particle size reduction on dissolution and oral absorption of a poorly water-soluble drug, cilostazol, in beagle dogs. J Control Release. 2006;111: 56–64.
35. Chen HS, Chang JH, Wu JS. Calcium bioavailability of nanonized pearl powder for adults. J Food Sci. 2008;73:H246–51.
36. Park HS, Ahn J, Kwak HS. Effect of nano-calcium-enriched milk on calcium metabolism in ovariectomized rats. J Med Food. 2008;11: 454–9.
37. Weaver CM, Heaney RP. Food sources, supplements, and bioavailability. In: Weaver CM, Heaney RP, editors. Calcium in human health. Totawa (NJ): Humana Press; 2006. p. 129–42.
38. Heaney RP. The natural history of vertebral osteoporosis. Is low bone mass an epiphenomenon? Bone. 1992;13 Suppl 2:S23–6.
39. Lips P. Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. Endocr Rev. 2001;22:477–501.
40. Won JH, Fukuda S, Sato R, Naito Y. Bone histomorphometric changes due to differences in calcium intake under metabolic acidosis in rats. J Vet Med Sci. 1996;58:611–6.
41. Breitman PL, Fonseca D, Ward WE. Combination of soy protein and high dietary calcium on bone biomechanics and bone mineral density in ovariectomized rats. Menopause. 2005;12:428–35.

42. Wronski TJ, Cintron M, Dann LM. Temporal relationship between bone loss and increased bone turnover in ovariectomized rats. Calcif Tissue Int. 1988;43:179–83.
43. Kalu DN, Liu CC, Hardin RR, Hollis BW. The aged rat model of ovarian hormone deficiency bone loss. Endocrinology. 1989;124: 7–16.
44. Yamazaki I, Yamaguchi H. Characteristics of an ovariectomized osteopenic rat model. J Bone Miner Res. 1989;4:13–22.
45. Ray R, Novotny NM, Crisostomo PR, Lahm T, Abarbanell A, Meldrum DR. Sex steroids and stem cell function. Mol Med. 2008;14:493–501.
46. Hawse JR, Subramaniam M, Ingle JN, Oursler MJ, Rajamannan NM, Spelsberg TC. Estrogen-TGFb cross-talk in bone and other cell types: role of TIEG, Runx2, and other transcription factors. J Cell Biochem. 2008;103:383–92.
47. Weitzmann MN, Pacifici R. Estrogen regulation of immune cell bone interactions. Ann N Y Acad Sci. 2006;1068:256–74.

ERRATUM

Shahnazari M, Martin BR, Legette LL, Lachcik PJ, Welch J, Weaver CM (2009). Diet calcium level but not calcium supplement particle size affects bone density and mechanical properties in ovariectomized rats. J Nutr. 139:1308–14.

The correct unit for the parameter "Stiffness" in Tables 2, 3, 4, and 5 should be "N/mm."

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