Reduction in Dietary Calcium/Phosphorus Ratio Reduces Bone Mass and Strength in Ovariectomized Rats Enhancing Bone Turnover

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To clarify the effects of the dietary calcium (Ca)/phosphorus (P) ratio on bone mineralization under the condition of estrogen deficiency, Wistar strain female rats were ovariectomized (OVX) at 12 weeks old. At 16 weeks old, the rats were divided into three dietary groups fed varying levels of P containing 0.5% Ca: 0.25% P, Ca/P = 2; 0.5% P, Ca/P = 1; and 1.0% P, Ca/P = 0.5 respectively. This study indicates that the reduction of the dietary Ca/P ratio impairs trabecular bone turnover accompanying the acceleration of bone formation in OVX rats.

Key words: dietary P; estrogen; ovariectomy; histomorphometry; bone formation

Menopause increases the overall rate of bone remodeling, and increased bone resorption contributes to a remodeling imbalance.2,3) Previous studies have identified several mediators stimulating osteoclastogenesis, down-regulated by estrogen.2,3) Additional support for a remodeling imbalance can be found in impairment of estrogen function, which leads to decreased calcium (Ca) absorption and negative Ca balance.4,5) On the other hand, the relative content of Ca against phosphorus (P) is recognized as a dietary regulator to maintain mineral homeostasis and bone metabolism. Epidemiologically, high P intake compared to Ca intake is associated with reduced bone mineral density (BMD) in perimenopausal women.6,7) In rats, chronic P supplementation decreases intestinal Ca absorption, leading to reduced serum Ca concentration and eventual hyperparathyroidism preserving vitamin D-dependent active Ca absorption.8–10) In the absence of vitamin D-dependent action, we have reported that restriction of dietary P reversed bone mineralization impaired in VDR knockout mice through an increase in intestinal Ca absorption.11) Accordingly, an optimum ratio of dietary Ca/P might be an essential factor in normalizing skeletal structure. Furthermore, we investigated the effect of dietary Ca/P ratio on bone mineralization and intestinal Ca absorption in ovariectomized (OVX) rats and sham-operated rats.12) In both sham and OVX rats, Ca absorption was decreased, and serum parathyroid hormone (PTH) and markers of bone turnover were increased by reducing the dietary Ca/P ratio. In contrast, these negative effects on bone mineralization and intestinal Ca absorption were improved by elevating the dietary Ca/P ratio. Especially in OVX rats, bone loss in the fifth lumbar (L5) was also suppressed by reducing the dietary Ca/P ratio.

In the present experiment, to evaluate the detail effects of dietary Ca/P on bone metabolism, systemic bone markers, mass and strength of bone, local bone turnover, and mineralization using histomorphometry, we examined OVX rats fed different ratios of dietary Ca/P.

Wistar strain female rats were ovariectomized at 12 weeks old and maintained on laboratory chow containing 1.2% Ca and 1.0% P (CE-2, Clea, Tokyo, Japan) for 4 weeks. At 16 weeks old, OVX rats were divided into three groups (n = 5 each), and fed one of three diets: 0.5% Ca and 0.25% P (Group 1, Ca/P = 2); 0.5% Ca and 0.5% P diet (Group 2, Ca/P = 1); or 0.5% Ca and 1.0% P diet (Group 3, Ca/P = 0.5). All test diets utilized CaCO3 and KH2PO4 as sources of dietary Ca and P, respectively. The amounts of Ca and P in the test diets were confirmed in advance. All animals were allowed unlimited access to the assigned diet and deionized water for 6 weeks, and were housed individually in metabolic cages in a room maintained at 22°C with a 12-h light-dark cycle. At the end of the experimental period, blood was collected from the carotid artery. Then, both tibiae, the left femur, and the L5 vertebra were obtained for further analysis. The experimental protocol was approved by the Tokyo University of Agriculture Animal Use Committee, and the animals were maintained in accordance with university guidelines for the care and use of laboratory animals.

The lengths of L5 and the left femur were measured using a micrometer. Bone mineral contents (BMC) and BMD values were measured in L5 and the left femur by Dual energy X-ray absorptiometry (DEXA: DCS-600, Aloka, Tokyo, Japan) adapted for use with small
animals. The volumes of the L5 and left femur were measured using a volumetric apparatus. According to a previous report, maximum compression load in the L5 and maximum bending load to failure were analyzed using a load tester. For bone histomorphometry, bone labeling was undertaken using intramuscular injection of calcein (6 mg/kg body weight) 7 and 3 days before sacrifice. The methods of sample treatment and analysis were described previously.

Histomorphometry was examined on the secondary spongiosa, which contained few cartilaginous cores, and a distinct mineralizing front was observed at the trabecular bone surface using Cosmoneuse 1S (Nikon, Tokyo, Japan). MMA sections with Villanueva’s staining were measured to determine the ratio of trabecular bone volume to tissue volume (BV/TV), trabecular bone thickness (Th.Th), and trabecular bone number (Th.N). Mineral apposition rate (MAR), bone formation rate to bone surface (BFR/BS), and mineralized bone surface (MS/BS) were measured as bone formation parameters. Trabecular osteoclast surface (Oc.S/BS) and osteoclast number (Oc.N/BS) were measured in sections subjected to TRAP-staining as bone resorption parameters.

Results were expressed as mean ± standard error of the mean (SEM). To determine the effects of diet on the OVX groups, data were evaluated by one-way analysis of variance (ANOVA), followed by the Bartlett test and Fisher’s PLSD test. In cases where significant differences were found between SEM values using the F-test and the Bartlett test, Mann–Whitney’s U-test was performed after Kruskall–Wallis testing. Values of p < 0.05 were considered significant. Analyses were performed using Stat View 5.0 software (Macintosh, Apple Computer, Cupertino, CA, U.S.A.).

Intake amounts of food, protein, calories, calcium, and magnesium did not differ among the three groups during 6 weeks of the experimental period (data not shown). P intake obviously increased dose-dependently according to dietary P in all groups. Body weights at the base line before the OVX operation, weights at the start and finish of the experiment, and the values for weight gain did not differ among the three groups.

The values of length, volume, BMC, BMD, and maximum load for the left femur did not differ among the three groups (Table 1). Compared to Group 1, the volume of L5 was significantly decreased in Group 2. The values for BMC, BMD, and maximum load of L5 were significantly smaller in Groups 2 and 3 than in Group 1. The values for BV/TV in the proximal tibia tended to decrease in a dose-dependent manner according to dietary P, although no statistical differences were found (Table 2). The values for Tb.N were significantly smaller in Groups 2 and 3 than in Group 1. The value for MS/BS was significantly greater in Group 3 than in Group 1. Compared to the respective values in Group 1, MAR in Groups 2 and 3 and BFR/BS in Groups 2 and 3 were significantly increased.

The dietary Ca/P ratio obviously changed local bone turnover in OVX rats, and reduction of the ratio promoted bone resorption while bone formation was increased in our previous study. Thus, further decreases in trabecular bone volume occurred in the present study.

We previously reported that high P feeding in young male rats decreased intake of food when dietary Ca levels were constant and that this resulted in decreased Ca intake. Increases in dietary P cause repression of growth promotion. In present study, reduction of the dietary Ca/P ratio by an increase in dietary P levels did not change food intake, due to the use of mature OVX rats. These results indicate the equality of supplemented nutrients that are important for bone growth, such as protein, energy, Ca, and Mg, except for P, in all rats fed diets composed of different Ca/P ratios. Body weight gain resulted reflecting dietary treatment, and showed similar values among the groups.

In the whole femur, parameters indicating bone mass, longitudinal bone growth, and BMC did not change with reduction of the dietary Ca/P ratio. In the lumbar vertebra, however, the values for BMC, BMD, and ultimate load decreased in Group 2 and 3 compared to Group 1. Generally, trabecular bone is rich in the lumbar compared to the femur. Therefore, the BMC

| Table 1. Bone Length, Bone Volume, Bone Mineral Content, Bone Mineral Density, and Ultimate Load of Left Femur and Fifth Lumbar Vertebra |
|---|---|---|---|---|
| Diet (Ca/P contents, Ca/P ratio) | Length (mm) | Volume (cm³) | BMC (mg) | BMD (mg/cm²) | Ultimate load (N) |
| Left femur | | | | | |
| Group 1 (0.5% Ca, 0.25% P Ca/P = 2) | 33.75 ± 0.25 | 0.506 ± 0.018 | 292.6 ± 11.6 | 162.2 ± 2.9 | 112.9 ± 5.3 |
| Group 2 (0.5% Ca, 0.5% P Ca/P = 1) | 33.78 ± 0.45 | 0.506 ± 0.020 | 287.2 ± 12.7 | 161.0 ± 2.6 | 113.5 ± 5.0 |
| Group 3 (0.5% Ca, 1.0% P Ca/P = 1/2) | 33.47 ± 0.24 | 0.517 ± 0.010 | 259.3 ± 10.7 | 154.0 ± 3.4 | 105.8 ± 6.3 |
| Fifth lumbar vertebra | | | | | |
| Group 1 (0.5% Ca, 0.25% P Ca/P = 2) | 7.85 ± 0.12 | 0.065 ± 0.001 | 26.44 ± 1.21 | 76.98 ± 1.60 | 271.3 ± 13.6 |
| Group 2 (0.5% Ca, 0.5% P Ca/P = 1) | 7.45 ± 0.11 | 0.056 ± 0.003A | 20.20 ± 2.16A | 71.06 ± 3.02A | 216.7 ± 16.2A |
| Group 3 (0.5% Ca, 1.0% P Ca/P = 1/2) | 7.64 ± 0.20 | 0.058 ± 0.03 | 20.90 ± 0.66A | 68.90 ± 0.99A | 201.6 ± 10.5A |

Data are mean ± SEM. AKoshihara et al. 13

*P < 0.05, **P < 0.01 vs Group 1 (Fisher’s PLSD test)

**P < 0.05, ***P < 0.01 vs Group 1 (Mann–Whitney U test after Kruskal–Wallis test)

**P < 0.05, ***P < 0.01 vs Group 2 (Fisher’s PLSD test)

*P < 0.05, **P < 0.01 vs Group 2 (Mann–Whitney U test after Kruskal–Wallis test)
and BMD were clearly decreased in lumbar vertebra due to the effects of the dietary Ca/P ratio.

In the histomorphometry of the proximal tibia, reduction of the dietary Ca/P ratio decreased the values for Tb.N and tended to decrease the values for BV/TV, and inversely increased the parameters of MS/BS, MAR, and BFR/BS. In the previous study, increased serum osteocalcin and PTH levels and urinary excretion of deoxypyridinoline were observed by reduction of the dietary Ca/P ratio. These results showed that the high bone turnover caused by rather high bone formation, not high bone resorption in this study.

On the other hand, estrogen deficiency increases the forming and resorbing surfaces in tibial histomorphometry. As assessed by bone densitometry, postmenopausal bone loss is much greater for cancellous than cortical bone. Trabecular bone loss has been observed in an estrogen-deficient condition. We therefore hypothesize that high turnover bone loss resulting from either ovariectomy or reduced dietary Ca/P appears only in enclosed bone formation, not in periosteal bone formation. In brief, the present results suggested that disadvantageous Ca nutrition with estrogen deficiency was emphasized by reduction of the dietary Ca/P ratio. We previously investigated that reduction of dietary Ca/P impaired intestinal Ca absorption in both OVX and sham operated rats. The absence of adequate Ca absorption resulted in decreased bone mineralization whereas the parameters of bone formation showed increment.

In conclusion, reduction of the dietary Ca/P ratio in estrogen deficiency decreased trabecular bone volume and increased the bone formation rate in the tibia, and BMD and bone strength in the lumbar vertebra of rats. A greater amount of cancellous bone exists in the lumbar vertebra than in the tibia and femur. The dietary Ca/P ratio with estrogen deficiency might have stronger effects on the lumbar vertebra than the tibia and femur.

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**References**

