The Effect of Different Amounts of Calcium Intake on Bone Metabolism and Arterial Calcification in Ovariectomized Rats

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Summary Low calcium (Ca) intake is one of the risk factors for both bone loss and medial elastocalcinosis in an estrogen deficiency state. To examine the effect of different amounts of Ca intake on the relationship between bone mass alteration and medial elastocalcinosis, 6-wk-old female SD rats were randomized into ovariectomized (OVX) control or OVX treated with vitamin D3 plus nicotine injection (VDN) groups. The OVX treated with VDN group was then divided into 5 groups depending on the different Ca content in their diet, 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% Ca intakes. After 8 wk of experimentation, the low Ca intake groups of 0.01% and 0.1% showed a low bone mineral density (BMD) and bone properties significantly different from those of the other groups, whereas the high Ca intake groups of 1.2% and 2.4% showed no difference compared with the OVX control. Only in the 0.01% Ca intake group, a significantly higher Ca content in the thoracic artery was found compared with that of the OVX control. Arterial tissues of the 0.01% Ca intake group showed an increase of bone-specific alkaline phosphatase (BAP) activity, a marker of bone mineralization, associated with arterial Ca content. However, the high Ca intake did not affect arterial Ca content nor arterial BAP activity. These results suggested that a low Ca intake during periods of rapid bone loss caused by estrogen deficiency might be one possible cause for the complication of both bone loss and medial elastocalcinosis.

Key Words estrogen deficiency, bone loss, medial elastocalcinosis, different calcium intake

Osteoporosis is a condition with bone fragility characterized by decreased bone mass and micro-architectural deterioration of bone tissue, and with a subsequent increase in risk of fractures (1). Postmenopausal women are prone to greater risks of osteoporosis due to a chronic decrease in circulating estrogen level and a low calcium (Ca) intake (2–4).

Primary postmenopausal osteoporosis has been now considered not only as a single musculoskeletal disorder but also as a multifactorial disorder. For instance, several recent epidemiological studies have demonstrated that there were positive correlations between bone loss, i.e., decreases in bone mineral density (BMD) or bone strength and vascular disorders, i.e., the progression of arterial calcification or arterial stiffness in post menopausal women (5–7). Moreover, the prevalence of arterial calcification such as Mönkeberg’s sclerosis particularly localized to the media mainly in women with ageing (8, 9). Since estrogen has been well known to play a crucial role for inhibition of both bone loss (10) and arterial calcification (11), it is suggested that estrogen deficiency might be involved in a possible mechanism of the occurrences of both bone loss and arterial calcification in postmenopausal women.

On the other hand, a low Ca intake might be responsible for promoting arterial calcification. According to several animal studies, an extraordinary low Ca intake around 0.01% induced an inhibitory effect on the pressure natriuresis response and increased sensitivity to the pressor response to angiotensin II, leading to the development of hypertension (12, 13). Elevating blood pressure induced an arterial stiffening, reducing its distensibility, which was associated with an increased Ca content in the artery (14, 15). However, it remained unclear if a low Ca intake might induce an arterial calcification in an estrogen deficiency state.

Ovariectomy (OVX) is an accepted female animal model to mimic postmenopausal osteoporosis. In fact, the OVX procedure promoted a decrease of bone loss; however it did not induce a clear increase of arterial stiffness nor arterial Ca content (17). On the other hand, vitamin D3 plus nicotine treatment (VDN) of young male rats produced vascular calcium overload (18). VDN treatment of young male rats caused Ca deposition of the arterial elastic site consisting mainly
of vascular smooth muscle cells (VSMCs) in the aorta, and it has been proposed as an animal model of medial elastocalcinosis and stiffness (19). Recently, we modified an alternative female rat model showing both pathologies of bone loss and medial elastocalcinosis in an estrogen deficiency state by the combined method of OVX surgery and VDN treatment (20, 21).

Although an adequate Ca intake implied a sustained reduction in the rate of bone loss after menopause (22–24), it remained unclear if the correcting or additional Ca intake could prevent or promote medial elastocalcinosis in estrogen deficiency state. The aim of the present study was to examine the effect of different Ca intakes on the relationship between medial elastocalcinosis and bone mass alteration by means of the modified rat model inducing both bone loss and medial elastocalcinosis.

**MATERIALS AND METHODS**

**Animals and protocol.** Female Sprague-Dawley rats, 6 wk old, were obtained (CLEA Experimental Animals Supply Co. Ltd., Japan) and cared for according to the “Guiding Principles for the Care and Use of Animals” based on the Helsinki Declaration of 1964. After a 5-d recovery period from the OVX operation assigned to all rats, they were divided into an ovariectomy (OVX) control group and 5 OVX groups treated with vitamin D3 plus nicotine injection (VDN), then those 5 groups were distributed by Ca content in their diet: 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% groups (for all groups distributed by Ca content in their diet; 0.01%, 0.1%, and 0.6% groups based on previous reports regarding bone mass alteration by means of the modified rat model inducing bone loss and medial elastocalcinosis.

Bone and arterial pathophysiology (17, 25, 26). Phosphorus (P) concentration for all groups was set at 0.6% of their diet. The preparation for vascular calcification was induced by VDN treatment using OVX rats, as described in our previous report (20), with minor modifications expecting further arterial Ca content by adding more one vitamin D3 injection. Briefly, the rats received vitamin D3 (300,000 IU/kg, i.m., cholecalciferol, Sigma Chemical Co.) treatment without nicotine was carried out. Afterward, no treatment was performed until finishing the experimental period. In the same way, the OVX control group received an injection of 0.15 m NaCl intramuscularly and a gavage of distilled water with oral administration. The experimental period was 8 wk from the beginning of the day of the 2nd injection of vitamin D3. The rats were kept in individual cages and allowed access to food and distilled water ad libitum. Food consumption and body weight gain were measured every second day. Room temperature was kept at 24 ± 1°C, and humidity at 50 ± 5%. Fluorescent lights were on from 8:00 a.m. to 8:00 p.m. Animal care and experimental procedures were approved by the Committee on Animal Research at the University of Tsukuba.

Calcium, phosphorus, and creatinine concentration. At the end of 8 wk after treatment, all rats were fasted overnight and sacrificed under ether anesthesia. Blood samples were obtained from the abdominal aorta. Serum was separated by centrifugation at 2,500 rpm for 15 min at 4°C and stored at −80°C. Serum Ca concentration was measured by Calcium E-test Wako (Wako Pure Chemical Industries, Japan) according to the man-

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**Table 1. Compositions of the experimental diets (g).**

| Constituents                        | 0.6% P | 0.6% P | 0.6% P | 0.6% P | 0.6% P |
|-------------------------------------|--------|--------|--------|--------|--------|
| Glucose monohydrate                 | 63.84  | 63.62  | 62.37  | 60.87  | 57.88  |
| Casein                              | 18.0   | 18.0   | 18.0   | 18.0   | 18.0   |
| Cystine                             | 0.2    | 0.2    | 0.2    | 0.2    | 0.2    |
| Cottonseed oil                      | 10.0   | 10.0   | 10.0   | 10.0   | 10.0   |
| CaCO₃                               | 0.016  | 0.240  | 1.490  | 2.988  | 5.984  |
| KH₂PO₄                              | 1.158  | 1.158  | 1.158  | 1.158  | 1.158  |
| K₂HPO₄                              | 1.492  | 1.492  | 1.492  | 1.492  | 1.492  |
| Roughege                            | 3.0    | 3.0    | 3.0    | 3.0    | 3.0    |
| Choline chloride                    | 0.2    | 0.2    | 0.2    | 0.2    | 0.2    |
| Water-soluble vitamin mixture       | 0.1    | 0.1    | 0.1    | 0.1    | 0.1    |
| Oil-soluble vitamin mixture         | ( )³   | ( )³   | ( )³   | ( )³   | ( )³   |
| Ca- and P-free salt mixture         | 2.0    | 2.0    | 2.0    | 2.0    | 2.0    |

1 Casein contained 0.22 mg calcium/g and 4 mg phosphorus/g.
2 The water-soluble vitamin mixture (in%): thiamin, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 2.8; nicotinamide, 2.0; inositol, 20.0; folic acid, 0.02; vitamin B₁₂, 0.002; biotin, 0.01; and glucose monohydrate, 73.7.
3 The rats received a supplement of the following oil-soluble vitamins in cottonseed oil three times a week: β-carotene, 70 μg; 2-methyl-1,4-naphthoquinone, 105 μg; α-tocopherol, 875 μg; and vitamin D₃, 525 IU.
4 Ca- and P-free salt mixture (in%): KCl, 57.7; NaCl, 20.9; MgSO₄, 17.9; FeSO₄·7H₂O, 3.22; CuSO₄·5H₂O, 0.078; NaF, 0.113; CoCl₂·6H₂O, 0.004; KI, 0.01; MnSO₄·5H₂O, 0.06; ZnSO₄·7H₂O, 0.44; and (NH₄)₂MoO₄·2H₂O, 0.005.
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Bone strength at the middle diaphysis of the femur was tested by measuring the mechanical properties. The bone strength at the middle diaphysis of the femur was tested by measuring the mechanical properties. The bone strength at the middle diaphysis of the femur was tested by measuring the mechanical properties.

Table 2. Final BW, BW gain, food intake, and food efficiency.

|                | Final BW (g) | BW gain (g/d) | Food Intake (g/d) | Food efficiency |
|----------------|--------------|---------------|-------------------|-----------------|
| OVX            | 351.17±27.68 | 2.81±0.49     | 18.45±1.38        | 0.15±0.02       |
| 0.01%          | 340.50±17.75 | 2.73±0.26     | 17.81±0.49        | 0.15±0.01       |
| 0.1%           | 354.70±23.88 | 3.04±0.35     | 19.08±1.36        | 0.16±0.02       |
| 0.6%           | 375.00±24.77 | 3.31±0.40     | 20.56±1.07        | 0.16±0.02       |
| 1.2%           | 360.00±14.13 | 3.04±0.26     | 19.29±1.02        | 0.16±0.01       |
| 2.4%           | 332.75±20.37 | 2.70±0.33     | 19.24±1.31        | 0.14±0.02       |

Values are expressed as means±SD. n=6 in each group. Food efficiency was calculated body weight (BW) gain (g/d)/Food intake (g/d). OVX control rats were fed with 0.6% Ca. Phosphorus (P) concentration for all groups was 0.6% of their diet.

Table 3. Ca, P, Cr concentration in serum and the excretion in urine.

|                | Serum | Urine |
|----------------|-------|-------|
|                | Ca (mg/dL) | P (mg/dL) | Cr (mg/dL) | Ca (mg/d) | P (mg/d) | Cr (mg/d) |
| OVX            | 10.78±1.00 | 4.66±0.53 | 1.56±0.08 | 0.71±0.16 | 61.32±20.62 | 8.32±2.34 |
| 0.01%          | 10.36±1.20 | 5.32±0.58 | 1.67±0.06 | 0.32±0.07<sup>abbccc</sup> | 75.14±25.99 | 9.67±1.97 |
| 0.1%           | 10.46±0.53 | 5.01±0.93 | 1.63±0.15 | 0.34±0.05 | 88.45±23.90 | 9.43±1.29 |
| 0.6%           | 10.75±0.22 | 5.18±1.38 | 1.58±0.15 | 0.73±0.18 | 58.05±22.68 | 9.36±1.29 |
| 1.2%           | 10.88±0.63 | 4.89±1.11 | 1.56±0.20 | 1.17±0.29 | 59.15±19.10 | 10.08±1.49 |
| 2.4%           | 10.28±0.29 | 4.59±1.11 | 1.57±0.15 | 1.79±0.48 | 45.49±12.03 | 11.88±2.54 |

Values are expressed as means±SD. n=6 in each group. OVX control rats were fed with 0.6% Ca. Phosphorus (P) concentration for all groups was 0.6% of their diet. *p<0.05 vs 0.6%, <sup>abb</sup>p<0.001 vs 1.2%, <sup>ccc</sup>p<0.001 vs 2.4%.

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Bone mineral density. The left and right tibiae and lumbar spine of each rat were isolated and freed from any muscles and connective tissue, and immersed in 70% ethanol solution for measurement of BMD. The BMD values of the tibia and L3–L6 lumbar spine were measured by dual-energy X-ray absorptiometry (DXA: Alok DCS-600R instrument) as previously described (29).

Bone mechanical properties. Femur samples were collected, freed from adhering connective tissues, and immediately tested by measuring the mechanical properties. The bone strength at the middle diaphysis of the femur was tested by measuring the mechanical properties. The bone strength at the middle diaphysis of the femur was tested by measuring the mechanical properties.

Bone metabolic markers. Serum bone-specific alkaline phosphatase (BAP) and tartrate-resistant acid phosphatase (TRAP) activities were measured as previously reported (30). For measurement of humeral BAP and TRAP activities, after completely removing marrow and blood in cortical region of the humerus, the fragment was ground in a mortar on dry ice and homogenized with Polytron in the buffer (10 m<sup>1</sup>H<sub>2</sub>O, 0.9% NaCl and 1% Triton X-100). After centrifugation at 3,000 rpm for 10 min, the protein content of supernatant and BAP activities were measured under the following conditions: the sample space was 1.0 cm, the plunger speed was 100.0 mm/min, the load range was 50.0 kg, and the chart speed was 120.0 cm/min.

Statistical analysis. All data were expressed as the mean±SD. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. The relationships...
between indices of bone metabolism markers and arterial calcium content were examined by Pearson’s correlation analysis. p values below 0.05, 0.01 or 0.001 were accepted as significant for all comparisons. All statistical analysis was prepared by SPSS Statistical Packages (SPSS Inc., Chicago, USA).

RESULTS

There were no significant differences for the final body weight, body weight gain, food intake, or food efficiency among groups (Table 2). The 0.01% and 0.1% Ca intake groups in OVX with VDN rats induced significantly lower urinary Ca excretion compared with the other groups. As one of the indices of kidney function, neither serum nor urinary Cr levels showed any significant effect by OVX and VDN treatment. No significant differences in serum Ca, P, and Cr levels were found between any groups (Table 3).

For BMD of tibia and lumbar spine (Fig. 1A and 1B), both the 0.01% and 0.1% Ca intake groups in OVX with VDN rats showed significant bone loss compared with the other groups. Subsequently, both groups also exhibited significantly lower urinary Ca excretion compared with the other groups. As one of the indices of kidney function, neither serum nor urinary Cr levels showed any significant effect by OVX and VDN treatment. No significant differences in serum Ca, P, and Cr levels were found between any groups (Table 3).

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![Fig. 1. Bone mineral density (BMD) and mechanical properties. A: BMD of tibia. B: BMD of lumbar spine. C: Breaking force of femur. D: Breaking energy of femur. Breaking force and energy represents the bone architectural function indicating mechanical properties of femur. Solid bars represent the groups with OVX and VDN, and the open bar represents the OVX control group. VDN, vitamin D3 and nicotine treatment; OVX, ovariectomy operation. ***p<0.001 vs. all groups except 0.1% Ca intake group in OVX and VDN; ###p<0.001 vs. all groups except 0.01% Ca intake group in OVX and VDN; *p<0.05 vs. 0.6% Ca intake; bbbp<0.001 vs. 1.2% Ca intake; ccpp<0.001 vs. 2.4% Ca intake; †p<0.05 vs. OVX control. Values are expressed as the means±SD, n=6 in each group.](image)

Table 4. Bone metabolic markers in serum and humerus.

|               | Serum | Humerus                  |
|---------------|-------|--------------------------|
|               | BAP (mU) | TRAP (mU) | BAP (mU/µg protein) | TRAP (mU/µg protein) |
| OVX           | 38.28±10.24 | 11.60±2.83 | 517.56±189.73 | 145.25±47.80 |
| 0.01%         | 59.23±10.26†,aa,cc | 16.30±3.42 | 644.90±146.66 | 186.40±43.13 |
| 0.1%          | 50.99±6.95 | 16.42±5.03 | 664.90±139.79 | 179.18±38.86 |
| 0.6%          | 40.84±13.05 | 14.19±3.98 | 545.23±114.68 | 154.20±51.72 |
| 1.2%          | 42.66±12.16 | 12.32±1.67 | 492.63±115.78 | 130.66±31.93 |
| 2.4%          | 35.99±6.99 | 11.57±1.11 | 534.80±140.71 | 134.77±51.49 |

Values are expressed as means±SD, n=6 in each group. BAP, bone alkaline phosphatase activity; TRAP, tartrate-resistant acid phosphatase activity. OVX control rats were fed with 0.6% Ca. Phosphorus (P) concentration for all groups was 0.6% of their diet. †p<0.05 vs OVX 0.6%, † † † p<0.01 vs 0.6%, † † † † p<0.001 vs 2.4%.
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ited lower femoral breaking force and energy regarding bone mechanical properties compared with the other groups (Fig. 1C and 1D). However, the 1.2% and 2.4% Ca intake groups resulted in no significant improvement on BMD or bone mechanical properties compared with the 0.6% Ca intake group in OVX with VDN rats.

The 0.01% Ca intake group in OVX with VDN rats showed significantly higher serum BAP activity, a marker of bone formation and mineralization, than that of OVX control or 0.6%, and 2.4% Ca intake groups in OVX with VDN rats, whereas TRAP activity, a marker of bone resorption, showed no significant differences among groups (Table 4). Although there were no significant variations with BAP or TRAP activity at the humerus, the data showed a similar trend toward high bone turnover in low Ca intake groups rather than high Ca intake groups.

As shown in Fig. 2A, only the 0.01% Ca intake group in OVX with VDN rats showed significantly higher serum BAP activity, a marker of bone formation and mineralization, than that of OVX control or 0.6%, and 2.4% Ca intake groups in OVX with VDN rats, whereas TRAP activity, a marker of bone resorption, showed no significant differences among groups (Table 4). Although there were no significant variations with BAP or TRAP activity at the humerus, the data showed a similar trend toward high bone turnover in low Ca intake groups rather than high Ca intake groups.

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content among all rats (Fig. 3B).

**DISCUSSION**

Our data demonstrated that a very low Ca intake of 0.01% induced remarkable bone loss and a significant increase of arterial Ca content associated with an increase of BAP activity in OVX with VDN rats. On the other hand, high Ca intakes of 1.2% and 2.4% did not demonstrate a further increase of bone mass compared with 0.6% Ca intakes in OVX with VDN rats while no alteration of arterial Ca content was observed.

In the present study, the 0.01% and 0.1% Ca intakes induced an apparent bone loss compared with the normal Ca intake group (0.6% Ca). As a possible mechanism underlying the bone loss, lower Ca intake might induce the secondary increase of circulating parathyroid hormone (PTH) level, associated with an increase of circulating vitamin 1,25-(OH)₂D₃ levels (32). We previously reported that 0.1% Ca intake induced an apparent bone loss with an increase of circulating vitamin 1,25-(OH)₂D₃ levels in young female rats (17). Because PTH has a crucial role in preosteoblast cell differentiation (33), the increase of circulating BAP levels in the present study might be due to a low Ca intake. In addition, less urinary Ca excretion was observed in the 0.01% and 0.1% groups, possibly because of an increase in renal tubular Ca re-absorption by the regulation of vitamin 1,25-(OH)₂D₃. However, despite the suppression in urinary Ca excretion and the injection of vitamin D doses in the VDN group, bone loss rapidly occurred in the low Ca intake groups and it seemed the consequence of insufficient Ca intake. Although it was unclear why no differences in BMD, bone mechanical properties, or urinary Ca excretions were exhibited between the 0.01% and 0.1% Ca intakes, it was probably due to a potential systemic defense mechanism against very low Ca intake. On the other hand, BMD and bone mechanical properties should show no significant differences among the 0.6%, 1.2%, and 2.4% Ca intake groups, suggesting a Ca intake of around 0.6% in their diet might be sufficient for maintaining a comparatively adequate bone status in an estrogen deficiency state.

The 0.01% low Ca intake promoted arterial Ca deposition which was positively associated with arterial BAP activity. Recent cellular studies regarding arterial Ca deposition have implicated a causal mechanism resembling bone modeling. For the primary medial elastocalcination, vascular smooth muscle cells (VSMCs) in the medial elastic site of aorta were shown to promote a certain osteoblastic gene expression such as alkaline phosphatase (ALP), and then VSMCs played an active role for bone-like mineralization in the medial elastic site (34, 35). Thus, there were positive correlations between the amount of Ca content and ALP activity in the aorta leading to medial elastocalcination (36–38). Even though it is still controversial which specific factors contribute to promote such osteoblastic gene expressions, the present study suggests that 0.01% low Ca intake might promote an ALP activity of VSMCs in the aorta, and cause a functional change in VSMCs which is associated with its mineralization. Therefore, 0.01% low Ca intake resulted in an arterial Ca deposition in an estrogen deficiency state.

In contrast to the results of very low Ca intake effecting an increase of arterial Ca content, a high Ca intake indicated no improvement of arterial Ca content. A previous animal experiment using a hypertensive rat model reported that a high 2.5% Ca diet attenuated the development of hypertension or cardiac hypertrophy (39), and it was supported by the view that higher Ca supplementation reduces blood pressure by decreasing peripheral arterial resistance (40). Subsequently, elevating blood pressure has been considered as one of the risk factors of arterial calcification and stiffness (12, 13). However, in this VDN rat model, we could not find any preventive effects of a high Ca intake on arterial Ca deposition.

The VDN treatment is an accepted procedure to increase the arterial sensitivity relative to arterial Ca overload in rodents (18). Several animal studies reported that VDN treatment brought 5- to 10-fold increase of arterial Ca content to young male Sprague-Dawley (SD) rats (36, 41, 42). However, our data unexpectedly showed a slight increase but no significant difference in the arterial Ca content of the 0.6% Ca intake group in OVX with VDN rats compared with the 0.6% Ca intake group in OVX without VDN. This discrepancy might be due to the sex difference of rats. Our previous result using young female SD rats with OVX and VDN may support slight arterial Ca deposition (2-fold) among female rats (20), even though the difference was statistically significant. It seemed that female rats showed a small increase of arterial Ca content with VDN treatment compared to male rats. Another possibility might be the strain difference of rats. According to previous studies using young Wistar male rats, arterial Ca content of more than 15-fold was found compared with young SD male rats (19, 43–45). It seemed to point toward higher arterial Ca deposition by means of young Wistar rats treated with OVX and VDN prospectively. Thus, further studies concerning local mineralization may be necessary, using young female Wistar rats and OVX and VDN treatment in the experimental design.

In conclusion, a very low Ca intake of 0.01% induced significant progression of arterial Ca deposition and BAP activity associated with remarkable bone loss in OVX and VDN rats. Accordingly, these results suggested that the amount of Ca intake might be involved in a mechanism underlying the relationship between bone loss and medial elastocalcination in an estrogen-deficiency state.

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