The Impact of Different Amounts of Calcium Intake on Bone Mass and Arterial Calcification in Ovariectomized Rats

Umon Agata¹, Jong-Hoon Park²,³, Satoshi Hattori¹, Yuki Aikawa¹, Yuya Kakutani¹, Ikuko Ezawa⁴, Takayuki Akimoto² and Naomi Omi¹,⁵,∗

¹Institute of Health and Sport Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305–8574, Japan
²Division of Regenerative Medical Engineering, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113–0033, Japan
³Department of Physical Education, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea
⁴Japan Women’s University, Bunkyo-ku, Tokyo 112–8681, Japan
⁵Health, Physical Education and Sport Sciences, University of Tsukuba, Tsukuba, Ibaraki 305–8574, Japan

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Summary Reduced estrogen secretion and low calcium (Ca) intake are risk factors for bone loss and arterial calcification in female rodents. To evaluate the effects of Ca intake at different amounts on bone mass changes and arterial calcification, 8-wk-old female Wistar rats were randomly placed in ovariectomized (OVX) control and OVX with vitamin D3 plus nicotine (VDN) treatment groups. The OVX with VDN rats were then divided into six groups to receive different amounts of Ca in their diets: 0.01%, 0.1%, 0.3%, 0.6%, 1.2%, or 2.4% Ca. After 8 wk of administration, low Ca intake groups with 0.01% and 0.1% Ca diets had significantly reduced bone mineral density (BMD) and bone mechanical properties as compared with those of the other groups, whereas high Ca intake groups with 1.2% and 2.4% Ca diets showed no differences as compared with the 0.6% Ca intake group. For both the 0.01% and 2.4% Ca intake groups, Ca levels in their thoracic arteries were significantly higher as compared with those of the 0.6% Ca diet group, and that was highly correlated with serum PTH levels. An increase in relative BMP-2 mRNA expression in the arterial tissues of the 0.01% and 2.4% Ca diet groups was also observed. These results suggested that extremely low Ca intake during periods of estrogen deficiency may be a possible risk for the complications of reduced BMD and arterial calcification and that extremely high Ca intake may promote arterial calcification with no changes in BMD.

Key Words ovariectomy, bone loss, arterial calcification, different calcium intake

Osteoporosis and arterial calcification often occur simultaneously with chronic systemic diseases and may remain undetected for several years (1). In addition, the incidence of these diseases increases with age (2). The phenomenon of developing both osteoporosis, caused by low bone mass, and arterial calcification, involving the deposition of bony particles and hydroxyapatite in vascular tissues (3, 4), is considered as the “calcification paradox” (5). Owing to the recent increase in the numbers of menopausal women who were found to have both low bone mass density (BMD) and arterial calcification (6), menopausal osteoporosis is now considered not only to be a singular skeletal disorder but also a pleiotropic disorder.

We previously reported that low calcium (Ca) intake was associated with low bone mass accompanied by accelerated arterial Ca deposition in female rats for which ovariectomy (OVX) was performed and vitamin D3 plus nicotine (VDN) were administered (7). Our hypothesis in that previous study was supported by findings indicating that bone loss and arterial calcification have common risk factors, such as reduced estrogen secretion (8–9) and low Ca intake (10, 11). Consequently, based on our findings, a high Ca diet administered to OVX- and VDN-treated rats may not have effectively prevented low bone mass and arterial calcification in vivo.

Although bone mass is altered by the combined activities of osteoblasts and osteoclasts, evidence derived from some cell studies has indicated that a causal mechanism underlying arterial calcification was similar to bone remodeling. Vascular smooth muscle cells (VSMCs) are normally present in vessel walls and regulate the medial elastic properties of the aorta. However, they also play an active role in bone-like mineralization of the media through their osteoblast-like Ca uptake into vessel walls by the process of calcification (12, 13). Although the expression of an osteoblast-like phenotype by VSMCs due to this calcification process was previously reported in several in vitro studies (14–17), to the best of our knowledge, there have been no in vivo studies for the effects of different amounts of Ca intake on an aortic phenotype, such as mRNA expression related to vascular bone-like mineralization.
Furthermore, although VDN is a well-known treatment for arterial calcification (18), our previous findings showed some limitations in that no Ca deposition occurred in arterial tissues even after OVX with VDN treatment (7). The inconsistency between the results of previous studies and our results were presumably because of using different animal models for in vivo studies. VDN treatment reportedly results in a 10-fold increase in arterial Ca levels in young male Wistar rats 8 wk of age (19–21), whereas this treatment results in <10-fold increase in arterial Ca levels in young male SD rats of various ages (22–24). In female rats, the investigation of the calcification paradox by means of OVX plus VDN treatment in vivo has not been studied, except in our previous studies with SD rats of 6 wk (7, 25). In addition, the examination of a Ca metabolic hormone such as parathyroid hormone (PTH) was not discussed in our previous study, and we could not have determined the effects of dietary Ca on bones through Ca metabolism. Thus, although the difference in physiological response to VDN treatment for local Ca deposition was unclear between male and female, reevaluating arterial Ca levels and bone in 8-wk-old female Wistar rats using OVX with VDN treatment is definitely required for investigating the calcification paradox systemically. Otherwise, this reevaluating study would possibly confirm the effects of dietary Ca on bones and arterial Ca levels, and newly reveal an alteration of arterial phenotypes which was different in parameter from our previous study in the situation of the accelerated calcification paradox.

The aim of this study was to determine the effects of Ca intake at different amounts on a local aortic Ca levels with the aortic phenotype, arterial osteoblast-related mRNA expression in addition to bone mass. Furthermore, we systemically evaluated the calcification paradox using bone metabolic markers and circulating PTH levels, which were undetermined in our previous study, by means of a modified protocol, providing the maximum effects of VDN treatment.

**MATERIALS AND METHODS**

**Animals and protocol.** Female Wistar rats (8-wk-old) were obtained from CLEA Japan, Inc., Japan, and cared for, according to the “Guiding Principles for the Care and Use of Animals” based on the Helsinki Declaration of 1964. After recovering for 1 wk following OVX, the rats were then divided into an OVX control group or OVX group with VDN administration. The OVX with VDN group was divided into six groups based on Ca concentrations for all groups were set at 0.6% of the diet, and a Ca diet of 0.6% was considered as the control condition. Phosphorus (P) concentrations for all groups were set at 0.6% of the diet, and a P diet of 0.6% was considered as the control condition. Preparations for vascular calcification were induced by VDN treatment using OVX rats, as described in our previous report (25). Briefly, rats received vitamin D$_3$ (300,000 IU/kg, i.m., cholecalciferol; Sigma Chemical Co.) plus nicotine (25 mg/kg, 5 mL/kg, p.o., nicotine hydrogen tartrate; Sigma Chemical Co.) at 9:00 a.m. No additional treatment was further administered during the experimental period. The OVX control group rats were administered 0.15 mL NaCl intramuscularly and distilled water orally. The experimental period was 8 wk beginning on the day when vitamin D$_3$ was injected.

The rats were kept in individual cages and allowed access to food and distilled water ad libitum. Food consumption was measured daily. The rats were killed by CO$_2$ and the aortas were excised and fixed with Bouin’s solution. The aortas were cut into 1-mm-thick segments and stained with von Kossa’s stain for calcification (26). The calcification scores were determined on a semi-quantitative basis as follows: 0, no calcification; 1, <10% calcification; 2, 10–20% calcification; 3, 20–30% calcification; 4, 30–40% calcification; 5, >40% calcification. The aortic calcification scores were determined in three segments from each rat, and the mean score was calculated as the final aortic calcification score of each rat. The rats were kept in individual cages and allowed access to food and distilled water ad libitum. Food consumption was measured daily. The rats were killed by CO$_2$ and the aortas were excised and fixed with Bouin’s solution. The aortas were cut into 1-mm-thick segments and stained with von Kossa’s stain for calcification (26). The calcification scores were determined on a semi-quantitative basis as follows: 0, no calcification; 1, <10% calcification; 2, 10–20% calcification; 3, 20–30% calcification; 4, 30–40% calcification; 5, >40% calcification. The aortic calcification scores were determined in three segments from each rat, and the mean score was calculated as the final aortic calcification score of each rat.

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

| Constituents | 0.01% Ca | 0.1% Ca | 0.3% Ca | 0.6% Ca | 1.2% Ca | 2.4% Ca |
|-------------|----------|---------|---------|---------|---------|---------|
| Glucose monohydrate | 63.84 | 63.62 | 63.12 | 62.37 | 60.87 | 57.88 |
| Casein$^1$ | 18 | 18 | 18 | 18 | 18 | 18 |
| Cystine | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Cotton seed oil | 10 | 10 | 10 | 10 | 10 | 10 |
| CaCO$_3$ | 0.016 | 0.240 | 0.740 | 1.490 | 2.988 | 5.984 |
| KH$_2$PO$_4$ | 1.158 | 1.158 | 1.158 | 1.158 | 1.158 | 1.158 |
| K$_2$HPO$_4$ | 1.482 | 1.482 | 1.482 | 1.482 | 1.482 | 1.482 |
| Choline chloride | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Water-soluble vitamin mixture$^2$ | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Oil-soluble vitamin mixture$^3$ | (3) | (3) | (3) | (3) | (3) | (3) |
| Ca- and P-free salt mixture$^4$ | 2 | 2 | 2 | 2 | 2 | 2 |

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$^1$ Casein contained 0.22 mg calcium (Ca)/g and 4 mg phosphorus (P)/g.

$^2$ Water-soluble vitamin mixture: thiamin, 0.5%; riboflavin, 0.5%; pyridoxine, 0.5%; calcium pantothenate, 2.8%; nicotinamide, 2.0%; inositol, 20.0%; folic acid, 0.02%; vitamin B$_12$, 0.002%; biotin, 0.01%; and glucose monohydrate, 73.7%.

$^3$ Rats received a supplement of the following oil-soluble vitamins in cottonseed oil three times a week: 2-methyl-1,4-naphthoquinone, 105 mg; a-tocopherol, 875 mg; and vitamin D$_3$, 525 IU.

$^4$ Ca- and P-free salt mixture: KCl, 57.7%; NaCl, 20.9%; MgSO$_4$, 17.9%; FeSO$_4$·7H$_2$O, 3.22%; CuSO$_4$·5H$_2$O, 0.078%; NaF, 0.113%; CoCl$_2$·6H$_2$O, 0.004%; Ki, 0.01%; MnSO$_4$·5H$_2$O, 0.06%; ZnSO$_4$·7H$_2$O, 0.44%; and (NH$_4$)$_2$Mo$_7$O$_24$·4H$_2$O, 0.0025%.
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         | 316.5±7.86   | 2.65±0.12     | 17.75±0.34        | 0.14±0.01       |
| 0.01%       | 313.9±7.80   | 2.59±0.13     | 16.56±0.36        | 0.16±0.01       |
| 0.1%        | 311.4±3.83   | 2.57±0.06     | 16.70±0.41        | 0.15±0.01       |
| 0.3%        | 317.0±7.24   | 2.64±0.13     | 16.67±0.37        | 0.16±0.01       |
| 0.6%        | 307.6±11.88  | 2.46±0.19     | 17.23±0.36        | 0.14±0.01       |
| 1.2%        | 308.0±7.75   | 2.51±0.13     | 16.96±0.36        | 0.14±0.01       |
| 2.4%        | 310.8±4.89   | 2.54±0.11     | 17.76±0.18        | 0.14±0.02       |

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

Table 3. Ca, P, and Cr excretion in urine.

|         | Urine       |           |           |           |
|---------|-------------|-----------|-----------|-----------|
|         | Ca (mg/d)   | P (mg/d)  | Cr (mg/d) |
| OVX     | 0.76±0.05   | 44.97±1.15| 11.11±0.32|
| 0.01%   | 0.27±0.01   | 68.12±2.93| 10.02±0.42|
| 0.1%    | 0.35±0.02   | 65.68±2.36| 10.29±0.29|
| 0.3%    | 0.50±0.04   | 59.29±0.58| 10.72±0.23|
| 0.6%    | 0.67±0.07   | 45.27±2.56| 10.01±0.33|
| 1.2%    | 1.12±0.14   | 29.19±3.38| 10.77±0.70|
| 2.4%    | 2.56±0.23   | 19.63±2.43| 11.36±0.67|

Values are expressed as means±SE, n=6 in each group. OVX control rats were fed 0.6% Ca. Phosphorus (P) concentration for all groups was 0.6% in their diet.

Bone mineral density. The left and right tibiae and lumbar spine of each rat were isolated, any remaining muscles and connective tissue were removed, and then specimens were immersed in a 70% ethanol solution for BMD measurements. BMD values of the tibia and L3–L6 lumbar spine were measured by dual-energy X-ray absorptiometry (Aloka DCS-600R instrument), as previously described (39).

Bone mechanical properties. Femur samples were collected. Adhering connective tissues were removed and then the samples were immediately measured for mechanical properties. Bone strength at the middle...
The diaphysis of the femur was tested by measuring its mechanical strength with an Iio DYN-1255 instrument, as previously reported (31). The force and energy necessary to produce a break at the center of the femur were determined using a sample space of 1.0 cm, plunger speed of 100.0 mm/min, load range of 50.0 kg, and chart speed of 120.0 cm/min.

**Results**

Among the different study groups, there were no significant differences in final body weights, weight gains, food intakes, or food efficiency (Table 2). As shown in Table 3, the 0.01%, 0.1%, and 0.3% Ca intake groups had significantly lower urinary Ca excretion and significantly higher P excretion than the 0.6% Ca intake group in OVX and VDN. Values are expressed as the means±SE, n=6 in each group.

**Table 4. Ca, P, BAP, TRAP and PTH concentration in serum.**

| Serum                      | Ca (mg/dL) | P (mg/dL) | BAP (mU) | TRAP (mU) | PTH (pg/mL) |
|----------------------------|------------|-----------|----------|-----------|-------------|
| OVX                        | 10.20±0.30 | 6.51±0.37 | 72.76±5.58 | 18.86±2.05 | 7.71±1.01   |
| 0.01%                      | 10.97±0.45 | 6.17±0.32 | 126.63±9.99 | 27.19±2.24 | 54.02±6.88   |
| 0.1%                       | 10.79±0.45 | 6.68±0.38 | 97.23±5.80 | 23.61±2.59 | 25.23±5.00   |
| 0.3%                       | 10.48±0.37 | 6.43±0.26 | 84.44±6.85 | 20.15±1.37 | 24.68±6.40   |
| 0.6%                       | 10.32±0.13 | 6.87±0.39 | 73.23±9.63 | 15.68±1.34 | 17.92±2.02   |
| 1.2%                       | 10.45±0.23 | 6.65±0.26 | 72.99±8.94 | 19.81±1.74 | 32.54±6.60   |
| 2.4%                       | 11.19±0.26 | 7.06±0.25 | 71.26±2.82 | 19.40±1.61 | 67.43±9.03   |

Values are expressed as means±SE, n=6 in each group. BAP: bone alkaline phosphatase activity; TRAP: tartrate-resistant acid phosphatase activity. OVX control rats were fed 0.6% Ca. Phosphorus (P) concentration for all groups was 0.6% in their diet.

*p<0.05 vs. 0.6% Ca diet group in OVX and VDN.

Statistical analysis. Results are presented as means±SE. Statistical comparisons were performed by one-way analysis of variance, followed by Tukey’s test for multiple comparisons. Possible associations between arterial mRNA expression and arterial calcium contents were determined by Pearson correlation analysis. A p-value of less than 0.05, 0.01, or 0.001 was considered significant. All statistical analyses were conducted using SPSS Statistical Packages (SPSS, Inc., Chicago, IL).

**RESULTS**

Among the different study groups, there were no significant differences in final body weights, weight gains, food intakes, or food efficiency (Table 2). As shown in Table 3, the 0.01%, 0.1%, and 0.3% Ca intake groups had significantly lower urinary Ca excretion and significantly higher P excretion than the 0.6% Ca intake group.
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The 1.2% and 2.4% Ca intake groups had significantly higher Ca excretion and significantly lower P excretion than the 0.6% Ca intake group among OVX with VDN rats. Although a slight increase in urinary Cr excretion was observed in the 2.4% Ca intake group, there were no significant differences among these groups.

The bone mineral density (BMD) of the tibia and lumbar spine (Fig. 1A and B) in both the 0.01% and 0.1% Ca intake groups showed significant bone loss compared with the other groups. For bone mechanical properties, both the 0.01% and 0.1% Ca intake groups exhibited a significantly lower femoral breaking force and breaking energy than the 0.6% Ca intake group (Fig. 1C and D). However, the 1.2% and 2.4% Ca intake groups showed no significant improvement in BMD or bone mechanical properties compared with the 0.6% Ca intake group. Those results were confirmatory for our previous study (7). On the other hand, in the 0.3% Ca intake group, the BMD of the lumbar spine was slightly lower than that in the 0.6% Ca intake group.

The results of laboratory analysis are shown in Table 4. The 0.01% Ca intake group showed significantly higher serum BAP activity, a marker of bone formation and mineralization, than that of the 0.6% Ca intake group. Serum TRAP activity, which is a marker of bone resorption, as well as serum BAP activity, were significantly higher in the 0.01% Ca intake group. Although the serum bone metabolism markers in all of the low Ca intake groups showed a trend toward high bone turnover, except for the high Ca intake groups, there were no significant differences in serum Ca and P levels among the groups. Additionally, the 0.01% and 2.4% Ca intake groups demonstrated significantly higher PTH levels than that of the 0.6% Ca intake group.

As shown in Fig. 2A, the 0.6% Ca intake group that underwent OVX with VDN treatment had significantly higher arterial Ca content than that of the group that underwent OVX alone. Furthermore, as we previously reported (7), the 0.01% and 2.4% Ca intake groups had significantly higher Ca contents in their thoracic arterial tissues as compared to the 0.6% Ca intake group. Regarding bone-like phenotypes of aortic tissues, although relative ALP mRNA expression were not significantly different among the groups (Fig. 2B), relative BMP-2 mRNA expression in both the 0.01% and 2.4% Ca intake groups were significantly higher as compared with those of the 0.6% Ca intake group (Fig. 2C). Both ALP and BMP-2 relative mRNA expression levels were positively correlated with arterial Ca contents for all rats (Fig. 3A and B). In addition, serum PTH levels were strongly correlated with arterial Ca contents (Fig. 3C).

**DISCUSSION**

Our data demonstrated that OVX with VDN treatment resulted in significant arterial Ca deposition as compared with rats that underwent OVX alone. Among the different amounts of Ca intake, low Ca intake of 0.01% resulted in both a remarkably low BMD and a marked increase in arterial Ca deposition. The group with the high Ca intake of 2.4% notably showed a significant increase in arterial Ca deposition without any changes in BMD or bone mechanical properties. Both low Ca intake of 0.01% and high Ca intake of 2.4% showed high serum PTH levels which was strongly correlated with arterial Ca content. Furthermore, both the 0.01% and 2.4% Ca intake groups showed a bone-like aortic phenotype that was characterized by increased BMP-2 mRNA expression as compared with the 0.6% Ca intake group, and these phenotypic alterations were strongly correlated with arterial Ca content.

In the present study, the low Ca intake groups dis-
Fig. 3. Correlation between arterial Ca content and the relative mRNA expression in the artery. A: The relationship between the arterial Ca content and the relative ALP mRNA expression. B: The relationship between the arterial Ca content and the relative BMP-2 mRNA expression. C: The relationship between the arterial Ca content and serum PTH concentration.

- ▲: 2.4% Ca intake group.
- ■: 0.01% Ca intake group.
- O: The other groups include 0.6% Ca intake (OVX control), 0.1% Ca intake (OVX with VDN), 0.3% Ca intake (OVX with VDN), 0.6% Ca intake (OVX with VDN), and 1.2% Ca intake (OVX with VDN). The values of the mRNA expression of ALP or BMP-2 were relative to GAPDH. n=6 in each group.
played a lower BMD than the normal Ca intake group (0.6% Ca). In addition, the urinary Ca excretion was lower in the 0.01%, 0.1%, and 0.3% Ca intake groups. Our previous study showed an increase in circulating active vitamin D$_3$, 1,25-(OH)$_2$D$_3$ levels in the low Ca intake group (26); on this basis, we believe the urinary Ca excretion may have been lower because of an increase in renal tubular Ca reabsorption caused by the regulatory effect of vitamin 1,25-(OH)$_2$D$_3$. However, despite the suppression in urinary Ca excretion and the administration of massive vitamin D$_3$ doses, low bone mass and disorientation of bone structure occurred equally in the low Ca intake groups, apparently the consequence of insufficient Ca intake. On the other hand, our data revealed no significant differences in BMD or bone mechanical properties among the 0.6%, 1.2%, and 2.4% Ca intake groups, suggesting a Ca intake of approximately 0.6% in the diet might be sufficient for maintaining a comparatively proper bone status.

As described in Fig. 2A, our study using young female Wistar rats that underwent OVX with VDN treatment confirmed the promotion of arterial Ca deposition. Based on previous reports (18–21), we modified the experimental model and determined that OVX plus VDN treatment promoted arterial Ca deposition in 8-wk-old Wistar rats. Although it was still unclear why physiological responses to OVX plus VDN treatment were different between SD rats and Wistar rats, this study suggested that using 8-wk-old Wistar rats was more effective for investigating the effects of dietary Ca on bone and arterial Ca contents. In addition, this study newly revealed that both 0.01% (low) Ca intake and 2.4% (high) Ca intake accelerated arterial Ca deposition, which correlated positively with significant local BMP-2 mRNA expression. Recent studies have reported that the mechanism of arterial Ca deposition is similar to that for bone remodeling. For primary arterial calcification, VSMCs in the medial elastic site of the aorta have been shown to promote the expression of some osteoblastic markers such as ALP or BMP-2 (14, 15). They also play an active role in bone-like mineralization in the medial elastic site, which leads to loss of elasticity of artery (12, 13). Therefore, the osteoblast-like phenotype of the aorta could be positively correlated with the arterial Ca deposition and lead to arterial calcification (16, 17, 22). Although both the 0.01% and 2.4% Ca intakes are outside the range of normal rodent Ca intake, our data suggest that the Ca metabolism of vascular tissue was altered by the different amounts of calcium intake.

Our data also revealed that a high serum PTH concentration accompanied significant arterial Ca deposition in both the 0.01% and 2.4% Ca intake groups. The mechanism by which the 2.4% (high) Ca intake group produces such a high serum PTH concentration is controversial; however, low Ca intake is well known to be an inducing factor for PTH secretion. Previous in vitro studies using VSMCs have shown that high concentrations of PTH cause a transient rise in intracellular Ca in the cells (33). In addition, Rostand and Drüeke (34) showed that elevated cytosolic-ionized Ca concentrations in cardiomyocytes could be reversed either by parathyroidectomy (PTX) or by treatment with felodipine, suggesting that prolonged exposure of arterial walls to high PTH increases the Ca content in VSMCs and the associated loss of arterial elastic function.

The high urinary Ca excretion in the 2.4% Ca intake group might be explained by Ca overload inducing a secondary increase in circulating PTH levels. In clinical settings, there is evidence that long-term high Ca intake induces intestinal fractional Ca absorption, which results in renal diseases such as nephrolithiasis in older women (35). Patients with impaired renal function are prone to secondary hyperparathyroidism and subsequently, a high risk of fracture (36). Conversely, some epidemiological studies claim that high Ca intake promotes calcification of the coronary arteries and subsequently increases the risk of myocardial infarction in older women (37–39). However, Samelson et al. (40) recently published the results of their prospective observational cohort study in which they found no evidence that additional Ca intake in dietary food sources had any effect on coronary artery calcification. The underlying mechanisms of the cardiovascular effects of high Ca intake remain open to debate, and an in vivo study is needed to further investigate the mechanism by which high Ca intake causes arterial Ca deposition.

There were some limitations for this study. First, although both the 0.01% and 2.4% Ca intake groups had significant increases in local BMP-2 mRNA expression, there were no changes in ALP mRNA expression. Although arterial Ca contents and ALP mRNA expression among the groups exhibited a trend similar to the correlation between arterial Ca contents and BMP-2 mRNA expression, further investigations will be required to determine the reason why only the arterial tissues in rats that underwent OVX with VDN treatment expressed a partial bone-like phenotype at the endpoint of this study. Second, the factors that link bone mass and arterial Ca contents remains controversial. The Ca intake amounts were different among the rats in this study and hence circulating PTH levels may have changed. Circulating PTH levels may be involved in arterial calcification processes (33, 34). However, our data suggest only a partial involvement and we could not determine if circulating PTH levels were the cause of arterial calcification. Besides, because this study could not explain the decrease of urinary P excretion despite high serum PTH levels, examination of other serum factors related to systemic mineral metabolism such as serum active vitamin D$_3$, 1,25-(OH)$_2$D$_3$ levels for kidney function and FGF-23 produced by bone tissue for phosphorus metabolism will be necessary. Third, although we have previously reported elastic degradation and Ca deposition in the medial layer of the thoracic aorta and confirmed those arteries with Ca deposition were associated with the increased arterial Ca content (25), further study might require the identification of Ca deposition in a histomorphological study. Finally, our study model used toxic levels of vitamin D and nicotine to induce arterial calcification. Thus, the nutritional relevance to humans.
is unclear and the possible effects of vitamin D and nicotine toxicity must be considered. Further investigations will be required to focus on the Ca metabolism of arterial tissues and VSMCs.

In summary, our study using young female Wistar rats that underwent OVX with VDN treatment confirmed the promotion of low bone mass and arterial Ca deposition in the extremely low Ca intake group, which has been described as the calcification paradox. In addition, we observed that a high Ca intake of 2.4% resulting in significant arterial Ca deposition associated with BMP-2 mRNA expression, but it did not affect bone mass. In both the groups, arterial Ca deposition results were strongly correlated with serum PTH levels. Thus, further investigations regarding the underlying mechanism will be necessary.

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Conflict of interest

The authors have no conflicts of interest.

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