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Effects of citric acid administration on femoral trabecular structures in ovariectomized mice

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Abstract
Objectives. To examine the effects of citric acid administration on trabecular structures.
Methods. Ovariectomized female ICR mice (n = 39), 8 weeks of age, were divided into four groups: ovariectomized control (OVX-control, n = 9, standard diet); citric acid (CIT, n = 10, citric acid 5 g/100 g diet); vitamin K_2 (menaquinone 7; VK2, n = 10, vitamin K_2 50 μg/100 g diet); and citric acid + vitamin K_2 (CIT + VK2, n = 10, citric acid 5 g/100 g diet and vitamin K_2 50 μg/100 g diet). After 12 weeks, the bone mass of the right femur was measured using peripheral quantitative computed tomography and the three-dimensional (3-D) trabecular structure of the right femur was assessed using microfocus computed tomography.
Results. The bone mineral density was significantly increased in the VK2 group versus the OVX-control group ($P < 0.05$). In the 3-D trabecular structure analysis, skeletal perimeter and number were significantly greater in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group. Skeletal separation, spacing, and trabecular bone pattern factor were significantly lower in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group. Almost all parameters in node–strut analysis were significantly greater in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group.
Conclusions. These results suggest that citric acid improved trabecular structures. However, simultaneous administration of citric acid and vitamin K_2 did not have any additional effect.

Key words Osteoporosis · Mice · Citric acid · Vitamin K_2

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Introduction

In 1993, osteoporosis was defined as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture."[1] In 2001, the NIH Consensus Development Panel defined osteoporosis as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality."[2]

Agents such as activated vitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone, vitamin K_2 (menaquinone 4), and bisphosphonate have been used in the treatment of osteoporosis.[3–6] However, these agents are administered for extended periods, and the financial and physical loads on patients are significant.[7] Recently, the scientific community has paid more attention to prevention rather than treatment of osteoporosis.

Citric acid increases the functional absorption of calcium in the intestinal tract owing to its ion-chelating action, and it is therefore thought to be a useful supplement in preventing osteoporosis.[8] However, while some studies have shown that citric acid increases calcium absorption in the intestinal tract, others have failed to show such an effect.[9,10] Consequently, no consensus has yet been reached on the effects of citric acid on calcium absorption.

While many studies have examined the effects of citric acid administration on intestinal tract calcium absorption,[11,12] few have examined changes in the internal structure of bone. The present study was performed to investigate the effects of citric acid administration on trabecular structures. This study also investigated the effects of combining citric acid with vitamin K_2 (menaquinone 7), which has been reported to lower the risk of fracture by acting on bone matrix proteins, without markedly improving bone mineral density.

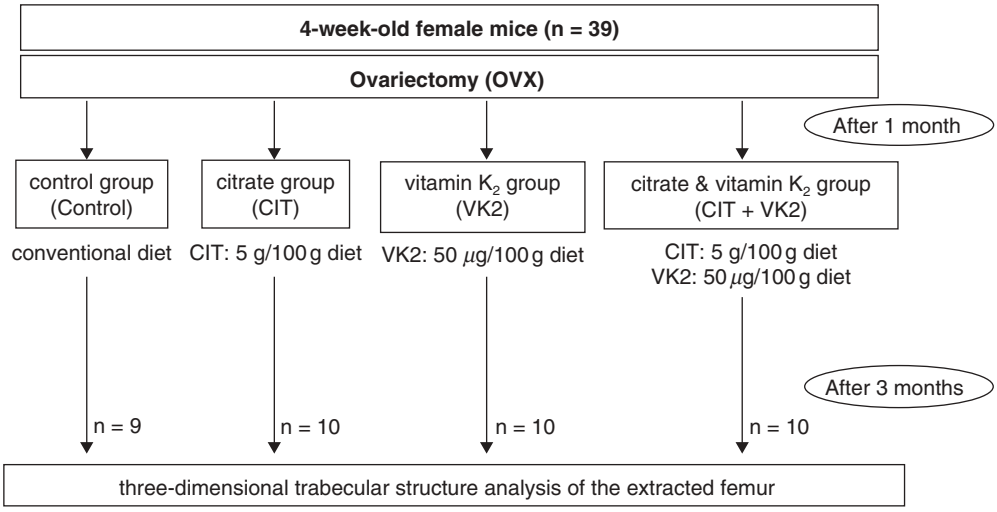


Fig. 1. Experimental protocol of feeding term and diet for the ovariectomized control (*OVX-control*), citrate (*CIT*), vitamin K_2 (*VK2*), and citrate and vitamin K_2 (*CIT + VK2*) groups

Materials and methods

Experimental animals

Ovariectomized female ICR mice ($n = 39$), aged 4 weeks, were obtained from Clea Japan (Tokyo, Japan) and were acclimated for 4 weeks before the experiments with a standard, synthetic diet containing 0.5% calcium per 100 g (CE-2; Clea Japan). Animals had access to distilled water ad libitum. Mice were 8 weeks old at the start of the experiment. The mice were kept in a room maintained at 23 ± 3°C and 55 ± 15% humidity, with a 12/12 h light/dark cycle.

The ethics committee of our institution approved all animal procedures, which complied with institutional guidelines for the care and handling of experimental animals.

Experimental design

The 39 mice were divided into the following four groups: ovariectomized control (OVX-control, $n = 9$); citric acid (CIT, $n = 10$); vitamin K_2 (menaquinone 7; VK2, $n = 10$); and citric acid + vitamin K_2 (CIT + VK2, $n = 10$). For 12 weeks, the standard diet was supplied to the OVX-control group, a diet containing citric acid (5 g/100 g citric acid) was supplied to the CIT group, a diet containing vitamin K_2 (50 μg/100 g vitamin K_2) was supplied to the VK2 group, and a diet containing citric acid (5 g/100 g citric acid) and vitamin K_2 (50 μg/100 g vitamin K_2) was supplied to the CIT + VK2 group. The body weight of each mouse was measured once per week. At the end of the experiment, the animals were killed under anesthesia with pentobarbital (Nembutal; Dainippon Pharmaceutical, Osaka, Japan), and blood samples were collected in heparin-containing tubes. After blood sampling, the right femur of each mouse was harvested. The bone mass of the right femur was measured using peripheral quantitative computed tomography (pQCT), and the three-dimensional (3-D) trabecular structure of the femur was assessed using microfocus computed tomography (μCT). The present study was conducted according to the procedures of Fujikawa et al.,[13] who documented significant differences in all trabecular structural parameters between sham-operated and OVX-control groups.[13] Figure 1 shows the experimental design of the study.

Plasma components

To separate plasma, the blood samples were centrifuged (175 G, 10 min, 4°C) with heparin sodium. We measured calcium (Ca) levels using the chelate color development method, phosphorus (P) levels with the direct molybdenum blue method, magnesium (Mg) levels with the xylidyl blue method, and alkaline phosphatase (ALP) activity using the phenyl phosphate substrate method. A clinical biochemical test kit (Wako Pure Chemical, Osaka, Japan) was used for these measurements.

Bone mass parameters

Bone mass parameters of the femur in the metaphysis region were measured by pQCT (XCT-μScope; Stratec, Birkenfeld, Germany). The diameter, voxel size, CT speed, and block number in tomographic imaging were 15 mm, 0.1 mm, 10 mm/s, and 1, respectively. The bone mineral content (BMC) was calculated from a tomographic image slice 3.0 mm medial to the growth plate. We verified that all system components were performing appropriately by running a hydroxyapatite standard embedded in acrylic plastic each day before scanning samples.

Three-dimensional trabecular structure analysis

A μCT system equipped with a microfocus X-ray tube (focus size 8 μm × 8 μm, MCT-100MF; Hitachi Medical, Tokyo, Japan) produced a 3-D image of each mandible from 201 image slices. The tube voltage, tube current, magnification, and voxel size were 40 kV, 100 μA, ×7, and 18.0 μm × 18.0 μm × 18.0 μm, respectively. Trabecular structure

analysis software (TRI/3D BON; Ratoc System Engineering, Tokyo, Japan) was used to calculate the 3-D trabecular structure parameters from the image information of 50 slices at the femoral metaphysis. In the procedure, the cortical bone and trabecular bone regions were separated by 3-D space filtration of the bone marrow cavity, and the data from the trabecular bone region of each slice were converted to binary by using a threshold obtained by discriminant analysis. That is, we assumed the pixel-value histograms of background and bone to be normally distributed. We chose as the threshold an intermediate pixel value, lying on the tails of the two normal distributions.

From the binary data, the bone volume (BV), bone surface area (BS), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular spacing (Tb.Spac), fractal dimension (FD), and trabecular bone pattern factor (TBPf), were measured. Tb.N, Tb.Sp, and Tb.Spac were measured by the parallel plate model.[14] The FD of the trabecular bone was measured as an indicator of complexity by the box-counting method.[15] The TBPf was measured as an indicator of the unevenness of the skeleton. The concept behind TBPf is that the connectedness of structures can be described by the relationships between convex to concave surfaces.[16] Trabecular connectivity was measured by using a node–strut analysis, as described by Garrahan et al.[17] We identified nodes (Nd, connective points among three or more trabeculae), and cortices (Ct, connective points between trabeculae and cortical bone) by the node–strut analysis, and the parameters of the number of Nd per tissue volume (N.Nd/TV), the number of Ct per tissue volume (N.Ct/TV), the number of struts between Nd and Nd (N.NdNd), the number of struts between Ct and Nd (N.CtNd), the number of struts between Ct and Ct (N.CtCt), the total strut length per tissue volume (TSL/TV), the strut length between Nd and Nd per tissue volume (NdNd/TV), and the strut length between Ct and Nd per tissue volume (CtNd/TV) were obtained by the node–strut analysis.

Statistical analyses

Values presented in the figures and tables are the means ± standard deviation (SD). Student's *t* test was used to determine whether differences between groups were significant ($P < 0.05$, 0.01).

Results

Body weight changes

Figure 2 shows changes in body weight during the study. At the end of the study, body weights in the OVX-control, CIT, VK2, and CIT + VK2 groups were 53.5, 48.5, 49.9, and 48.7 g, respectively. There were no significant differences between the groups. Thus, the bone changes in the present study were not due to the effects of body weight.

Biochemical tests

Table 1 shows the blood component results. The diets did not markedly alter serum Ca, P, or Mg levels. The ALP level, a marker of bone turnover, in the OVX-control group was significantly higher than that in the CIT or VK2 groups ($P < 0.01$), suggesting that CIT and VK2 decreased bone turnover. However, there was no significant difference between the CIT + VK2 and OVX-control groups.

Bone mass parameters

Figure 3 shows the bone mass parameter results. The BMC was significantly increased in the VK2 group compared with in the OVX-control group ($P < 0.05$). The BMC tended to

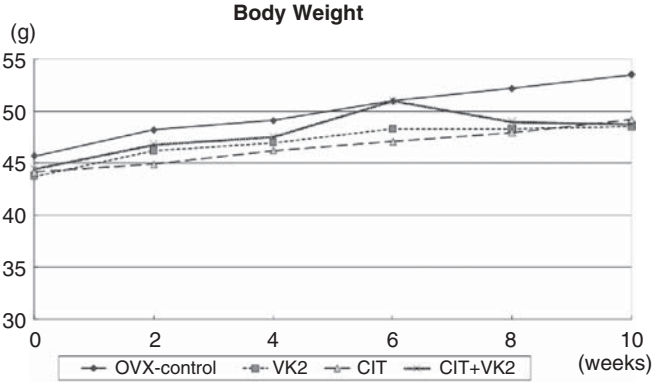


Fig. 2. Changes in body weight in the OVX-control, CIT, VK2, and CIT + VK2 groups

Table 1. Effects of citric acid and vitamin K_2 on plasma calcium, phosphorus, magnesium, and alkaline phosphatase activity after 12 weeks of feeding with supplemented diet

	Plasma			
	Ca (mg/dl)	P (mg/dl)	Mg (mg/dl)	ALP (KA-U/dl)
OVX-Control	7.75 ± 0.90	7.02 ± 0.20	2.53 ± 0.22	4.25 ± 0.25
CIT	7.55 ± 0.54	6.95 ± 0.10	2.55 ± 0.37	3.68 ± 0.26**
VK2	7.74 ± 0.84	6.62 ± 0.41	2.85 ± 0.68	3.84 ± 0.11**
CIT + VK2	7.68 ± 0.54	7.48 ± 0.54	2.78 ± 0.55	4.40 ± 0.20

Values represent means ± SD ($n = 9$ or 10)
CIT, citric acid; VK2, vitamin K_2; ALP, alkaline phosphatase; OVX-control, ovariectomized control; Ca, calcium; P, phosphorus; Mg, magnesium
** $P < 0.01$ *vs.* OVX-control group (Student's *t* test)

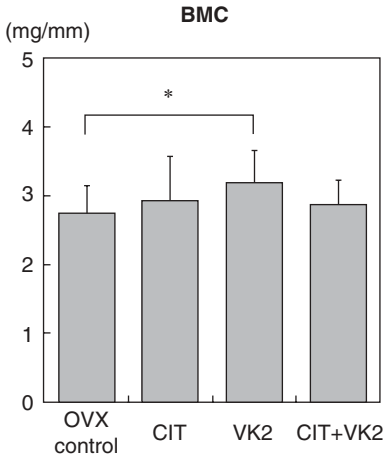


Fig. 3. Mean values of bone mineral content (*BMC*) in the OVX-control, CIT, VK2, and CIT + VK2 groups

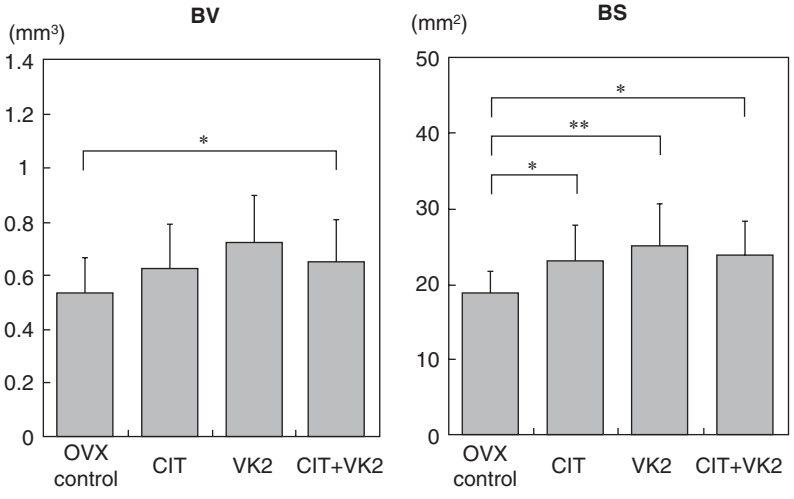
be greater in the CIT and CIT + VK2 groups than in the OVX-control group, but the difference was not significant.

Three-dimensional trabecular structure analysis

Figure 4 shows the results of 3-D trabecular structure analysis. BV was significantly higher in the CIT + VK2 group than in the OVX-control group. BS was highest in the VK2 group and was significantly higher than that in the OVX-control group ($P < 0.01$). BS was also significantly higher in the CIT and CIT + VK2 groups than in the OVX-control group ($P < 0.05$).

Figure 5 shows the results of the parallel plate model analysis. Tb.N was significantly greater, and Tb.Sp and Tb.Spac were significantly lower in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group (all $P < 0.05$). These results indicate that citric acid and vitamin K_2

Fig. 4. Comparison of bone volume (*BV*) and bone surface (*BS*) between OVX-control and CIT, VK2, and CIT + VK2 groups. *$P < 0.05$; **$P < 0.01$



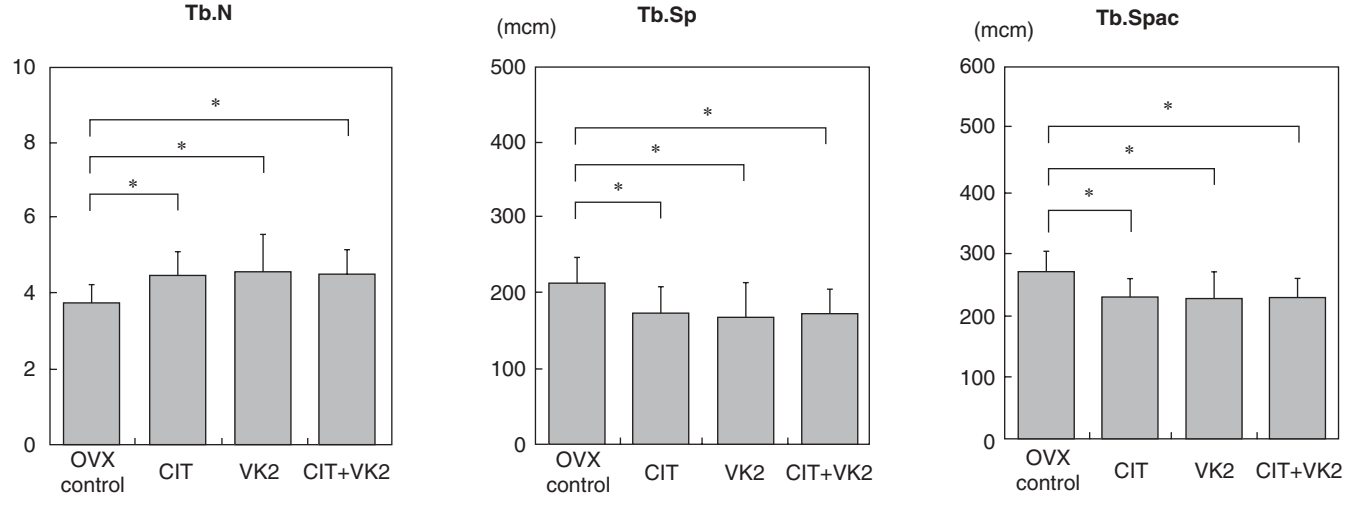


Fig. 5. Comparison of trabecular number (*Tb.N*), trabecular separation (*Tb.Sp*), and trabecular spacing (*Tb.Spac*) between OVX-control and CIT, VK2, and CIT + VK2 groups

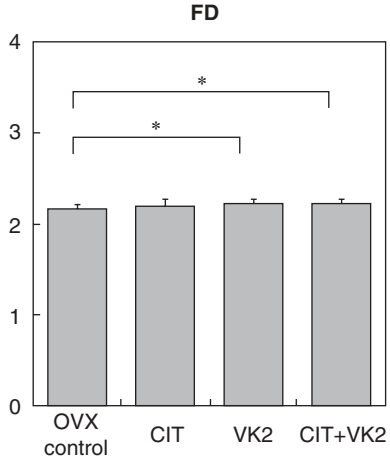


Fig. 6. Mean values of fractal dimension (*FD*), indicating complexity of bone trabecular structure, between OVX-control and CIT, VK2, and CIT + VK2 groups. $*P < 0.05$

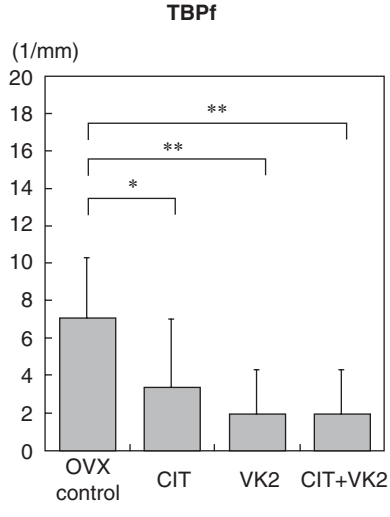


Fig. 7. Mean values of trabecular bone pattern factor (*TBPf*), indicating irregularity of bone trabecular structure, between OVX-control and CIT, VK2, and CIT + VK2 groups. $*P < 0.05$; $**P < 0.01$

administration increased the density of the trabecular structure of the femur. Administration of a combination of citric acid and vitamin K_2 did not result in a further increase in the density.

Figure 6 shows the FD, an indicator of trabecular complexity. In comparison with the OVX-control group, FD was significantly greater in the VK2 and CIT + VK2 groups (both $P < 0.05$). However, there was no significant difference between the OVX-control and CIT groups. This result indicated that VK2 affected trabecular complexity.

Figure 7 shows the results for TBPf, an indicator of trabecular irregularity. The TBPf values in the CIT, VK2, and CIT + VK2 groups were significantly lower ($P < 0.05$, 0.01, 0.01, respectively) than in the OVX-control group, and especially pronounced effects were seen in the VK2 and CIT + VK2 groups.

Figure 8 shows the node–strut analysis results. N.Nd/TV, N.Ct/TV, N.NdNd, N.CtNd, and N.CtCT (number-related parameters) were significantly greater in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group. Similarly, TSL/TV, NdNd/TV, and CtNd/TV (length-related parameters) were significantly greater in the CIT, VK2, and CIT + VK2 groups than in the OVX-control group. These results indicate that citric acid and vitamin K_2 individually increased trabecular connectivity in the femur. Further, combined administration of citric acid and vitamin K_2 increased trabecular connectivity similarly, without any additive effect.

Figure 9 shows typical 3-D images from each group. Compared with the OVX-control group, internal structures appeared visually denser in the CIT, VK2, and CIT + VK2 groups.

Discussion

With the increase in the elderly population, the prevalence of osteoporosis is becoming an important public health problem. The decreased bone strength associated with osteoporosis is due to a decrease in bone density or quality. The major clinical signs of osteoporosis are fractures, caused by bone fragility, and accompanying dysfunction and chronic pain. Fractures are likely to occur in the vertebral body, femoral neck, distal forearm, proximal humerus, and ribs, and they significantly lower the quality of life (QOL). Hip fractures, in particular, are one of the main causes of patients becoming bedridden, and significantly reduce both QOL and vital prognosis.[18]

Prevention of osteoporosis is thus an important social issue. The present study was performed to investigate the effects of citric acid on bone structure, because citric acid has been suggested to facilitate calcium transfer by its ion-chelating action. Furthermore, we compared the effects of citric acid with those of vitamin K_2, which has been reported to modulate the proliferation and function of osteoblastic cells.[19] As vitamin K_2 and citric acid are nutrients with different mechanisms of action, these agents were administered both separately and in combination.

Ovariectomized mice were used in this study as animal models of osteoporosis. Mice were prepared by using the methods described by Fujikawa et al.[13] Parameters such as bone volume fraction, BS, BS/BV, Tb.N, Tb.Th, Tb.Sp, and FD in this model are significantly reduced in comparison with sham-operated controls. This report shows the usefulness of this animal model of osteoporosis.

Three-dimensional trabecular structure analysis showed significant improvements in trabecular structures in the CIT, VK2, and CIT + VK2 groups, compared with in the OVX-control group. Citric acid administration did not bring about marked changes in bone mineral content but significantly improved trabecular structure. By its chelating action, citric acid facilitates calcium absorption in the intestinal tract. Calcium is a nutrient with a low intestinal absorption rate, especially in osteoporosis patients.[20] The majority of dietary calcium is known to be excreted without being absorbed. Thus, increasing calcium absorption via citric acid intake has important implications. Lacour et al.[8]

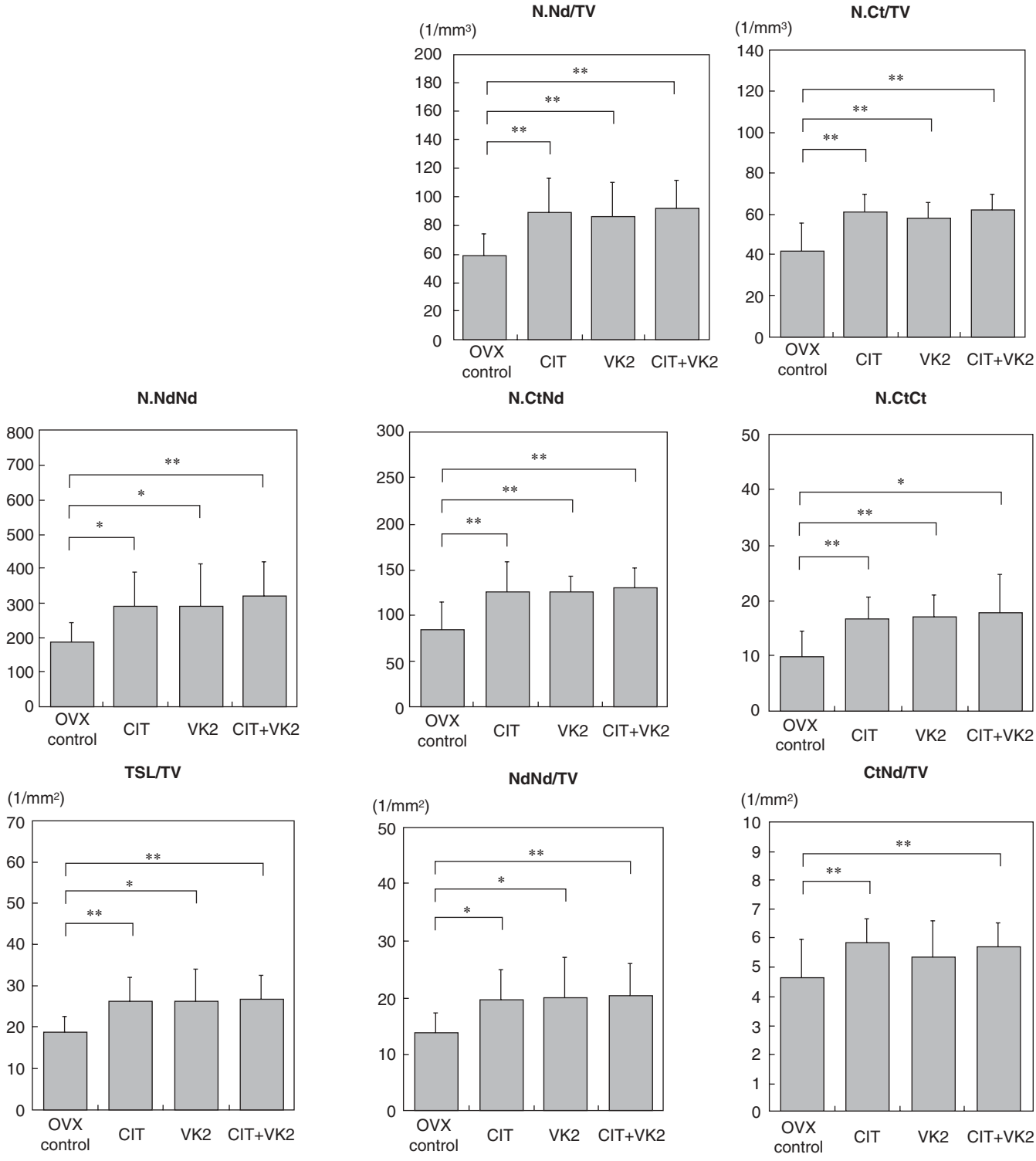


Fig. 8. Comparison of node–strut analysis parameters N.Nd/TV, N.Ct/TV, N.NdNd, N.CtNd, N.CtCt, TSL/TV, NdNd/TV, and CtNd/TV between OVX-control and CIT, VK2, and CIT + VK2 groups. *$P < 0.05$; **$P < 0.01$. *N.Nd*, number of nodes; *N.Ct*, number of cortices; *TV*, tissue volume; *N.NdNd*, number of struts between nodes; *N.CtNd*, number of struts between a node and a cortex; *N.CtCt*, number of struts between cortices; *TSL*, total strut length; *NdNd/TV*, strut length between nodes in relation to tissue volume; *CtNd/TV*, strut length between cortices and nodes in relation to tissue volume

provided a calcium-rich diet containing citric acid to rats and reported significant increases in calcium absorption.[8] In the present study, citric acid administration facilitated calcium absorption. Furthermore, we investigated the effects of absorbed calcium on bone structure, and showed that citric acid administration improved trabecular structures. Thus, absorbed calcium was effective at maintaining trabecular structures.

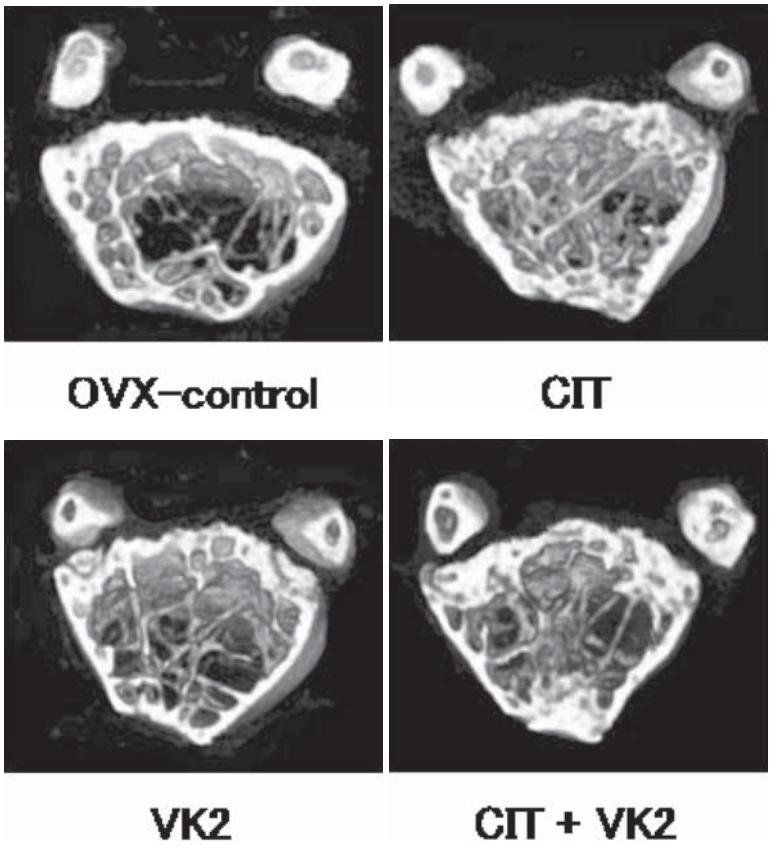


Fig. 9. Reconstructed three-dimensional images of the femur bone trabecular structure in the OVX-control, CIT, VK2, and CIT + VK2 groups

Compared with the OVX-control group, bone mineral content was significantly greater in the VK2 group and tended to be higher in the CIT and CIT + VK2 groups, although these latter differences were not significant. The results were similar for serum ALP, a marker of bone turnover. These observations suggest that VK2 suppresses decreases in bone mineral content due to high turnover in osteoporosis. Menaquinone 4 (MK_4), a chemically synthesized VK2, is difficult to use as a supplement. In the present study, we used menaquinone 7 (MK_7), a naturally occurring VK2, purified from fermented soybean yeast. MK_7 and MK_4 have comparable molecular structures, and recent studies have shown that both affect bone similarly.[13,21] VK2 acts as an essential cofactor for the gamma-carboxylation of bone matrix proteins. For example, VK2 causes gamma-carboxylation of osteocalcin and matrix Gla protein (MGP) in osteoblasts and is involved in calcification. However, in recent years, VK2 has been shown to increase the expression of marker proteins involved in bone formation, such as ALP, osteoprotegerin, osteopontin, MGP, and COL1A1, by transcriptional regulation via the nuclear receptor SXR.[22]

No additive effect of coadministration of CIT and VK2 was seen with any of the parameters assessed. Trabecular structure parameters with single administration were comparable to those with coadministration. This result agreed with those of a previous study of drugs for osteoporosis treatment, which indicated that combination therapy with two drugs did not show significantly better effects than either drug alone.[23] While the reasons for this are unclear, agent selection and order of administration can have a marked impact on therapeutic effects when administering multiple agents together. Furthermore, VK2 is a fat-soluble vitamin and citric acid is water-soluble, and the conditions under which these two agents are absorbed most effectively in the intestinal tract are different. It is possible that fat-soluble agents may not be absorbed under conditions in which a water-soluble agent would be absorbed effectively, and when administering such diverse agents, administration at different times may promote more efficient uptake.

In trying to prevent osteoporosis, proper nutrients must be taken in an appropriate manner. The results of the present study indicated that CIT improved trabecular structure. In addition, VK2 suppressed decreases in bone mineral content and improved trabecular structure. However, simultaneous administration had no additive effect. These observations suggested that both CIT and VK2 are effective at preventing osteoporosis and that when both agents are administered together, the methods of administration must be improved, possibly by their being taken at different times.

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