Effects of Vitamin K₂ (Menatetrenone) on Calcium Balance in Ovariectomized Rats

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ABSTRACT—Vitamin K₂ (menatetrenone) has been used for the treatment of osteoporosis in Japan. We investigated the effects of ovariectomy (OVX) and vitamin K₂ on the calcium (Ca) balance in 20-week-old female Fischer rats. Vitamin K₂ (31 mg/kg per day) was given to animals as a dietary supplement. At weeks 4 and 8 after OVX, a Ca balance study was performed for 5 days. The intestinal Ca transport was determined using the everted gut-sac technique at week 9. The Ca balance was poorer in the OVX-control group than in the sham-control group at weeks 4 and 8 after OVX. The Ca balance improved significantly in the vitamin K₂ groups as compared with the sham- and OVX-control groups. The intestinal Ca transport decreased due to OVX and was higher in the vitamin K₂ administration groups than in the sham- and OVX-control groups, but not to a significant extent. The bone mineral density in the femoral metaphysis as well as the cortical area and cortical thickness in the femoral diaphysis in the OVX-control group were lower than in the sham-control group. The administration of vitamin K₂ significantly inhibited an OVX-induced decrease in cortical area and cortical thickness in the femur. These findings suggest that the poor Ca balance observed in ovariectomized rats may be improved by vitamin K₂; vitamin K₂ may be involved in preventing bone loss *in vivo*.

Keywords: Vitamin K₂, Ovariectomy, Calcium absorption, Bone mineral density

Ovarian hormone deficiency is commonly presumed to be a major risk factor for osteoporosis in postmenopausal women (1). This type of osteoporosis is often associated with the intestinal malabsorption of calcium (Ca), which is believed to aggravate the negative Ca balance that may occur with aging and to contribute to bone loss in postmenopausal women (2–4).

Menatetrenone, vitamin K₂ with 4 isoprene units, has high γ -carboxylation activity in hypoprothrombinemic rats (5). A specific protein containing γ -carboxyglutamic acid (gla) was found in bone and termed osteocalcin (6). Since this protein is synthesized only by osteoblasts, the serum osteocalcin concentration is regarded as a biochemical marker for bone turnover (7, 8). Vitamin K is essential for the γ -carboxylation of osteocalcin, and noncarboxylated osteocalcin cannot bind to hydroxyapatite (9, 10). Therefore, much attention has been paid to the role of vitamin K in bone metabolism. Previously, we studied the effects of vitamin K₂ on bone metabolism and found that the drug prevents bone loss induced by ovariectomy (OVX) of or

prednisolone administration to rats (11–13). *In vitro* studies indicated that vitamin K₂ inhibits bone resorption in an organ culture system, inhibits the osteoclast-like cell formation in bone marrow culture and co-culture systems and enhances mineralization in human osteoblasts (14–16). Moreover, we previously reported that vitamin K₂ improves the poor Ca balance and intestinal Ca absorption induced by sodium loading in rats (17).

The OVX rat model is considered to be appropriate for investigating the changes related to postmenopausal bone loss, and many investigators have tried to clarify the effects of OVX on the intestinal Ca absorption in rats (18, 19). However, the results thereof are not always in agreement with those seen clinically (20, 21). In this study, therefore, we investigated the effects of OVX on the Ca balance and whether vitamin K₂ improves these changes in rats.

MATERIALS AND METHODS

Experimental protocol

We used 8-week-old female Fischer rats (Clea Japan Inc., Tokyo). Animals were acclimated for 12 weeks prior to the study, in which they were allowed to take a synthetic

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diet (Clea Japan, Inc.), which contained 0.5% calcium, 0.66% phosphorus and 24 IU vitamin D₃/100 g, and deionized drinking water ad libitum. Animals were housed in groups and were then bred according to the following environmental conditions: lighting condition of 12-h light/dark cycle and temperature of $23 \pm 2^\circ\text{C}$. At 20 weeks of age, animals were allotted into 4 groups: sham-operated-control (sham-control), sham-vitamin K₂, OVX-control and OVX-vitamin K₂ groups. Under pentobarbital anesthesia, rats in the OVX groups were subjected to bilateral ovariectomy, and rats in the sham groups subjected to sham surgery. All animals were fed a synthetic diet. Vitamin K₂ (menatetrenone; Eisai Co., Ltd., Tokyo) was given to animals as a dietary supplement for 8 weeks. The vitamin K₂ content of the diet in the sham- and OVX-vitamin K₂ groups was 58 mg per 100 g of diet. The dose level of the vitamin K₂ administration groups was 31 mg/kg of body weight, which was calculated by the mean body weight and daily diet intake of rats that were measured four times during the study. All animal procedures were approved by the ethical committee at our laboratories and were performed in compliance with the institutional guidelines for the care and handling of experimental animals. A calcium balance test was performed 4 and 8 weeks after OVX. After the second calcium balance test, these rats were sacrificed under pentobarbital anesthesia. Blood was collected from the abdominal aorta, and the small intestine was isolated to measure the intestinal calcium transport. Subsequently, the femur was harvested.

Calcium balance measurement

At weeks 4 and 8 after OVX, animals were housed individually in the metabolic cage for 5 days and were fed a known amount of diet (10.1 g/day) every day and were allowed to take deionized water. The feces and urine of the animals were daily collected for 5 days. The carmine (Wako Pure Chemical Industries, Ltd., Osaka)-added diet (25 mg/100 g of diet) was given to rats instead of the regular diet on the first and last days of the study in order to mark the onset and termination of the fecal collection. The residual diet was weighed to calculate the amount of the diet that was fed to rats for 5 days. Food samples and the feces were hydrolyzed with 6 N-HCl, and calcium in these hydrated solutions and urine was measured with a commercial kit (Calcium C test Wako; Wako Pure Chemical Industries, Ltd.).

The calcium absorption and calcium balance were calculated by the following equations:

Calcium absorption (mg/day) = Calcium intake (mg/day) – fecal calcium (mg/day)

Calcium balance (mg/day) = Calcium intake (mg/day) – {fecal calcium (mg/day) + urinary calcium (mg/day)}

Calcium transport

The intestinal calcium transport was determined by everted gut-sac techniques. The small intestine was isolated to measure the intestinal calcium transport. The small intestine isolated at sacrifice was immediately rinsed with saline, and the first 10-cm portion of the intestine distal to the pyloric valve was dissected. A large portion of the mesentery was trimmed free, and the intestine was everted as described below. First, an everting rod was inserted into the distal end of the segment. The intestine was then ligated at a level just distal to the pyloric valve. Subsequently, the intestine was trimmed to a length of 4.0 cm, was filled with 0.4 ml of incubation medium using a syringe with a blunt needle and was then ligated. The incubation medium consisted of 125 mM CaCl₂, 10 mM fructose, 30 mM Tris-HCl, and 0.25 mM CaCl₂ · 2H₂O. Subsequently, the everted gut-sac was then placed into a 25 ml Erlenmeyer flask containing 10 ml of the same incubation medium and was then incubated at 37°C for 60 min. A gas mixture (95% O₂, 5% CO₂) was continuously bubbled through the incubation medium, and the flask was shaken on a shaker at 60 rpm. The gut-sac was displaced from the flask 60 min later, and most of the external liquid was allowed to drip off. The gut-sac was cut after the final drop was carefully blotted away, and the content was drained from the pyloric end into a centrifuging tube (liquid side). The calcium concentration of the fluid inside and outside of the gut-sac (external liquid) was determined with a commercial kit (Calcium-C test Wako). The intestinal calcium transport was expressed as the ratio (S/M) of the calcium concentration in the serosal medium (inside the sac) to that in the mucosal medium (outside the sac).

Determination of bone parameters

The right femur, from which adhering connective tissue and muscle were removed, was placed into 70% ethanol. Peripheral quantitative computed tomography (pQCT) images were obtained from the femur using the XCT-960 system (Stratec, Birkenfeld, Germany).

The femur was immersed into 70% ethanol in an acrylic plastic tube (75 mm × 12 mm). After a scout scan was obtained and the growth plate was identified, transverse image sets of two slices were obtained at 3.0 mm distal to the growth plate as the metaphysis and those at 12 mm distal as the diaphysis. For the analysis of data, the following conditions were set: 0.148 mm in voxel size; separation mode 1 with a threshold of 0.930 for cortical bone; contour mode 2; and peel mode 20 with 52% of total area for cancellous bone. Quality assurance measurements were made daily using the hydroxyapatite standard embedded in acrylic plastic to check all system components before sample scans were performed.

Plasma assays

Blood samples were centrifuged (4°C, 3000 rpm, 10 min) to obtain the plasma. Calcium and inorganic phosphorus concentrations and alkaline phosphatase activity in plasma were immediately measured with commercial kits (Calcium C-test Wako, P-test Wako, Alkaline-phosphate K test Wako). Plasma concentrations of parathyroid hormone (PTH) were determined with a rat PTH-specific RIA-kit (Nichols Institutes Diagnostics, San Juan Capistrano, CA, USA). Plasma concentrations of 1,25(OH)₂ vitamin D₃ (1,25(OH)₂D₃) and 25(OH) vitamin D₃ (25(OH)D₃) were analyzed as follows: these substances were extracted from the plasma with acetonitrile and separated by high pressure liquid chromatography, and the separated samples were then measured using a competitive protein binding assay (Teijin Bio Inc., Tokyo).

Statistical analyses

All data are expressed as the mean $\pm$ S.E.M. Comparison between the sham-operated-control and OVX-control groups was performed by Student's *t*-test. Analysis of variance was performed in the 4 groups, and the significance of difference was tested in sham-operated and ovariectomized rats between the control and vitamin K₂ administration groups according to the orthogonal contrast procedure. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Calcium balance

The calcium intake, as well as the fecal and urinary excretions of calcium in the sham- and OVX-control groups are shown in Table 1. At week 8 after OVX, the calcium intake, as well as the fecal and urinary excretions of calcium were significantly lower in the OVX-control

group than in the sham-control group. At week 8 after OVX, the fecal calcium excretion in both sham-operated and ovariectomized rats in the vitamin K₂ administration group was significantly lower as compared with the respective control group. At weeks 4 and 8 after OVX, the urinary calcium excretion was higher in the OVX-vitamin K₂ group than in the OVX-control group.

Figure 1 shows the calcium absorption in the sham- and OVX-control groups at weeks 4 and 8 after OVX. At week 4 after OVX, the calcium absorption in the OVX-control group was reduced to 45% of the value in the sham-control group. At week 8 after OVX, the calcium absorption in the sham-control group provided a negative value, and the calcium absorption was significantly lower in the

Fig. 1. Effects of ovariectomy (OVX) and vitamin K₂ (V.K₂) on calcium (Ca) absorption 4 and 8 weeks after operation. Ca absorption was calculated by the following equations: Ca absorption (mg/day) = Ca intake (mg/day) – fecal Ca (mg/day). Each column and bar represent the mean $\pm$ S.E.M. of 10 animals.

Table 1. Effects of ovariectomy (OVX) and vitamin K₂ on mean calcium intake and mean urinary and fecal calcium excretion 4 and 8 weeks after operation

	4 weeks after operation			8 weeks after operation		
	Ca intake	Urinary Ca excretion	Fecal Ca excretion	Ca intake	Urinary Ca excretion	Fecal Ca excretion
	(mg/day)	(mg/day)	(mg/day)	(mg/day)	(mg/day)	(mg/day)
Sham						
Control	37.7 $\pm$ 1.1	0.89 $\pm$ 0.19	32.6 $\pm$ 1.1	35.2 $\pm$ 1.0	2.00 $\pm$ 0.14	35.5 $\pm$ 1.6
Vitamin K ₂	34.2 $\pm$ 1.4	0.53 $\pm$ 0.05	27.2 $\pm$ 1.4 ^{††}	32.7 $\pm$ 1.1	1.61 $\pm$ 0.18	24.8 $\pm$ 1.6 ^{††}
OVX						
Control	33.2 $\pm$ 1.6	0.43 $\pm$ 0.08	31.1 $\pm$ 2.0	25.5 $\pm$ 0.9 ^{**}	0.73 $\pm$ 0.10 ^{**}	29.8 $\pm$ 0.9 ^{**}
Vitamin K ₂	35.8 $\pm$ 1.1 ^{††}	1.91 $\pm$ 0.22 [†]	30.4 $\pm$ 1.3	26.8 $\pm$ 1.1	1.28 $\pm$ 0.23 [†]	22.2 $\pm$ 1.1 ^{††}

Each value represents the mean $\pm$ S.E.M. of 10 animals. *P<0.05, **P<0.01 vs sham-control group; [†]*P*<0.05, ^{††}*P*<0.01 vs respective control group.

OVX-control group than in the sham-control group. At week 4 after OVX, the calcium absorption was significantly higher in the OVX-vitamin K₂ group than in the control group. At week 8 after OVX, in particular, the calcium absorption in both sham- and ovariectomized rats in the vitamin K₂ administration groups changed to provide positive values. The effects of vitamin K₂ on the calcium balance in sham-operated and ovariectomized rats are shown in Fig. 2. The calcium balance changed in almost the same pattern as did the calcium absorption. The effects of OVX or vitamin K₂ administration on the calcium absorption and balance were more remarkable at week 8 than at week 4 after OVX.

Fig. 2. Effects of ovariectomy (OVX) and vitamin K₂ (V.K₂) on calcium (Ca) balance 4 and 8 weeks after operation. Ca balance was calculated by the following equations: Ca absorption (mg/day) = Ca intake (mg/day) – fecal Ca (mg/day), Ca balance (mg/day) = Ca absorption (mg/day) – urinary Ca. Each column and bar represent the mean $\pm$ S.E.M. of 10 animals.

Intestinal calcium transport

The intestinal calcium transport was determined by the everted gut-sac technique and was expressed as the ratio of the calcium concentration in the serosal medium (inside the sac) to that in the mucosal medium (outside the sac) after 60 min of incubation. The effects of OVX and vitamin K₂ on the intestinal calcium transport are shown in Fig. 3. OVX provoked a significant decrease in S/M ratio at week 9. The ratio in both sham-operated and ovariectomized rats were slightly higher in the vitamin K₂ treatment groups than in their respective control groups.

Body weight, uterine weight and plasma assay

The effects of OVX and vitamin K₂ on body weight, uterine weight and calcium-regulating hormones in plasma at week 9 after OVX are shown in Table 2. At the termination of the study, OVX provoked an increase in body weight and a decrease in uterine weight.

Fig. 3. Effects of ovariectomy (OVX) and vitamin K₂ (V.K₂) on intestinal calcium transport 9 weeks after operation. Each column and bar represent the mean $\pm$ S.E.M. of 10 animals.

Table 2. Effects of ovariectomy (OVX) and vitamin K₂ on body weight, uterine weight, and plasma calcium 25(OH)D₃, 1,25(OH)₂D₃ and PTH levels 9 weeks after operation

	Body weight (g)	Uterine weight (mg)	Plasma			
			Ca (mg/dl)	25(OH)D ₃ (ng/dl)	1,25(OH) ₂ D ₃ (pg/ml)	PTH (pg/ml)
Sham						
Control	184 $\pm$ 2	679 $\pm$ 65	9.04 $\pm$ 0.18	14.6 $\pm$ 1.2	20.9 $\pm$ 2.2	22.7 $\pm$ 3.1
Vitamin K ₂	191 $\pm$ 3	750 $\pm$ 42	9.12 $\pm$ 0.21	16.9 $\pm$ 1.9	25.8 $\pm$ 3.5	21.1 $\pm$ 4.3
OVX						
Control	211 $\pm$ 5**	160 $\pm$ 14**	8.66 $\pm$ 0.14	18.8 $\pm$ 0.9*	34.0 $\pm$ 2.4**	27.8 $\pm$ 3.2
Vitamin K ₂	219 $\pm$ 3	159 $\pm$ 18	8.70 $\pm$ 0.15	19.2 $\pm$ 0.7	38.4 $\pm$ 3.9	23.7 $\pm$ 3.8

Each value represents the mean $\pm$ S.E.M. of 10 animals. * P <0.05, ** P <0.01 vs sham-control group.

Table 3. Effects of ovariectomy (OVX) and vitamin K₂ on total density and trabecular density at the metaphysis and cortical area, cortical thickness, outer perimeter and endo perimeter at the diaphysis in the femur 9 weeks after operation

	Metaphysis		Diaphysis			
	Total density (mg/cm ³)	Trabecular density (mg/cm ³)	Cortical area (mm ²)	Cortical thickness (mm)	Outer perimeter (mm)	Endo perimeter (mm)
Sham						
Control	611.3 ± 9.4	287.8 ± 12.7	4.22 ± 0.04	0.591 ± 0.005	9.21 ± 0.06	5.29 ± 0.06
Vitamin K ₂	632.8 ± 12.8	327.2 ± 17.8	4.24 ± 0.04	0.599 ± 0.004	9.25 ± 0.09	5.20 ± 0.05
OVX						
Control	469.5 ± 4.9**	79.5 ± 8.8**	4.01 ± 0.04**	0.548 ± 0.007**	9.26 ± 0.08	5.59 ± 0.08**
Vitamin K ₂	480.7 ± 3.0	90.6 ± 9.5	4.13 ± 0.04 [†]	0.566 ± 0.004 [†]	9.19 ± 0.07	5.52 ± 0.05

Each value represents the mean ± S.E.M. of 10 animals. **P < 0.01 vs sham-control group; [†]*P* < 0.05 vs respective control group.

Plasma concentrations of both 25(OH)D₃ and 1,25(OH)₂D₃ were significantly higher in the OVX-control group than in the sham-control group. However, OVX did not affect plasma concentrations of PTH and calcium. Vitamin K₂ treatment did not affect these parameters.

Bone parameters in the femur

The bone parameters in the femoral metaphysis and diaphysis, which were determined using the pQCT method, are shown in Table 3. At week 9 after OVX, the total density and trabecular density in the femoral metaphysis of animals in the OVX-control group were markedly low as compared with the sham-control group. In the femoral diaphysis, the cortical area and cortical thickness of animals in the OVX-control group were significantly lower than those in the sham-control group. Vitamin K₂ treatment improved these changes.

DISCUSSION

To confirm the effects of vitamin K₂ on the intestinal Ca absorption in a rat model for osteoporosis, we used OVX rats because they represent an animal model of ovarian hormone deficiency in postmenopausal women (18, 19). Clinically, women with postmenopausal osteoporosis often have impaired calcium absorption (2–4). Despite many reports describing the effects of OVX on the intestinal Ca absorption in rats, however, the results thereof are not always in agreement with those seen clinically. Thomas et al. have demonstrated that both the growth rate of bone and the intestinal Ca absorption increased in 6-week-old rats at week 3 after OVX (22). In contrast, Kalu and Orhii have reported that the intestinal Ca absorption decreased gradually in 12-month-old, ovariectomized, 0.5% Ca-fed rats at weeks 8 and 12 after OVX (23). O'Loughlin and

Morris have also reported that the intestinal Ca absorption decreased in 10- to 24-month-old OVX rats at weeks 12 and 24 after OVX (24). We speculate that the rationale for such contradictory findings is associated with the age of rats. Therefore, we used aged rats to investigate the effects of OVX and vitamin K₂ on the Ca absorption at weeks 4 and 8 after OVX. The Ca absorption and Ca balance were significantly lower in the OVX-control group than in the sham-control group at weeks 4 and 8 after OVX. Namely, the Ca intake and fecal Ca excretion in the OVX-control group reduced to 88% and 94% of the values in the sham-control group, respectively, at week 4 after OVX; these parameters reduced to 73% and 84% of the values in the sham-control group, respectively, at week 8 after OVX. The extent of decrease was greater at week 8 than at week 4 after OVX. Eight weeks after surgery, calcium absorption showed a negative value and was significantly lower than that at week 4 in both the sham- and OVX-control groups. Calcium absorption is known to be extensively affected by food intake. Food intake is calculated by deducting the total of the remaining and fallen food from the total volume of food given to the animals. Structurally, the metabolic cage that we used does not allow animals to take food that falls into the tray. In this study, the lot of food used in the calcium balance test at postoperative week 4 was different from that at week 8. The food used at week 8 was more fragile than that given at week 4, so the amount of fallen food was thought to have increased compared to that at week 4. Therefore, we consider that a decrease in apparent food intake was the causative factor for negative calcium absorption at week 8 after surgery.

Both the Ca absorption and the Ca balance in the vitamin K₂ administration groups increased as compared with the sham- and OVX-control groups at weeks 4 and

8 after OVX. These effects of vitamin K₂ are considered to be due to a vitamin K₂-induced decrease in fecal Ca excretion. The ameliorating effect of vitamin K₂ on the Ca balance was more remarkable at week 8 than at week 4 after OVX. The intestinal Ca transport was measured at week 9 after OVX to clarify the mechanism by which vitamin K₂ improves the Ca balance. Regarding the effects of OVX on the intestinal Ca transport, Hope et al. have reported that OVX provoked an increase in Ca transport (25), Arjmandi et al. (26) and O'Loughlin and Morris (27) have reported that OVX provoked a decrease, and Lindgren and DeLuca have reported that OVX provoked no change (28). In the present study, we found the OVX provoked a decrease in intestinal Ca transport in rats, which well reflected an OVX-induced decrease in both fecal Ca excretion and Ca balance. These findings were consistent with the report of Arjmandi et al. (26), and O'Loughlin and Morris (27). We have already reported that vitamin K₂ improved a decrease in intestinal Ca transport which was induced by sodium loading in rats (17). In this study, however, the intestinal Ca transport was higher in the vitamin K₂ administration groups than in the OVX-control group, but not to a significant extent. In our previous study, the Ca transport was measured in 11-week-old SD rats at week 6 of sodium loading. The S/M ratio in the sham-treatment group was 3.37. In this study, in contrast, the S/M ratio in the sham-treatment group was lower (1.18) than that in our previous study (17). This result is considered due to the use of very aged (29-week-old) Fischer rats. Therefore, we consider that such a low S/M ratio may be primarily responsible for our failure in clarifying the effects of vitamin K₂ on the intestinal Ca absorption in this study. Increased plasma concentrations of both 25(OH)D₃ and 1,25(OH)₂D₃ in the OVX-control group may be due to an OVX-induced decrease in intestinal Ca absorption. Vitamin K₂ treatment slightly increased plasma concentrations of 25(OH)D₃ and 1,25(OH)₂D₃. Therefore, we speculate that the improving effect of vitamin K₂ on the Ca balance may be partly mediated by an elevation of circulating blood concentrations of vitamin D₃.

At week 9 after OVX, the total density and trabecular density in the femoral metaphysis of animals in the OVX-control group decreased to 77% and 28% of the values in the sham-control group, respectively. The cortical area and cortical thickness were significantly lower in the OVX-control group than in the sham-control group. Vitamin K₂ treatment inhibited these decreases induced by OVX. We studied the effects of menatetrenone on the bone metabolism and reported that the drug prevents bone loss induced by OVX or prednisolone administration to rats (11–13). Vitamin K₂ has been shown to inhibit bone resorption in the mouse calvaria culture system (14), to inhibit an increase in pit formation area which is induced by isolated osteoclasts

on dentine slices and to induce apoptosis of osteoclasts (29). The effects of vitamin K₂ on the calcium balance may constitute a mechanism for the prevention of bone loss.

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