Inhibitory Effect of Vitamin K₂ (Menatetrenone) on Bone Resorption in Ovariectomized Rats: A Histomorphometric and Dual Energy X-Ray Absorptiometric Study

Yasuhiro Akiyama¹, Kuniko Hara¹, Masatoshi Kobayashi¹, Takashi Tomiuga¹ and Tetsuya Nakamura²

¹Department of Applied Drug Research, ²Vitamin E Information and Technology Section, Eisai Co., Ltd., Koishikawa 4–6–10, Bunkyo-ku, Tokyo 112–8088, Japan

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ABSTRACT—To clarify how vitamin K₂ prevents bone loss in vivo, it was given to ovariectomized 20-week-old rats for 2 weeks. Bone mineral density (BMD) in the whole femur and in 7 specific portions (F1 to F7 from the proximal to the distal end) was determined by dual-energy X-ray absorptiometry, and histomorphometry was also performed in proximal tibial metaphysis. Ovariectomy (OVX) resulted in significant decreases in the BMD in the whole femur and the F1, F2, F6 and F7 portions. Histomorphometrical analysis of the tibia showed that the bone volume / tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) were decreased, while trabecular separation (Tb.Sp) and osteoclast number / bone surface (Oc.N/BS) were increased by OVX. The parameters for bone formation were not changed by OVX. These data indicate that the bone loss within 2 weeks is due to the enhancement of bone resorption. Vitamin K₂ at 50 mg/kg inhibited the decrease in the BMD of the whole femur together with the F6 and F7 portions. Vitamin K₂ also inhibited the decrease in Tb.N and the increases in Tb.Sp, Oc.N/BS and osteoclast surface / bone surface (Oc.S/BS) caused by OVX. These results suggest that vitamin K₂ prevents bone loss through the inhibition of bone resorption and osteoclast formation in vivo.

Keywords: Vitamin K₂, Ovariectomy, Bone mineral density, Histomorphometry, Osteoclast number

Many studies suggesting a relationship between vitamin K and bone metabolism have been reported; e.g., the circulating level of vitamin K (1–4) or undercarboxylated osteocalcin (5, 6) is related to the risk of fractures in osteoporotic patients, and vitamin K supplementation in postmenopausal women (7) or vitamin K-depleted rats (8) induces a decrease in the urinary excretion of calcium and hydroxyproline.

Menatetrenone, vitamin K₂ with 4 isoprene units, has high γ-carboxylation activity in hypoprothrombinaemic rats (9). Therefore, we studied the effect of menatetrenone on bone metabolism and found that it prevents bone loss induced by ovariectomy (10) or prednisolone administration (11) in rats. Moreover, menatetrenone has a significant therapeutic effect on involutional osteoporosis (12). In vitro studies indicated that vitamin K₂ inhibits bone resorption in an organ culture system (13), inhibits osteoclast-like cell formation in bone marrow culture (14) and co-culture systems (15) and enhances human osteoblast-induced mineralization (16). However, it has not yet been demonstrated that these in vitro effects of vitamin K₂ actually contribute to bone metabolism in vivo. It is generally accepted that estrogen deficiency in rats induces an immediate increase in bone turnover, as evidenced by rapid increases in osteoclast number, resulting in a rapid loss of trabecular bone (17–20). Therefore, in this study, to clarify the inhibitory effect of vitamin K₂ on bone resorption in vivo, we measured bone mineral density in the femur and histomorphometrically compared bone resorption and bone formation in the proximal tibia metaphysis in rats treated with vitamin K₂ during the early period after ovariectomy.

MATERIALS AND METHODS

Experimental protocol
A group of 40 female Fischer rats, 10 weeks of age, were obtained from Clea Japan, Inc. (Tokyo) and acclimated for 10 weeks prior to the start of the experiment with a standard diet (CE-2; Clea Japan, Inc.). At 20 weeks of age, they were divided into 4 groups: sham, ovariectomy (OVX)-control, OVX-vitamin K₂ 5 mg/kg
and OVX-vitamin K₂ 50 mg/kg. Under pentobarbital anesthesia, rats in the 3 groups were bilaterally ovariec-
tomized and the remainder were subjected to sham sur-
geries. All animals were fed a synthetic diet containing
0.5% calcium, 0.78% phosphorus and 240 IU/100 g vi-
tamin D₃ (Clea Japan, Inc.). Vitamin K₂ (menatetrenone;
Eisai Co., Ltd., Tokyo) was given as a dietary supplement
for 2 weeks. The vitamin K₂ content of the diet in the
OVX-vitamin K₂ 5 and 50 mg/kg groups was 9.4 and 85.0
mg per 100 g diet, respectively, calculated by the mean
body weight (178.8 g) and daily food intake/rat (10.1 g).
In this study, food intake of all the rats was restricted
to the same amount of food, 10.1 g per rat per day, to
prevent the hyperphagia that occurs in ovariec-
tomized animals. Distilled water was given ad libitum. They
were kept in a room maintained at 24±2°C with a 12-hr light-
dark cycle. At days 11 and 5 before sacrifice, these rats
were given 2 subcutaneous injections of tetracycline
(Teramycin®; Pfizer, Tokyo) at a dose of 25 mg/kg in
order to label the sites of active bone formation. The body
weights of all animals were measured at the time of ovari-
ectomy and at sacrifice. Two weeks after the ovariec-
tomy, these rats were sacrificed under pentobarbital anesthesia,
and the success of the ovariec-toomy was confirmed by
the weights of the uterine horns. Then bilateral femora
and tibiae were harvested.

**Bone mass measurements**

After the length of the left femur was measured,
femoral bone mineral content (BMC), bone mineral den-
sity (BMD) and the area of the whole femur were mea-
sured by dual-energy X-ray absorptiometry (DXA, QDR-
1000; Hologic Inc., Waltham, MA, USA) using special
software developed for small animals, set to a high
resolution mode. Both intra- and interassay coefficients
of variation (CVs) for BMD were less than 1%. As the
length of the femora in the 4 groups was almost the same,
the scan image of the femur was divided into 7 portions
equal length (Fig. 1), and BMC and BMD in each portion
were calculated using this software.

**Preparation of specimens**

The tibiae were removed and dissected from the adher-
ing connective tissue and muscle before being cut 10 mm
from the proximal end. The proximal portions of the
right tibiae were fixed with 70% alcohol and stained using
Villanueva bone stain (Maruto Instrument Co., Ltd.,
Tokyo) for 7 days. They were then dehydrated with se-
quential changes (70%, 95% and 100%) of an ethanol
solution and embedded in methyl-methacrylate (Wako
Pure Chemical Industries, Ltd., Osaka) without decalc-
cification, and 8-μm-thick longitudinal sections were ob-
tained with a Jung rotary microtome (Leica Instruments
GmbH, Heidelberg, Germany). Some of these sections
were then stained by Goldner’s trichrome method for
measurements of static parameters. Those sections
without Goldner’s stain were used for the measurement
of dynamic parameters.

The proximal portions of the left tibiae were fixed with
10% formalin in phosphate-buffered saline. They were
derhydrated, embedded in glycolmethacrylate (2-hydroxy-
ethyl methacrylate EM; TAAB Laboratories Equipment,
Ltd., Berks, England) and longitudinally sectioned at the
center to yield 8-μm frontal undecalcified sections. These
sections were stained with tartrate-resistant acid phospha-
tase (TRAP) as a marker of osteoclast.

**Histomorphometric analysis**

Bone histomorphometry of the secondary spongiosa of
the proximal metaphysis between 0.8 and 3.2 mm distal
to the growth plate-epiphysial junction (Fig. 2) was
performed using an automated image analysis system
as reported previously (21). The system consisted of an
epifluorescent microscope (OPTIPHOTO-2; Nikon Co.,
Tokyo), a CCD color camera (HCC-3600; Flovel, Tokyo),
a real time image analyzer (Luzex-F; Nireco Co., Tokyo),
an autoscanner (MicroScanner; Sapporo Breweries Ltd.,
Tokyo) and a Macintosh computer.

Measurements and calculations-related static param-
eters were as follows: total metaphysial area (TV, mm²);
metaphysial area between 0.8 and 3.2 mm distal to the
growth plate-epiphysial junction without the cortices,
trabecular bone area (BV, mm²): total area of trabecular
within TV, trabecular bone surface (BS, mm): length of
RESULTS

**BMC and BMD in femur**

The BMC and BMD in the whole femur and the 7 divided portions are shown in Fig. 3, Table 1 and Table 2, respectively. The BMC in the whole femur in the OVX-control group tended to be lower than that in the sham group (P<0.1), while that in the vitamin K\textsubscript{2} 50 mg/kg group was significantly higher than that in the OVX-control group (P<0.05). Ovariectomy resulted in a decrease in the BMC of the F2 and F6 portions, and the BMC in the F6 portion of the vitamin K\textsubscript{2} 50 mg/kg group was significantly higher than that in the OVX-control group.

The BMD in the whole femur from the OVX-control group was significantly lower than in the sham group (P<0.01), while in the vitamin K\textsubscript{2} 50 mg/kg group, it was significantly higher than in the OVX-control group.

**Statistical analyses**

All data are expressed as the mean±standard error of the mean (S.E.M.). Comparison of 2 groups (sham-operated vs OVX-control) was performed by Student’s t-test. Comparison of OVX-control and vitamin K\textsubscript{2}-treated groups was performed by Fisher’s least significant different test.
Table 1. Effect of vitamin K<sub>2</sub> on bone mineral content (BMC) in 7 portions of the femur 2 weeks after ovariectomy

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>50.4±1.1</td>
<td>48.5±0.7</td>
<td>38.0±0.7</td>
<td>32.6±0.7</td>
<td>30.9±0.8</td>
<td>44.2±1.2</td>
<td>69.9±1.9</td>
</tr>
<tr>
<td>OVX Control</td>
<td>48.3±1.0</td>
<td>46.4±0.7*</td>
<td>37.2±0.8</td>
<td>32.1±0.7</td>
<td>31.2±0.7</td>
<td>38.5±1.2**</td>
<td>66.3±1.1</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;2&lt;/sub&gt; 5</td>
<td>48.5±0.8</td>
<td>46.5±0.8</td>
<td>38.3±0.7</td>
<td>33.2±0.5</td>
<td>31.0±0.5</td>
<td>38.7±1.4</td>
<td>67.9±1.4</td>
</tr>
<tr>
<td>50</td>
<td>51.3±1.5</td>
<td>47.7±0.8</td>
<td>38.5±0.7</td>
<td>33.5±0.7</td>
<td>32.4±0.9</td>
<td>42.0±0.8†</td>
<td>69.0±2.0</td>
</tr>
</tbody>
</table>

Vitamin K<sub>2</sub> (menatetrenone) was given as a dietary supplement. A whole femur was divided into 7 equal portions, F1 to F7 from the proximal to distal end. Each value represents the mean±S.E.M. (n=10). *P<0.05, **P<0.01 vs Sham; †P<0.05 vs OVX-Control.

Table 2. Effect of vitamin K<sub>2</sub> on bone mineral density (BMD) in 7 portions of the femur 2 weeks after ovariectomy

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>228.9±1.9</td>
<td>249.5±3.2</td>
<td>226.2±2.7</td>
<td>220.1±2.5</td>
<td>206.9±2.3</td>
<td>232.8±4.9</td>
<td>303.9±4.3</td>
</tr>
<tr>
<td>OVX Control</td>
<td>218.4±3.6*</td>
<td>235.7±2.2**</td>
<td>219.9±4.4</td>
<td>219.1±3.0</td>
<td>207.3±2.5</td>
<td>203.8±5.4**</td>
<td>278.5±3.6**</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;2&lt;/sub&gt; 5</td>
<td>216.1±2.2</td>
<td>237.7±3.3</td>
<td>222.7±2.0</td>
<td>220.1±2.1</td>
<td>206.6±2.5</td>
<td>205.1±5.6</td>
<td>286.1±4.3</td>
</tr>
<tr>
<td>50</td>
<td>222.1±3.0</td>
<td>241.1±3.3</td>
<td>229.1±3.6</td>
<td>227.0±3.1</td>
<td>214.4±3.5</td>
<td>220.0±3.3†</td>
<td>291.5±4.3†</td>
</tr>
</tbody>
</table>

Vitamin K<sub>2</sub> (menatetrenone) was given as a dietary supplement. A whole femur was divided into 7 equal portions, F1 to F7 from the proximal to distal end. Each value represents the mean±S.E.M. (n=10). *P<0.05, **P<0.01 vs Sham; †P<0.05 vs OVX-Control.

(P<0.05, Fig. 3). The BMDs of the F1, F2, F6 and F7 portions in the OVX-control group was lower than those in the sham group, but those of the F3, F4 and F5 portions were not affected by OVX (Table 2). In the vitamin K<sub>2</sub> 50 mg/kg group, the BMDs of the F6 and F7 portions were significantly higher than those in the OVX-control group (Table 2). In the vitamin K<sub>2</sub> 5 mg/kg group, the BMC and BMD were slightly higher than those in the OVX-control group, but not significantly.

Bone histomorphometry

Figure 4 shows typical microphotographs of longitudinal sections of the proximal tibial metaphysis 2 weeks after ovariectomy. Trabecular bone in ovariectomized rats was markedly decreased compared to that in the sham group. In the vitamin K<sub>2</sub> 50 mg/kg group, the decrease induced by ovariectomy was not observed. Table 3 summarizes the static histomorphometric parameters, BV/TV, Tb.Th, Tb.N and Tb.Sp, in the 4 groups. In the OVX-control group, BV/TV, Tb.Th and Tb.N were decreased, and Tb.Sp was increased compared to those in the sham group. Treatment with 50 mg/kg of vitamin K<sub>2</sub> significantly inhibited these changes in Tb.N and Tb.Sp induced by ovariectomy. These values in the 50 mg/kg of vitamin K<sub>2</sub> group were 96% and 110% of those in the sham group, respectively, and no significant difference was observed between these two groups (Student's t-test). Treatment with 5 mg/kg of vitamin K<sub>2</sub> also slightly inhibited the changes induced by ovariectomy.

Dynamic parameters and bone resorption parameters were determined in the sham, the OVX-control and the OVX-K<sub>2</sub> 50 mg/kg groups since a significant effect of vitamin K<sub>2</sub> on bone loss was observed at the dose of 50 mg/kg, but not at 5 mg/kg. Neither ovariectomy nor treatment with vitamin K<sub>2</sub> affected the dynamic histomorphometric parameters, Irl.Th, sLS/BS, dLS/BS, MAR and BFR/BS (Table 4). The parameters for bone resorption, Oc.N/BS and Oc.S/BS, in the TRAP-stained longitudinal sections of proximal tibia metaphysis were markedly higher in the OVX-control group than in the sham group (P<0.01), while those in the OVX-vitamin K<sub>2</sub> 50 mg/kg group were significantly lower than in the OVX-control group (Fig. 5).

DISCUSSION

To understand the mechanism for how an agent prevents bone loss, it is important to clarify whether it inhibits bone resorption or enhances bone formation. In this study, to demonstrate that vitamin K<sub>2</sub> prevents bone
Fig. 4. Typical microphotographs of longitudinal sections of the proximal tibial metaphysis 2 weeks after ovariectomy. Tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. After embedding in methylmethacrylate, they were sectioned longitudinally at 8-μm thickness and stained by the Villanueva Goldner's trichrome method.

Table 3. Effects of vitamin K₂ on bone histomorphological parameters in the longitudinal sections from the proximal tibia 2 weeks after ovariectomy

<table>
<thead>
<tr>
<th></th>
<th>Dose (mg/kg)</th>
<th>BV/TV (%)</th>
<th>Tb.Th (μm)</th>
<th>Tb.N (/mm)</th>
<th>Tb.Sp (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td>21.7±1.3</td>
<td>64.4±1.9</td>
<td>3.35±0.15</td>
<td>239.3±14.4</td>
</tr>
<tr>
<td>O VX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.1±0.9**</td>
<td>53.0±1.7**</td>
<td>2.83±0.12*</td>
<td>306.2±16.3**</td>
<td></td>
</tr>
<tr>
<td>Vitamin K₂</td>
<td>5</td>
<td>17.1±0.7</td>
<td>55.5±0.9</td>
<td>3.07±0.09</td>
<td>278.8±14.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>17.7±1.1</td>
<td>54.8±1.7</td>
<td>3.20±0.12*</td>
<td>262.6±15.2*</td>
</tr>
</tbody>
</table>

Vitamin K₂ (menatetrenone) was given as a dietary supplement. Two weeks after ovariectomy, tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. They were stained by Villanueva bone stain, dehydrated and embedded in methyl-methacrylate. Next, they were sectioned longitudinally at 8-μm thickness and stained by Goldner's trichrome method. As bone histomorphological parameters, the bone volume / tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were determined. Each value represents the mean±S.E.M. (n=10). *P<0.05, **P<0.01 vs Sham; 1P<0.05 vs O VX-Control.

loss partly due to the inhibition of bone resorption in vivo, we used a bone loss model 2 weeks after ovariectomy in rats and evaluated the effect of vitamin K₂ by bone mass measurement and histomorphometric analysis.
Table 4. Effects of vitamin K₂ on the parameters of bone formation in the longitudinal section of the proximal tibia 2 weeks after ovariectomy

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>LrLTh (μm)</th>
<th>sLS/BS</th>
<th>dLS/BS</th>
<th>MAR (μm/day)</th>
<th>BFR/BS (μm²/μm²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.63±0.50</td>
<td>0.110±0.013</td>
<td>0.068±0.026</td>
<td>1.090±0.072</td>
<td>0.155±0.042</td>
</tr>
<tr>
<td>OVX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.14±0.51</td>
<td>0.094±0.011</td>
<td>0.035±0.009</td>
<td>1.163±0.073</td>
<td>0.104±0.017</td>
</tr>
<tr>
<td>Vitamin K₂</td>
<td>50</td>
<td>0.088±0.017</td>
<td>0.048±0.009</td>
<td>1.018±0.058</td>
<td>0.094±0.011</td>
</tr>
</tbody>
</table>

Vitamin K₂ (menatetrenone) was given as a dietary supplement. Rats were subcutaneously treated with 25 mg/kg of tetracycline 11 and 5 days before sacrifice. Two weeks after ovariectomy, tibias were cut 10 mm from the proximal end and fixed with 70% alcohol. They were embedded in methyl-methacrylate and sectioned longitudinally at 8-μm thickness. As the parameters for bone formation, the inter labeled thickness (IrLTh), single labeled surface / bone surface (sLS/BS), double labeled surface / bone surface (dLS/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) were determined. Each value represents the mean±S.E.M. (n=9–10).

Fig. 5. Effects of vitamin K₂ on osteoclast number/bone surface (Oc.N/BS) and osteoclast surface/bone surface (Oc.S/BS) in longitudinal sections from the proximal tibia 2 weeks after ovariectomy. Vitamin K₂ (menatetrenone) was given as a dietary supplement. Tibias were cut 10 mm from the proximal end and fixed with 10% formalin in phosphate-buffered saline. After embedding in glycolmethacrylate, they were sectioned longitudinally at 8-μm thickness and stained with tartrate-resistant acid phosphatase stain. Each column and bar represent the mean±S.E.M. (n=10).

In the bone histomorphometry, Oc.S/BS and Oc.N/BS, as parameters of bone resorption, in the OVX-control group were 4.3 and 3.7 times as high as those in the sham group, respectively (Fig. 5). BV/TV and Tb.N in the OVX-control group were decreased to 69.6% and 84.5% of those in the sham group, respectively (Table 3). There were no significant differences in MAR and BFR/BS, parameters of bone formation, in the 2 groups (Table 4). Yamaura et al. (18) observed histomorphometrical changes in proximal tibial metaphyses during the early period after ovariectomy and indicated that OVX resulted in significant increases in Oc.S/BS and Oc.N/BS at the 3rd day, which were maintained thereafter, but dLS/BS and BFR/BS were not changed until 14 days after OVX and then increased at the 19th day. Dempster et al. (17) also observed histomorphometrical changes in the proximal tibial metaphysis every 5 days and reported that Oc.S/BS was increased on the 5th day, but BFR/BS was increased on the 15th day. These data indicate that the loss of trabecular bone during the early periods after ovariectomy, especially within 2 weeks, is due to the enhancement of bone resorption, and they agree with the histomorphometrical data in this study.

At 2 weeks after ovariectomy, BMC and BMD in the whole femur in the OVX-control group were both decreased to 95% of those in the sham group (Fig. 3). Moreover, the decreases in the F6 portion reached 87%, whereas in the F3, F4 and F5 portion, these changes were not observed (Tables 1 and 2). These data indicate that bone loss occurs early after ovariectomy, particularly in trabecular-bone-abundant portions. Geusens et al. (23) reported that OVX resulted in a decrease in the distal end
at 20% of the length of the femur, which contains more trabecular bone, but not in the middle portion, which contains cortical bone, after 3 months. A recent histomorphometrical study in femora showed that bone loss in the metaphysis was more rapid than in the epiphysis or diaphysis after ovariectomy (20). The F7 portion in this study corresponds to epiphysis, and the F6 portion to metaphysis. So the decrease in the BMC and BMD in the F6 portion after ovariectomy is thought to be caused by the increase in bone resorption.

Vitamin K2 at 50 mg/kg significantly inhibited the decrease in BMC and BMD both in the whole femur and the F6 portion and the decrease in Tb.Sp in the tibial metaphysis. As mentioned above, bone loss 2 weeks after ovariectomy is due to increase in bone resorption, especially in the metaphysis, so vitamin K2 is thought to cause an inhibitory effect on bone loss through the inhibition of bone resorption. Moreover, the inhibition of the increase in Oc.N/BS and Oc.S/BS caused by vitamin K2 indicated that vitamin K2 precisely inhibited bone resorption in the cell level.

We have already reported that vitamin K2, \(3 \times 10^{-6} - 3 \times 10^{-5} \) M, inhibits bone resorption induced by PGE2 and IL-1α in a mouse calvaria organ culture system dose-dependently (13). Moreover, osteoclast-like cell formation caused by 1,25(OH)2D3 was strongly inhibited by bone resorption. In these studies, it was shown that these effects after such a high dose of vitamin K2 were not due to cell toxicity (13, 24). Sano et al. (25) reported that after once daily oral administration at a dose of 4 mg/kg for 10 days in female rats, the radioactivity concentration of vitamin K2 in the bone marrow of the femur was higher than that in the plasma, reaching to \(10^{-5} \) M. This level is consistent with the effective concentration of vitamin K2 in vitro studies. In this study, we did not determine the concentrations of vitamin K2 in plasma or bone marrow, but the concentration of vitamin K2 at 50 mg/kg administration seemed to reach the pharmacologically effective level in bone.

In conclusion, the data presented here show that the effect of vitamin K2 on bone resorption reported in vitro actually contributes to its effect on bone metabolism in vivo.

Acknowledgments
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