

Effect of vitamin K₂ (menaquinone-7) in fermented soybean (*natto*) on bone loss in ovariectomized rats

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Abstract: The effect of dietary vitamin K₂ (menaquinone-7) on bone loss in ovariectomized (OVX) rats was investigated. OVX rats were freely given experimental diets containing menaquinone-4 (MK-4; 12 mg/100 g diet) or menaquinone-7 (MK-7; 18.1 mg/100 g diet) for 24 days; MK-4 and MK-7 were equal in molar concentrations. This feeding caused a remarkable increase of MK-4 and MK-7 concentrations in the serum and femur of OVX rats. OVX-induced decrease in the femoral dry weight and femoral calcium content was prevented by the feeding of dietary MK-4 or NK-7. In separate experiments, OVX rats were freely given experimental diets containing the fermented soybean (*natto*; including 9.4 μg MK-7/100 g diet) without or with added MK-7 (37.6 μg/100 g diet) for 77 days. Feeding produced a significant elevation of MK-4 and MK-7 concentrations in the serum of OVX rats. In this case, a significant increase in the femoral MK-4 content was observed but MK-7 was not detected in the femoral tissues. OVX-induced decreases in the femoral dry weight and femoral calcium content were significantly prevented by the feeding of diets containing *natto* with MK-7 added (37.6 μg/100 g diets). This study demonstrates that the intake of dietary MK-7 has a preventive effect on bone loss caused by OVX. This effect may be partly caused by MK-4, which is formed by degradation of MK-7.

Key words: Vitamin K₂, menaquinone-4, menaquinone-7, bone metabolism, ovariectomy, osteoporosis

Introduction

There is growing evidence that vitamin K₂ may play a role in the regulation of bone metabolism. Vitamin K₂ is

essential for the γ-carboxylation of osteocalcin, a calcified tissue protein containing γ₂-carboxyglutamic acids, which is synthesized only in osteoblasts [1,2]. Noncarboxylated osteocalcin cannot bind to hydroxyapatite in mineralized tissues [2,3]. Much attention has been paid to the role of vitamin K in bone metabolism, because its supplementation may be important as a therapeutic tool for osteoporosis.

There are two types of vitamin K: vitamin K₁ and vitamin K₂. Vitamin K₁ is a single compound, but vitamin K₂ is a series of vitamers with multiisoprene units (one to four) at the 3-position of the naphthoquinone. Several reports have indicated the effects of vitamin K₁ on bone metabolism [4,5]. In contrast, the effect of vitamin K₂ on bone metabolism has not attracted notice. Like vitamin K₁, vitamin K₂ (menatetrenone), with four isoprene units, not only enhances mineralization but also increases the amount of osteocalcin in cultured human osteoblasts [6]. Moreover, it has been reported that menatetrenone inhibits bone resorption, which may be related to its side chain [7], and that the compound inhibits bone loss in rats induced by ovariectomy [8]. However, the effect of vitamin K₂ (menaquinone-7), with seven isoprene units, on bone metabolism has not been fully clarified.

Recently, it has been demonstrated that vitamin K₂ (menaquinone-7) can directly stimulate calcification in the femoral metaphyseal tissues obtained from normal rats in vitro [9,10]. The action of menaquinone-7 (MK-7) on bone calcification has been shown to have the same effect as menaquinone-4 (MK-4) [10]. MK-7 is highly contained in the fermented soybean (*natto*) [10]. A preventive effect of dietary MK-7 on osteoporosis is not unknown so far. Therefore, we investigated the preventive effect of dietary MK-7 and the fermented soybean (*natto*) containing MK-7 on ovariectomy (OVX)-induced bone loss. We found that the intake of dietary MK-7 can prevent OVX-induced bone loss.

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Materials and methods

Chemicals

Vitamin K₂ (menaquinone-7; 96.8% purity) was supplied by Honen (Tokyo, Japan), which was highly purified from the fermented soybean (*natto*). Menaquinone-4 (99.5% purity) was obtained from Nishin Seifun (Tokyo, Japan). Menaquinone-4 (MK-4) or menaquinone-7 (MK-7) were dissolved in ethanol solution (99.5%). Other chemicals were reagent grade from Wako (Osaka, Japan).

Animals

Female Wistar rats (5 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The six animals in each group were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% Ca, and 1.1% P at room temperature of 25°C, and were given distilled water freely. Rats were given a sham ovariectomy or bilateral ovariectomy under ether anesthesia [11]. The sham-operated animals were fed matched amount of the chow described for 1 week, and then changed to experimental diets.

Experimental procedures

Effect of dietary MK-4 and MK-7. Animals in group 1 (sham ovariectomy) and in group 2 (ovariectomy) were freely given the experimental diets not containing vitamin K₂. Animals (ovariectomy) in group 3 or 4 were freely given the experimental diets containing MK-4 (12 mg/100 g diet) or MK-7 (18.1 mg/100 g), respectively. Dietary MK-4 and MK-7 were equal in molar concentration. All animals were freely fed matched amounts of the chow as described with distilled water for 24 days, and then killed by bleeding.

Effect of the fermented soybean (natto) containing MK-7. Freeze-dried *natto* powder usually contained 45.0% protein, 24.2% lipids, 23.4% carbohydrate, 0.23% Ca, 0.06% P, 0.0021% MK-7, and 0.2033% isoflavone (ISFL). The experimental diets contained freeze-dried *natto* powder; the *natto* content was 0.452%. MK-7 and ISFL content in the experimental diets containing *natto* powder was 9.4 µg/100 g diet and 915 µg/100 g diet, respectively. MK-7 and ISFL were removed from the *natto* powder by extracting with 80% hot ethanol solution; MK-7 alone was removed from *natto* powder by extracting with water.

Animals in group 1 (sham ovariectomy) and in group 2 (ovariectomy) were freely given experimental diets not containing *natto*. Animals (ovariectomy) in group 3 to 7 were freely given diets containing either *natto* with-

out both MK-7 and ISFL (group 3), *natto* without MK-7 (group 4), usual *natto* including MK-7 (9.4 µg/100 g diet) and ISFL (915 µg/100 g diet) (group 5), *natto* with more MK-7 added (9.4 µg/100 g) (group 6; total MK-7 content was 18.8 µg/100 g), or *natto* with more added MK-7 (37.6 µg/100 g) (group 7; total MK-7 content was 47.0 µg/100 g of the diet). All animals were freely fed matched amounts of the chow with distilled water freely available for 77 days, and then killed by bleeding.

Analytical procedures

After feeding of experimental diets, rats were killed by cardiac puncture under light anesthesia with ether, and the blood and femur were removed immediately. Blood samples were centrifuged 30 min after collection. The serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis [12]. Serum γ -carboxylated osteocalcin was assayed by a double-antibody method of enzyme-linked immunosorbent assay (ELISA) using KIT (Takara Syuzou, Osaka, Japan).

The femur was removed after bleeding and soaked in ice-cold 0.25 mol/l sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and epiphysis (containing metaphyseal tissue) were separated and dried for 16 h at 110°C and weighted. The femoral tissues were digested for 24 h at 110°C. Femoral calcium was determined by atomic absorption spectrophotometry [13]. Calcium content was expressed as milligrams per gram dry bone.

Vitamin K₂ (MK-4 and MK-7) concentration in the serum and femur of rats fed experimental diets was measured by HPLC assay. Powdered femoral tissues and serum were added to 66% isopropanol solution and homogenized. After extraction with hexane addition to the homogenate, the hexane phase was dried. Resulting pellets were dissolved in hexane, and it was eluted through Sep-Pak silica using hexane-diethylether. The eluted samples were dried and dissolved in ethanol. This ethanol solution was filtered, and the filtration was injected to HPLC. For calculations, the standard materials of MK-4 and MK-7 were injected to HPLC. MK-4 or MK-7 concentrations in the serum and femoral tissues were expressed as picomoles per milliliter of serum or picomol per gram of wet bone tissues, respectively.

Statistical analysis

The significance of difference between values was estimated using Student's *t*-test. *P* values of less than 0.05 were considered to show a statistically significant difference. Also, we used a multiway ANOVA and Turkey-Kramer multiple comparison test to compare the treatment groups.

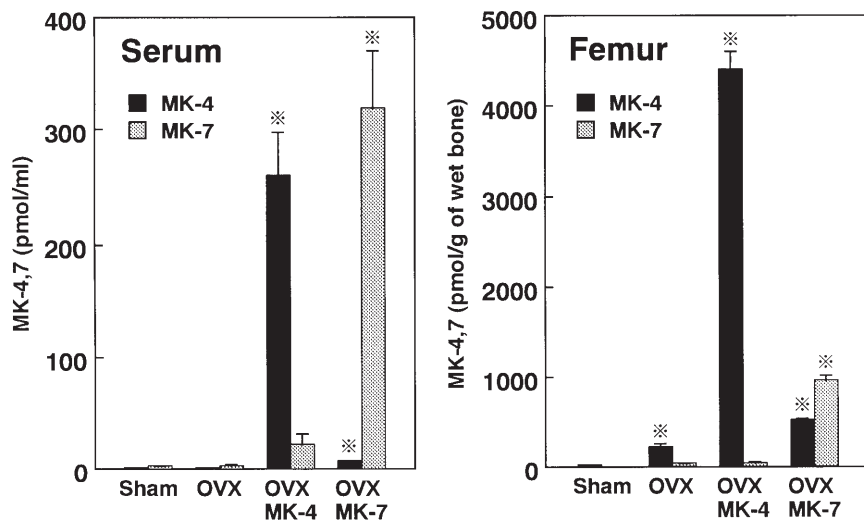


Fig. 1. Alteration in menaquinone-4 (MK-4) (black bars) and menaquinone-7 (MK-7) (stippled bars) concentrations in the serum (left) and femur (right) of rats fed diets containing MK-4 or MK-7. Sham-operated (group 1) and ovariectomized (OVX; group 2) rats were fed experimental diets not containing vitamin K₂ for 24 days. OVX animals in group 3 and group 4 were fed experimental diets containing MK-4 (12mg/100g diet) or MK-7 (18.1mg/100g diet) for 24 days. Each value is the mean \pm SEM of six animals. *, $P < 0.01$ as compared with the value of sham-operated rats

Results

Effect of dietary MK-4 and MK-7 on bone loss in OVX rats

Rats were freely given the vitamin K₂-containing experimental diets for 24 days. Body weight of OVX rats (group 2) was not significantly altered by the feeding of diets containing MK-4 (group 3) or MK-7 (group 4); animals in group 2, group 3, and group 4 were 204.3 ± 5.5 , 205.3 ± 5.5 , and 199.8 ± 2.9 g (mean \pm SEM), respectively. Serum calcium concentration in OVX rats (group 2) was not significantly altered by the feeding of dietary MK-4 or MK-7 (data not shown).

The concentrations of MK-4 or MK-7 in the serum or femur of rats fed experimental diets is shown in Fig. 1. The serum and femoral MK-4 and MK-7 concentrations in sham-operated and OVX rats were negligible. The serum MK-4 or MK-7 concentration in OVX rats was markedly elevated by the feeding of dietary MK-4 or MK-7, respectively. The femoral MK-4 concentration in OVX rats was markedly increased by dietary MK-4. The feeding of dietary MK-7 affected the femur of OVX rats; the result indicated that MK-7 produced a significant increase in MK-4 and that MK-7 may be degraded to MK-4.

The concentration of serum γ -carboxylated osteocalcin of rats fed dietary vitamin K₂ is shown in Fig. 2. OVX did not cause a significant alteration in serum γ -carboxylated osteocalcin concentration as compared with that of sham-operated rats. The feeding of dietary MK-4 or MK-7 produced a slight increase of serum osteocalcin levels in OVX rats.

The femoral dry weight was significantly decreased by OVX (Fig. 3). However, the bone dry weight was not

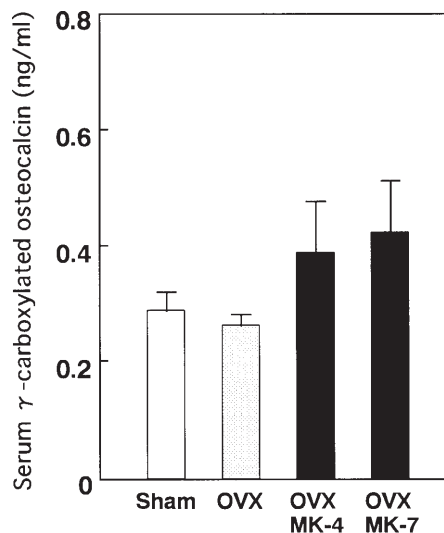


Fig. 2. Alteration in serum γ -carboxylated osteocalcin of rats fed diets containing menaquinone-4 (MK-4) or menaquinone-7 (MK-7). Animals were fed as described in legend of Fig. 1. Each value is the mean \pm SEM of six animals. Data were not significant

appreciably reduced by the feeding of dietary MK-4 or MK-7 to OVX rats. Thus, dietary MK-4 or MK-7 had a preventive effect on OVX-induced bone loss.

Calcium content in the femoral metaphyseal tissues was significantly decreased by OVX (Fig. 4). This decrease was completely prevented by the feeding of dietary MK-4 or MK-7 to OVX rats. In the femoral diaphysis, the calcium content was slightly decreased by OVX. This reduction was significantly prevented by the feeding of dietary MK-7 in OVX rats, although such a preventive effect was also seen by dietary MK-4.

Effect of fermented soybean (natto) containing MK-7 in OVX rats

Rats were freely given the *natto*-containing experimental diets for 77 days. Body weight of OVX rats (group 2) was 222.6 ± 4.0 g (mean \pm SEM of six rats). Feeding of experimental diets in groups 3 to 7 did not cause a

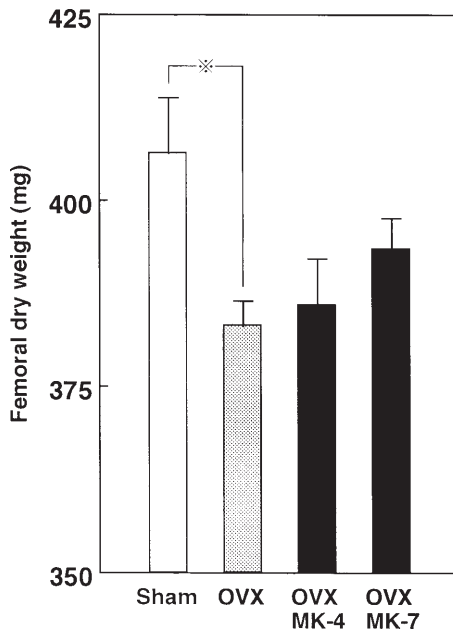


Fig. 3. Alteration in femoral dry weight of rats fed diets containing menaquinone-4 (MK-4) or menaquinone-7 (MK-7). Animals were fed as described for Fig. 1. Each value is the mean \pm SEM of six animals. ※, $P < 0.01$ as compared with the value of sham-operated rats

significant alteration in body weight as compared with that of OVX rats (group 2). OVX (group 2) caused a significant decrease ($P < 0.01$) in serum calcium concentration in comparison with that of sham-operated rats (group 1); serum values in group 1 and 2 were 9.88 ± 0.22 and 8.62 ± 0.07 mg/100ml (mean \pm SEM of six rats), respectively. This decrease by OVX was significantly restored by the feeding of *natto*-containing diet with the addition of MK-7 ($37.6 \mu\text{g}/100$ g diet) (group 7); serum value was 9.31 ± 0.32 mg/100 ml (mean \pm SEM of six rats). Such restoration was not seen in other groups (groups 3 to 6).

The alteration in serum MK-7 or MK-4 concentration of OVX rats fed the experimental diets containing *natto* is shown in Fig. 5. Serum MK-7 concentration of OVX rats in groups 5, 6, and 7 fed the *natto* diets including MK-7 was significantly increased as compared with that of sham-operated rats (group 1). Interestingly, the serum MK-4 concentration in OVX rats (group 7) fed the *natto* diets with MK-7 added was significantly elevated as compared with that of sham-operated rats (group 1) or OVX control rats (group 2). In the femur of OVX rats fed the *natto* diets including MK-7 (groups 5, 6, and 7) MK-7 was not detected, although the femoral MK-4 concentration was significantly increased. This result may indicate that MK-4 degraded from MK-7 is accumulated in bone tissues.

The alteration in serum γ -carboxylated osteocalcin concentration of rats fed dietary MK-7 is shown in Fig. 6. Serum osteocalcin was significantly decreased by OVX. This reduction was not restored by the feeding of diets containing *natto* from which MK-7 was removed, although it was significantly prevented by the *natto* diets with MK-7 added.

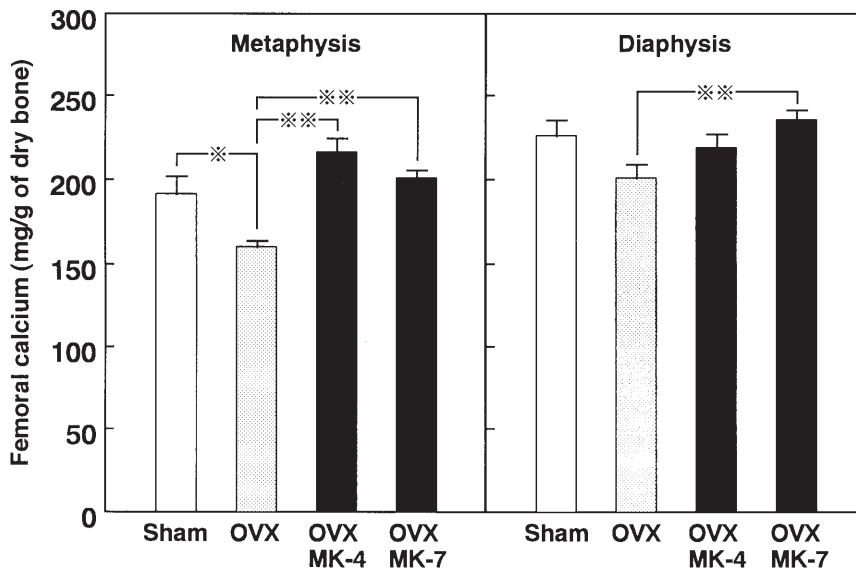


Fig. 4. Alteration in calcium content of the femoral metaphyseal (*left*) and diaphyseal (*right*) tissues of rats fed diets containing menaquinone-4 (MK-4) or menaquinone-7 (MK-7). Animals were fed as described for Fig. 1. Each value is the mean \pm SEM of six animals. ※, $P < 0.05$; ※※, $P < 0.01$ as compared with the value of sham-operated rats

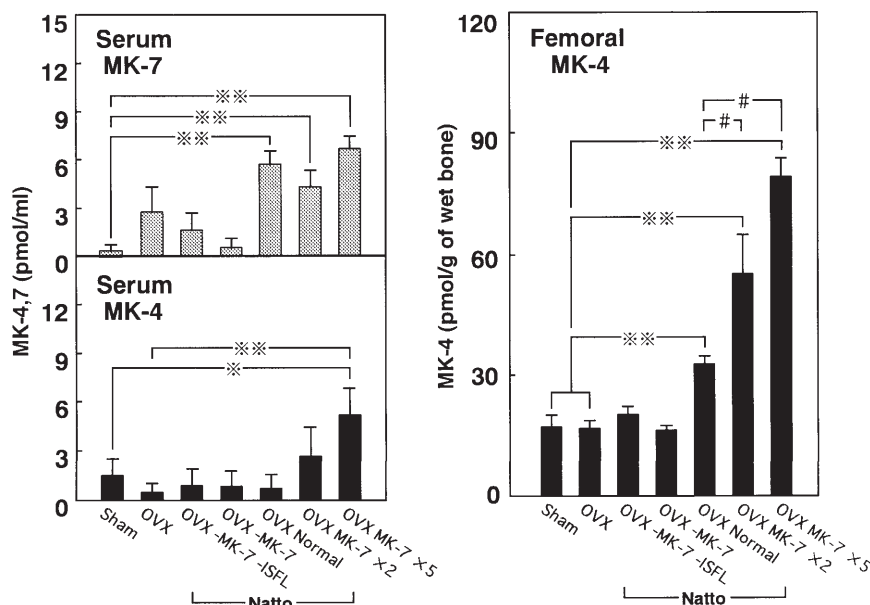


Fig. 5. Alteration in menaquinone-4 (MK-4) and menaquinone-7 (MK-7) concentrations in the serum (left) and femur (right) of rats fed diets containing fermented soybean (*natto*). Rats were freely given the experimental diets for 77 days. Animals in group 1 (sham operation) and group 2 (OVX) were given the experimental diets without *natto* addition. OVX rats in group 3 to 7 were given the experimental diets containing *natto*: group 3, *natto* without both MK-7 (9.4 μ g/100g diet) and isoflavone (ISFL; 915 μ g/100g diet);

group 4, *natto* without MK-7 (9.4 μ g/100g diet) alone; group 5, usual *natto* with MK-7 and isoflavone; group 6, *natto* with more MK-7 (9.4 μ g/100g diet) added and *natto* with more MK-7 (37.6 μ g/100g diet) added. Each value is the mean \pm SEM of six animals. *, $P < 0.05$; **, $P < 0.01$ as compared with the value obtained from sham group or OVX control group; #, $P < 0.01$ as compared with the value obtained from OVX rats fed *natto* containing MK-7 and isoflavone

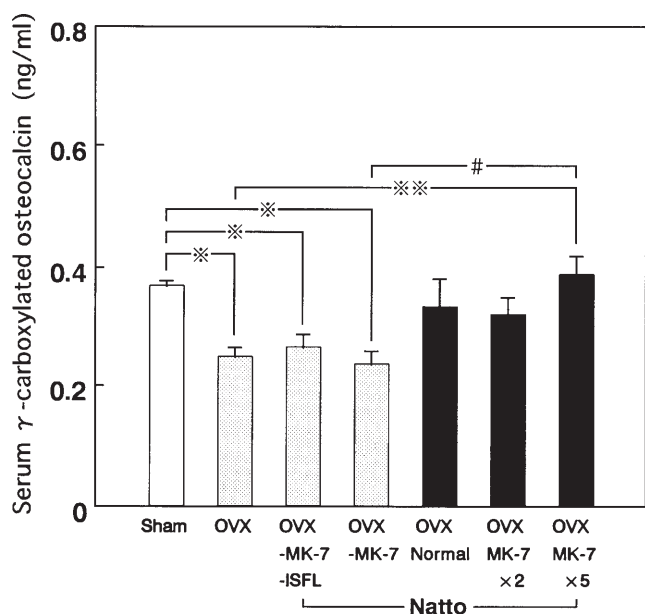


Fig. 6. Alteration in serum γ -carboxylated osteocalcin of rats fed diets containing the fermented soybean (*natto*). Rats were fed as described in the legend of Fig. 5. Each value is the mean \pm SEM of six animals. *, $P < 0.05$ as compared with the value of sham-operated rats; **, $P < 0.01$ as compared with the value of OVX control rats; #, $P < 0.05$ as compared with the value of OVX rats fed *natto* without MK-7

Femoral dry weight was significantly decreased by OVX (Fig. 7). This decrease was significantly prevented by the feeding of *natto* diets including MK-7. Meanwhile, calcium contents in the femoral metaphyseal and diaphyseal tissues were significantly reduced by OVX (Fig. 8). These decreases were significantly prevented by the feeding of *natto* diets including MK-7. With the higher content of added MK-7, however, the preventive effect was not further enhanced.

Discussion

The effect of vitamin K₂ (menaquinone-7, MK-7) on bone metabolism has not been fully clarified. Recently, it has been reported that MK-7 can stimulate calcification in the femoral metaphyseal tissues obtained from normal rats in vitro [9,10]. Furthermore, the present study was undertaken to clarify whether dietary MK-7 has a preventive effect on bone loss in OVX rats. Meanwhile, menaquinone-4 (MK-4) is used as the therapeutic tool for the prevention of osteoporosis [4–6,14,15]. The effect of dietary MK-4 and MK-7 on bone loss in OVX rats was compared.

When the experimental diets containing MK-4 were fed to OVX rats for 24 days, MK-4 markedly increased

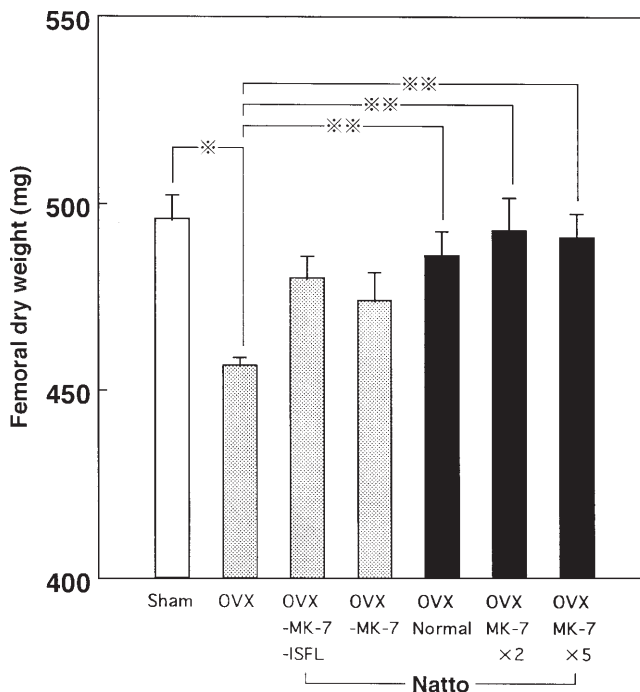


Fig. 7. Alteration in femoral dry weight of rats fed diets containing fermented soybean (*natto*). Rats were fed as described for Fig. 5. Each value is the mean \pm SEM of six animals. *, $P < 0.01$ as compared with the value of sham-operated rats; **, $P < 0.01$ as compared with the value of OVX rats without *natto* addition

in the serum and femoral tissues. Meanwhile, the feeding of dietary MK-7 caused a remarkable increase of the serum MK-7 concentration in OVX rats. However, both MK-7 and MK-4 were significantly elevated in the femoral tissues. Presumably, MK-4 is accumulated in the femoral tissues by degradation of MK-7.

OVX caused a significant decrease in the femoral dry weight and the femoral metaphyseal and diaphyseal calcium content. These decreases were prevented by the feeding of diets with added MK-4 or MK-7. Especially, the femoral diaphyseal calcium content in OVX rats was significantly increased by dietary MK-7, although such a effect was not seen in the case of dietary MK-4. Thus, dietary MK-4 and MK-7 may prevent bone loss in OVX rats. MK-4 has been demonstrated to have an inhibitory effect on bone resorption and a stimulatory effect on bone calcification [6–8]. It has been also demonstrated that MK-7 can stimulate bone formation and mineralization in bone tissue culture in vitro [9,10]. MK-7, as well as MK-4, when accumulated in the femoral tissues may directly stimulate the functions of osteoblastic cells.

Vitamin K is essential for the γ -carboxylation of osteocalcin, which is synthesized only in osteoblasts [1,2]. Noncarboxylated osteocalcin cannot bind to hydroxyapatite in bone tissues [2,3]. MK-4 and MK-7 may partly exert a stimulatory effect through the γ -carboxylated osteocalcin. In fact, the serum γ -carboxylated of osteocalcin concentration in OVX rats

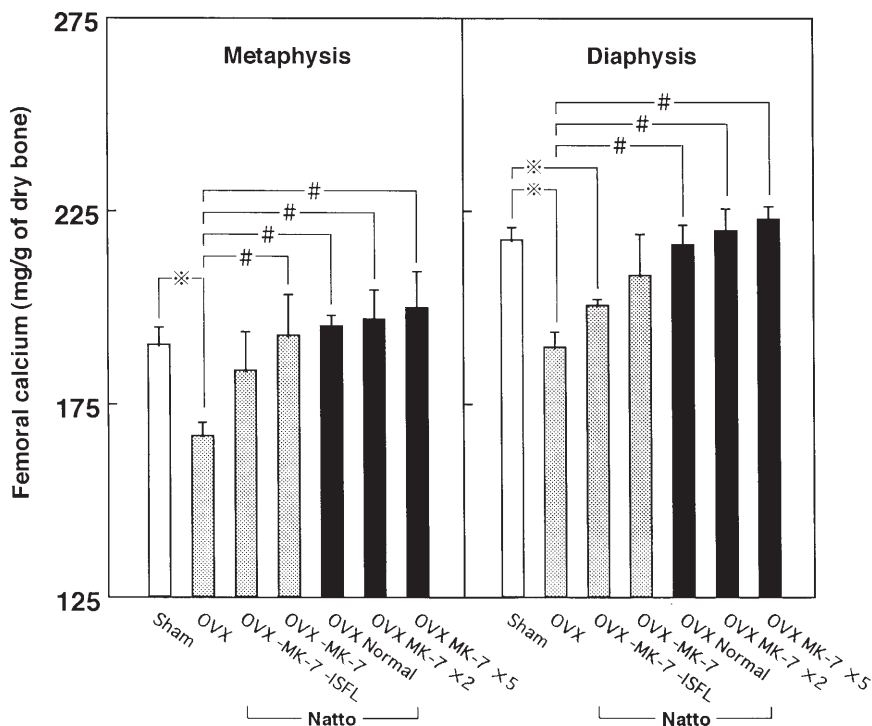


Fig. 8. Alteration in calcium content in the femoral metaphyseal (*left*) and diaphyseal (*right*) tissues of rats fed diets containing the fermented soybean (*natto*). Rats were fed as described for Fig. 5. Each value is the mean \pm SEM of six animals. *, $P < 0.05$ as compared with the value of sham-operated rats; #, $P < 0.01$ as compared with the value of OVX rats without *natto* addition

was slightly increased by the feeding of diets with added MK-4 or MK-7.

Natural MK-7 is contained at a high level in the fermented soybean (*natto*) [10]. Moreover, we investigated whether OVX-induced bone loss is prevented by the feeding of diets containing *natto* (9.4 µg MK-7/100 g diet) for 77 days. The feeding of *natto* diets to OVX rats caused a significant increase in the serum MK-7 concentration. However, MK-7 was not detected in the femoral tissues of OVX rats fed *natto* diets without or with the addition of MK-7 (9.4 and 37.6 µg/100 g diet), although the femoral MK-4 content was significantly increased. This result suggests that the feeding of *natto* diets containing a comparatively lower concentration of MK-7 causes the femoral accumulation of MK-4, which is degraded from MK-7.

The serum γ -carboxylated osteocalcin concentration was significantly decreased in OVX rats given the experimental diets without added *natto* for 77 days. This decrease was significantly prevented by the feeding of *natto* diets with added MK-7 (37.6 µg/100 g diet), suggesting the stimulatory effect of vitamin K₂ on the γ -carboxylation of osteocalcin in osteoblastic cells.

OVX-induced decrease in femoral dry weight and femoral metaphyseal and diaphyseal calcium content were significantly prevented by the feeding of *natto* diets including MK-7 for 77 days, although such an effect was not seen by feeding of diets containing *natto* without MK-7 or both MK-7 and isoflavone. This result suggests that the MK-7 contained in *natto* diets has a role in the prevention of OVX-induced bone loss. However, it cannot exclude the possibility that an interactive effect of MK-7 (and/or MK-4) and isoflavone (including genistin, genistein, daidzin, and daizein) may play a role in the preventive effect on OVX-induced bone loss. This remains to be elucidated.

In conclusion, it has been demonstrated that dietary MK-7 can prevent bone loss in OVX rats, and that the prolonged administration of *natto* diets containing MK-7 has a role in the prevention of OVX-induced bone loss. Dietary MK-7 may be important as a nutritional factor in the prevention of osteoporosis.

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