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 MK-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 γ-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 on osteoporosis. We found that the intake of dietary MK-7 can prevent OVX-induced bone loss.
Materials and methods

Chemicals

Vitamin K$_2$ (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 is group 1 (sham ovariectomy) and in group 2 (ovariectomy) were freely given the experimental diets not contained vitamin K$_2$. Animals (ovariectomy) in group 3 or 4 were freely given the experimental diets containing MK-4 (12 mg/100g diet) or MK-7 (18.1 mg/100g), 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 IFL content in the experimental diets containing *natto* powder was 9.4 µg/100g diet and 915 µg/100g 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 containing either *natto* without both MK-7 and ISFL (group 3), *natto* without MK-7 (group 4), usual *natto* including MK-7 (9.4 µg/100g diet) and ISFL (915 µg/100g diet) (group 5), *natto* with more MK-7 added (9.4 µg/100g) (group 6; total MK-7 content was 18.8 µg/100g), or *natto* with more added MK-7 (37.6 µg/100g) (group 7; total MK-7 content was 47.0 µg/100g 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 K$_2$ (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-diethyl ether. 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 pmol per ml of serum or pmol 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.