Food Sci. Technol. Res., **6** (4), 288–290, 2000

Note

Effect of Vitamin K_2 (Menaquinone-7) and Soybean Isoflavone Supplementation on Serum Undercarboxylated Osteocalcin in Female Long-Distance Runners

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Received March 27, 2000; Accepted August 22, 2000

Fractures of the lower limbs due to fatigue often occur in female long-distance runners. We examined the effect of simultaneous supplementation of 1.35 mg/day of vitamin K_2 (MK-7+MK-6) and 72 mg/day of soybean isoflavones for 30 days on bone metabolism in these athletes. Despite the daily intake of 200–400 μg of vitamin K, the vitamin was found to be subclinically deficient in athletes on the basis of γ-carboxyglutamic acid formation of osteocalcin, because supplementation of 1.35 mg/day of vitamin K_2 markedly decreased serum undercarboxylated osteocalcin level by 81%. This suggests that 200 μg/day of vitamin K was not sufficient for γ-carboxylation of osteocalcin. Therefore, supplementation of vitamin K and soybean isoflavones may help reduce the risk of bone fracture in female long-distance runners.

Keywords: vitamin K, isoflavone, athletes, osteocalcin, bone metabolism

There are two naturally occurring forms of vitamin K: vitamins K_1 and K_2. Vitamin K_1 (phylloquinone) is formed in plants, whereas vitamin K_2 (menaquinone, MK-*n*) is primarily synthesized by bacteria. Vitamin K is a cofactor required for the post-translational conversion of specific glutamic acid residues into γ-carboxyglutamic acid (Gla) in Gla-containing proteins (Shearer, 1990). Osteocalcin, a bone-specific protein synthesized by osteoblasts, is γ-carboxylated vitamin K-dependently. Noncarboxylated or undercarboxylated osteocalcin (ucOC) cannot bind to hydroxyapatite in mineralized tissues (Price, 1985). Szulc *et al.* (1993) have shown that ucOC could be a marker for the risk of bone hip fracture, and ucOC was attributed to vitamin K deficiency. Their report was reinforced by the results of their three-year follow-up study (Szulc *et al*., 1996). Poor vitamin K status in the blood has been reported to be associated with bone fracture (Hodges *et al*., 1993; Kaneki *et al*., 1995). Furthermore, supplementation of vitamin K_2 (MK-4) was shown to reduce the loss of bone mass among Japanese women (Orimo *et al*., 1992). Several *in vitro* studies showed that MK-4 not only enhances mineralization in human osteoblasts (Koshihara *et al*., 1996), but also inhibits bone resorption (Hara *et al*., 1995). The fermented soybean (*natto*), which is popular in Japan, contains MK-7 at a high concentration. Recently, the effect of MK-7 on bone metabolism has been demonstrated to be equal to the effect of MK-4 (Sato *et al*., 1996; Yamaguchi *et al*., 1999).

Soybean isoflavones were also found to improve bone metabolism (Anderson & Garner, 1997; Yamaguchi & Gao, 1998). Soybean isoflavones include six isomers of isoflavone; three glycons (daidzein, glycitein, genistein) and three glucosides (daidzin, glycitin, genistin). Soybean isoflavones are also called phytoestrogen, because they have estrogen-like activity. Estrogen deficiency at menopause results in rapid bone loss, therefore, the primary effect of soybean isoflavones on bone metabolism is thought to be that of countering estrogen deficiency at menopause. Toda *et al*. (1996) reported that the intake of isoflavone is related to a decrease in the levels of a bone resorption marker, deoxypyridinoline, in urine in Hawaiian women.

In female long-distance athletes, particularly marathon runners, stress fractures of the lower limbs often occur. Hypoestrogenism induced by strenuous exercise has been thought to be one of the causes of rapid loss of bone mass. Recently, Craciun *et al*. (1998) reported that a supplementation of 10 mg/day of vitamin K_1 to female elite athletes resulted in improved bone metabolism.

Here we investigated the effects of simultaneous supplementation of vitamin K_2 and soybean isoflavone on bone metabolism in elite Japanese female long-distance runners.

Materials and Methods

Study design Studies were carried out according to the Declaration of Helsinki, and informed consent was signed by all participants.

Vitamin K_2 and soybean isoflavones were obtained from Honen Corporation (Tokyo). Four 20- to 24-year-old female long-distance runners were administered 1350 μg of vitamin K_2 (1299 μg of MK-7 and 51 μg of MK-6) and 72 mg of soybean isoflavones (43.2 mg of daidzin, 15.9 mg of glycitin, 9.3 mg of genistin, 2.3 mg of daizein, 0.8 mg of glycitein, 0.5 mg of genistein) each day for 30 days. As control, five athletes were not given supplementation. Physical and anthropometrical characteristics of the subjects are listed in Table 1. Blood and urine samples were collected 30 days after the first blood and urine

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Table 1. Physical and anthropometrical characteristics of female athletes.

Number of subjects	Control group		Supplementation group	
	5		4	
	Range	Mean	Range	Mean
Age	20–24	22	20–24	22
Height (cm)	156–168	161	157–160	158
Weight (kg)	42–50	46	42–48	44
Best time for 5 km (min: s)	15 : 42–16 : 40	16:04	15 : 41–16 : 32	16:05

sampling prior to the supplementation. All of the subjects lived in a dormitory. Two daily meals, except for the luncheon meal and supplementation, were strictly controlled by a nutritionist, and the daily intake of nutrients is shown in Table 2. The lunch menus were carbohydrate-rich foods such as pasta, noodles or rice, which had about 800 kcal/meal. Subjects ran 15–30 km every day.

Biochemical analyses Commercial kits for determination of concentrations of total osteocalcin (OC) and undercarboxylated osteocalcin (ucOC) in serum were purchased from Mitsubishi Chemicals (Tokyo) and Takara (Kyoto), respectively. Concentration of albumin was determined by a kit (Wako, Osaka). Free deoxypyridinoline (DPD) concentration in urine was measured by ELISA (Sumitomo Pharmaceuticals, Osaka). Concentrations of calcium in serum and urine, and creatinine in urine were also determined using commercially available kits (Daiya Shiyaku, Tokyo). Inorganic phosphorus concentration in urine was measured using a kit (Kyowa Medix, Tokyo). Values of free DPD, calcium and inorganic phosphorus in urine were expressed as DPD/urinary creatinine (DPD/uCr), Ca/uCr and P/uCr, respectively.

Table 2. Daily intake of nutrients from breakfast and supper.

Nutrient (g)	Range	Mean
Protein (g)	70–90	80
Fats (mg)	40–52	46
Calcium (mg)	1000–1200	1097
Magnesium (mg)	180–220	213
Phosphorus (mg)	1000–1500	1370
Vitamin C (mg)	150–250	177
Vitamin K (μg)	200–400	312
Vitamin D (IU)	200–400	310
Energy (kcal)	1500–1550	1529

Two daily meals except for luncheon meal and supplementation, were under the supervision of a nutritionist. Values described above were calculated according to the Standard Tables of Food Composition in Japan (Resources Council, Science and Technology, Japan, 1996). The lunch menus were carboxylate-rich foods such as pasta, noodle or rice, which had about 800 kcal/meal.

Results and Discussion

We investigated the effects of vitamin K_2 and soybean isoflavones on bone metabolism in female long-distance runners, who were members of one of the highest-level athletic teams in Japan. About half of these athletes have experienced bone fracture due to fatigue. As shown in Table 1, the running ability of the athletes tested here is very high (their average best time for 5 km was 16 min 04 s; the Japanese record for 5 km was 15 min 03 s as of March, 2000). These athletes underwent strenuous training and ran 15–30 km every day. Four of them took a supplementation of 1.35 mg of vitamin K_2 and 72 mg of soybean isoflavones every day, and another five athletes on the same athletic team acted as controls. Physical and anthropometrical data of the subjects was almost the same in the groups with and without supplementation (Table 1). All subjects lived in the same dormitory, and daily intake of nutrients except for the luncheon meal was strictly controlled and checked by a nutritionist (Table 2). To burn energy quickly during the afternoon training, the subjects took carbohydrate-rich lunch such as pasta, noodles or rice. Since the vitamin and mineral content of those lunches were not high, the athletes' actual daily intake of vitamins and minerals was slightly higher than the amounts shown in Table 2. Therefore, the intake of minerals, and vitamins D and K was thought to be enough for the recommended intake of nutrients for adult women issued by the Ministry of Welfare and Health in Japan (Ca: 600 mg/day, P: 700 mg/day, Mg: 250 mg/day, Vitamin D: 100 IU, Vitamin K: 55 μg/day).

Before supplementation and after 30 days, various bone metabolism markers were measured (Table 3). Values of the bone for-

Table 3. Effects of supplementation of vitamin K_2 and soybean isoflavones on the levels of bone markers in female athletes.

Number of subjects	Control			Supplementation		
	5			4		
Marker tested	day 0	day 30	% change	day 0	day 30	% change
In serum						
Total OC (ng/ml)	3.7±0.79	4.4±0.92	+19	3.5±0.38	4.3±0.47	+21
ucOC (ng/ml)	3.2±0.72	1.7±0.38	−47	3.1±1.1	0.60±0.32	−81
Albumin (mg/ml)	44.4±0.93	44.2±0.66	±0	47.6±1.28	47.0±0.71	−1
Ca (μg/ml)	97±2.5	94±1.8	−3	99±2.2	95±1.9	−4
In urine						
Free DPD/uCr (nM/mM Cr)	8.5±1.3	8.1±1.1	−5	6.6±1.7	5.4±0.93	−18
Ca/uCr (mg/g Cr)	89.2±20.2	60.5±8.81	−32	110.6±14.4	113.3±1.13	+2
P/uCr (mg/g Cr)	811.4±93.35	459.5±69.4	−43	843.7±123.9	525.2±147.9	−38

Data were expressed as mean±SE.
Abbreviations: OC, osteocalcin; ucOC, undercarboxylated osteocalcin; DPD/uCr, deoxypyridinoline/urinary creatinine; Cr, creatinine.

mation marker, OC, and a bone resorption marker, DPD, were measured. An ucOC in serum is a marker of substantial vitamin K deficiency, and it has been reported to be a marker for the risk of bone fracture (Szulc *et al.* 1993; 1996).

The ucOC concentration on day 0 in both groups was high compared to total OC level (Table 3). This result corresponded well with the report of Craciun *et al.* (1998), who investigated the effect of vitamin K_1 on bone metabolism using a small number of elite athletes. They showed that the athletes were biochemically vitamin K deficient as deduced from the calcium binding capacity of OC. In their study, however, dietary intake of vitamin K from meals was not clear. In our study, the subjects took 200–400 μg /day of vitamin K (K_1+K_2) before and throughout the study which they obtained from daily meals, mainly from green leafy vegetables (Table 2). The amount was much higher than the present recommended dietary allowance of vitamin K for adults in the U. S. A., which is 1 μg/(kg body wt·day), and the recommended adequate intake of vitamin K in Japan, which is 55 μg/day for 15–69 year old females. These values are based on the amount required for the maintenance of plasma prothrombin concentrations. After supplementation of 1.35 mg of vitamin K_2 for 30 days, the concentration of ucOC in our subjects was drastically decreased by 81% (Table 3), suggesting that 200 μg of vitamin K, mainly vitamin K_1, from dairy meals is not sufficient for maturation of OC. This may be partly related to the bioavailability of membrane-bound forms of vitamin K_1 in green leafy vegetables. Further studies are required to determine the adequate intake of vitamin K in regard to γ-carboxylation of osteocalcin.

Serum ucOC correlates with hip bone mineral density in elderly women (Szulc *et al.*, 1994). Furthermore, ucOC was demonstrated to be a marker for the risk of bone fracture (Szulc *et al.*, 1993; 1996). In this study, supplementation of vitamin K_2 markedly decreased the concentration of serum ucOC in athletes. Therefore, supplementation of vitamin K may help reduce the risk of bone fracture in athletes.

In female long-distance athletes, hypoestrogenism induced by strenuous exercise has been thought to be one of the causes of rapid loss of bone mass. Since soybean isoflavone has an estrogen-like activity, the inhibitory effect of bone resorption was expected. Furthermore, isoflavone in combination with vitamin K_2 exhibited an additive inhibitory effect of bone resorption (Notoya *et al.*, 1995). In the current study, urinary free DPD, a bone resorption marker, seemed to be slightly decreased by supplementation (Table 3). It was also found that the balance of Ca and P in urine was different between the two groups, with or without supplementation (Table 3). These effects might be caused by isoflavone and/or vitamin K_2, but they must be clarified by further studies.

In this study, only the effect of vitamin K_2 on serum ucOC concentration was shown. The effect of isoflavones or the synergistic effect of vitamin K_2 and isoflavones was not clear. Apparently, the number of subjects tested here was too small to determine the effect of supplementation on bone metabolism. Further studies are required using a larger number of subjects to obtain meaningful results, which may be difficult because high-level athletes such as those tested here are few in Japan.

Acknowledgement This work was supported by a grant from The Japanese Research and Development Association for New Functional Foods.

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