Oral Administration of *Cal K₂* Containing Menaquinone-4 (Vitamin K₂) Enhances Serum *r*-Carboxylated Osteocalcin and Biochemical Components in the Femoral Tissues of Rats

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The effects of menaquinone-4 (MK-4; vitamin K₂) supplemented egg shell calcium (*Cal K₂*) on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats was investigated. *Cal K₂* contained calcium carbonate (Ca 100 mg/g) and MK-4 (593 µg/g). Rats were orally administered a solution of *Cal K₂* (10, 25, or 50 mg/ml/100 g body weight) or placebo (without MK-4, 50 mg/ml/100 g body weight) once daily for 7 days. The serum *r*-carboxylated osteocalcin concentration, which is produced in osteoblastic cells, was significantly increased after the administration of *Cal K₂* (50 mg/100 g body weight) as compared with that in the control group or placebo-control group without MK-4. Calcium content and alkaline phosphatase activity in the femoral-diaphyseal tissues were significantly increased after *Cal K₂* (25 or 50 mg/100 g) as compared with that in the placebo-control group. Femoral-metaphyseal calcium content was significantly increased after the administration of *Cal K₂* (25 or 50 mg/100 g) as compared with that in the control group. DNA content in the femoral-diaphyseal and -metaphyseal tissues was significantly increased after the administration of *Cal K₂* (50 mg/100 g) as compared with that in the control or placebo-controlled group. This study demonstrates that the oral administration of *Cal K₂* containing MK-4 has anabolic

effects on bone components in rats. Supplemental *Cal K₂* may have a role in the prevention of bone loss with aging.

Key words — menaquinone-4, bone metabolism, *r*-carboxylated osteocalcin, *Cal K₂*, rat femur

INTRODUCTION

Bone mass decreases with aging. Osteoporosis is induced with a decrease in bone mass, and it is widely recognized as a major public health problem. Postmenopausal osteoporosis, resulting from estrogen deficiency, induces a remarkable decrease in bone mass with aging. The most dramatic expression of the disease is represented by fractures of the proximal femurs.¹⁻⁴⁾ A decrease in bone mass may be due to decreased osteoblastic bone formation and increased osteoclastic bone resorption. Pharmacologic and nutritional factors may prevent bone loss with aging.^{5,6)} Food and nutritional factors may be important in the prevention of osteoporosis.

Vitamin K, which is a nutritional factor, plays a role in the regulation of bone metabolism. 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 vitamins with multiisoprene units (one to four) at the 3-position of the naphthoquinone. Vitamin K₁ has an effect on bone metabolism.^{7,8)} Vitamin K₂ (menaquinone) is essential for the *r*-carboxylation of osteocalcin, a bone matrix protein containing *r*-carboxyglutamic acid which is synthesized in osteoblast of bone tissues.⁹⁻¹¹⁾ Menaquinone-4 (MK-4) with four isoprene units has been shown to have a preventive effect on bone loss induced in rats by ovariectomy.¹²⁻¹⁴⁾ MK-4 is used as a therapeutic tool for osteoporosis. The effects of the supplemental intake of MK-4, however, have not been fully clarified.

This study was undertaken to determine whether the oral administration of MK-4 supplemented egg shell calcium (*Cal K₂*) has anabolic effects on bone components in the femoral tissues of rats *in vivo*.

MATERIALS AND METHODS

Chemicals — Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled. *Cal K₂* was prepared

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Table 1. Nutritional Composition in *Cal K₂*

Ingredients	Content
Protein	33.3 mg
Carbohydrate	700.0 mg
Water	33.3 mg
Ash	233.3 mg
Calcium	100 mg
Menaquinone-4	593 μ g

Each ingredient indicates the amount per gram of *Cal K₂*.

in the Q.P. Corporation (Tokyo, Japan) and its composition is shown in Table 1. *Cal K₂* contained egg shell calcium (Cal-Hope; Q.P. Corporation, Ca 100 mg/g) and MK-4 (*Vitamin K₂ KYOWA H*, KYOWA HAKKO KOGYO Co., Ltd., Tokyo, Japan, MK-4 593 μ g/g).

Animals — Male Wistar rats (conventional) weighting 90–100 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus at a room temperature of 25°C, with free access to distilled water.

Administration Procedures — *Cal K₂* was suspended in distilled water. The solution of *Cal K₂* (10, 25, or 50 mg/ml/100g body weight) was orally administered to rats through a stomach tube once daily for 7 days. Control rats received distilled water (1.0 ml/100 g body weight), and placebo-control rats were administered *Cal K₂* without MK-4 (50 mg/100 g). Rats were killed 24 hr after the last administration of *Cal K₂*, and the blood and femur were removed immediately.

Analytical Procedures — Blood samples obtained by cardiac puncture were centrifuged for 30 min after collection, and the serum was separated. Serum was frozen at –80°C until assay. Serum calcium and inorganic phosphorus concentrations were determined using an assay kit (Wako Pure Chemical Industries). The serum undercarboxylated osteocalcin or *r*-carboxylated osteocalcin concentration was assayed using a Rat Glu-type osteocalcin (Glu-OC) and Rat Gla-type osteocalcin (Gla-OC) EIA kit (Takara Shuzou, Shiga, Japan).

The diaphyseal or metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined using atomic absorption spectrophotometry.¹⁵⁾ Calcium content in bone tissues is expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the dia-

physeal or metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.5 mM (pH 7.4), cut into small pieces, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at 600 $\times$ g for 5 min was used to measure enzyme activity. Enzyme assay is carried out under optimal conditions. Alkaline phosphatase activity was determined using the method of Walter and Schutt.¹⁶⁾ Enzyme activity was expressed as μ mol of *p*-nitrophenol liberated per minute per milligram of protein. The protein concentration was determined using the method of Lowry *et al.*¹⁷⁾

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr after homogenization of the bone tissues.¹⁸⁾ After alkali extraction, the samples were centrifuged at 10000 $\times$ g for 5 min, and the supernatant was determined using the method of Ceriotti.¹⁹⁾ and expressed as the amount of DNA (milligrams)/gram wet weight of bone tissue.

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 indicate statistically significant differences. We also used multiple analysis of variance (ANOVA) to compare the treatment groups.

RESULTS

Change in Serum Findings in Rats Administered *Cal K₂*

The suspensions of *Cal K₂* (10, 25, or 50 mg/100 g body weight) were orally administered to rats once daily for 7 days, and the animals were killed 24 hr after the last administration. The body weight and serum inorganic phosphorus concentration did not change significantly after *Cal K₂* administration (Table 2). The serum calcium concentration was significantly increased after the administration of *Cal K₂* (25 or 50 mg/100 g body weight) as compared with that in the control group, while it was not significantly changed as compared with that of placebo-control group.

Serum *r*-carboxylated osteocalcin was significantly increased after the administration of *Cal K₂* (50 mg/100 g) as compared with that in the control group or placebo-control group without MK-4 (Fig. 1). The administration of *Cal K₂* (25 mg/100 g) caused a significant increase in serum *r*-carboxylated osteocalcin concentration as compared with that in

Table 2. Body Weight, Serum Calcium, and Inorganic Phosphorus Concentrations in Rats Orally Administered *Cal K₂*

Treatment	Body weight (g)	Serum concentration (mg/dl)	
		Calcium	Inorganic phosphorus
Control	125.6 ± 2.80	10.72 ± 0.20	8.81 ± 0.14
Placebo control	128.6 ± 2.21	11.31 ± 0.26	9.11 ± 0.06
10 mg/100 g	127.3 ± 2.51	11.20 ± 0.20	9.14 ± 0.24
25 mg/100 g	128.7 ± 1.58	11.45 ± 0.06*	9.15 ± 0.15
50 mg/100 g	125.8 ± 3.22	11.57 ± 0.16 [#]	9.16 ± 0.15

Rats were orally administered *Cal K₂* (10, 25, or 50 mg/100 g body weight) or placebo-control *Cal K₂* (without MK-4, 50 mg/100 g) for 7 days and killed 24 hr after the last administration. Each value is the mean ± SEM of 6 rats. **p* < 0.025, and [#]*p* < 0.01 compared with control (none) value. Differences were not significant as compared with the value from the placebo-control group.

Fig. 1. Change in Serum *r*-Carboxylated Osteocalcin in Rats Orally Administered *Cal K₂*

Rats were orally administered either vehicle (distilled water), placebo-control *Cal K₂* (without MK-4, 50 mg/100 g body weight), or *Cal K₂* (10, 25, or 50 mg/100 g) once daily for 7 days and killed 24 hr after the last administration. Each value is the mean ± SEM of 6 rats. **p* < 0.025 compared with the value in the control (none) or placebo-control groups.

the placebo-controlled group. The serum osteocalcin (undercarboxylated osteocalcin) concentration was not significantly changed after the administration of *Cal K₂* (10, 25, or 50 mg/100 g).

Effects of Administration of *Cal K₂* on Bone Components in Rats

The calcium content in the femoral-diaphyseal tissues was significantly increased after the administration of *Cal K₂* (25 or 50 mg/100 g body weight) as compared with that in the placebo-control group (Fig. 2). The femoral-metaphyseal calcium content was significantly increased after the administration of *Cal K₂* (25 or 50 mg/100 g) as compared with that in the control group.

Alkaline phosphatase activity in the femoral-dia-

physeal tissues was significantly increased after the administration of *Cal K₂* (25 or 50 mg/100 g) as compared with that in the control or placebo-control group (Fig. 3). Femoral-metaphyseal alkaline phosphatase activity was not significantly changed after *Cal K₂* administration.

The DNA content in the femoral-diaphyseal and -metaphyseal tissues was significantly increased after the administration of *Cal K₂* (50 mg/100 g) as compared with that in the control or placebo-control group (Fig. 4).

Thus MK-4 in *Cal K₂* had anabolic effects on bone components in the femoral tissues of rats.

Fig. 2. Change in Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered *Cal K₂*

Rats were orally administered either vehicle (distilled water), placebo-control *Cal K₂* (without MK-4, 50 mg/100 g body weight), or *Cal K₂* (10, 25, or 50 mg/100 g) once daily for 7 days and killed 24 hr after the last administration. Each value is the mean $\pm$ SEM of 6 rats. * p < 0.05 compared with the control (none) value. # p < 0.01 compared with the value in the placebo-control group.

Fig. 3. Change in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered *Cal K₂*

Rats were orally administered either vehicle (distilled water), placebo-control *Cal K₂* (without MK-4, 50 mg/100 g body weight), or *Cal K₂* (10, 25, or 50 mg/100 g) once daily for 7 days and killed 24 hr after the last administration. Each value is the mean $\pm$ SEM of 6 rats. * p < 0.01 compared with the value in the control (none) or placebo-control group.

Fig. 4. Change in DNA Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered *Cal K₂*

Rats were orally administered either vehicle (distilled water), placebo-control *Cal K₂* (without MK-4, 50 mg/100 g body weight), or *Cal K₂* (10, 25, or 50 mg/100 g) once daily for 7 days and killed 24 hr after the last administration. Each value is the mean $\pm$ SEM of 6 rats. * p < 0.01 compared with the value in the control (none) or placebo-control group.

DISCUSSION

Recent studies have shown that the chemical factors in food and plants have anabolic effects on bone components in femoral tissues of rats, suggesting a role in the prevention of bone loss with aging.^{20–30)} Whether the supplemental intake of MK-4 (vitamin K₂) as nutrient has anabolic effects on bone components has not been fully determined, although MK-4 is used as therapeutic tool for osteoporosis treatment in the clinical setting. *Cal K₂* was developed as a new MK-4 supplement containing calcium. This study demonstrates that the supplemental intake of MK-4 supplemented egg shell calcium (*Cal K₂*) has anabolic effects on bone components in the femoral tissues of rats *in vivo*.

Vitamin K₂ (menaquinone) is essential for the *r*-carboxylation of osteocalcin, a bone matrix protein containing calcium-binding *r*-carboxyglutamic acid, which is synthesized in osteoblasts of bone tissues.^{9–11)} The serum *r*-carboxylated osteocalcin concentration was significantly increased after the oral administration of *Cal K₂* to normal rats, suggesting that the intake of *Cal K₂* enhances newly synthesized *r*-carboxylated osteocalcin in osteoblastic cells in the bone tissues. Takeuchi *et al.* have reported that the intake of *Cal K₂* tablet increases serum *r*-carboxylated osteocalcin concentration in middle-aged women.³¹⁾ MK-4, which is contained in *Cal K₂*, may stimulate the production of *r*-carboxylated osteocalcin in osteoblastic cells in bone tissues.

Alkaline phosphatase is a marker enzyme of osteoblasts and the enzyme participates in bone mineralization.³²⁾ DNA content is an index of bone growth and the number of bone cells.³³⁾ The administration of *Cal K₂* caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral tissues of rats. The intake of *Cal K₂* may stimulate the proliferation and function of osteoblastic cells in bone tissues. *Cal K₂* may have a stimulatory effect on osteoblastic bone formation *in vivo*.

Cal K₂ contains protein, carbohydrate, ash, calcium, and MK-4 as shown in Table 1. Of these ingredients, MK-4 contributes to the anabolic effects on bone component after *Cal K₂* intake in rats.

In conclusion, it has been demonstrated that the intake of *Cal K₂* containing MK-4 (vitamin K₂) has an anabolic effect on bone components in the femoral tissues of rats *in vivo*.

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