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Low-Dose Daily Intake of Vitamin K_2 (Menaquinone-7) Improves Osteocalcin γ-Carboxylation: A Double-Blind, Randomized Controlled Trials

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Summary Vitamin K is essential for bone health, but the effects of low-dose vitamin K intake in Japanese subjects remain unclear. We investigated the effective minimum daily menaquinone-7 dose for improving osteocalcin γ-carboxylation. Study 1 was a double-blind, randomized controlled dose-finding trial; 60 postmenopausal women aged 50–69 y were allocated to one of four dosage group and consumed 0, 50, 100, or 200 μg menaquinone-7 daily for 4 wk, respectively, with a controlled diet in accordance with recommended daily intakes for 2010 in Japan. Study 2 was a double-blind, randomized placebo-controlled trial based on the results of Study 1; 120 subjects aged 20–69 y were allocated to the placebo or MK-7 group and consumed 0 or 100 μg menaquinone-7 daily for 12 wk, respectively. In both studies, circulating carboxylated osteocalcin and undercarboxylated osteocalcin were measured. The carboxylated osteocalcin/undercarboxylated osteocalcin ratio decreased significantly from baseline in the 0 μg menaquinone-7 group, in which subjects consumed the recommended daily intake of vitamin K with vitamin K_1 and menaquinone-4 (Study 1). Menaquinone-7 increased the carboxylated osteocalcin/undercarboxylated osteocalcin ratio dose dependently, and significant effects were observed in both the 100 and 200 μg groups compared with the 0 μg group. Undercarboxylated osteocalcin concentrations decreased significantly, and the carboxylated osteocalcin/undercarboxylated osteocalcin ratio increased significantly in the 100 μg menaquinone-7 group compared with the placebo group (Study 2). Daily menaquinone-7 intake ≥100 μg was suggested to improve osteocalcin γ-carboxylation.

Key Words carboxylated osteocalcin, undercarboxylated osteocalcin, blood coagulation, recommended daily intake

Vitamin K is a fat-soluble vitamin with a naphthoquinone skeleton and various lipophilic side chains (*1*, *2*). There are two main vitamin K compounds, which differ with respect to their side chain. Vitamin K_1 has a phytyl group and is found mainly in leafy green vegetables and vegetable oils (*3*). Menaquinones (MKs) have isoprenoid side chains with 4–14 repeats and are found in animal products; they are also produced in various bacterial fermentation processes and are, therefore, found in fermented products such as cheese and pickles (*3*). Vitamin K acts as a cofactor for post-translational carboxylation, in which γ-glutamyl carboxylase converts certain protein-bound glutamate residues into γ-carboxy glutamate (Gla) (*2*, *4*). At least 14 types of proteins with glutamate residues, designated vitamin K-dependent Gla-proteins, have been discovered. Well-known Gla-proteins are involved in blood coagulation (factors VII, IV, and X), which are synthesized in the liver (*4*, *5*). Gla-proteins that are not involved in coagulation include osteocalcin, a bone modulator (*1*), and matrix Gla protein, an inhibitor of vascular calcification (*6*). Osteocalcin has a structural function wherein it binds to hydroxyapatite because of γ-carboxylation (*7*), depositing calcium on bone for bone formation.

Observational studies in Japan showed that fracture frequency was inversely correlated with high consumption levels of natto (fermented soybean), which contains large amounts of menaquinone-7 (MK-7) produced by *Bacillus subtilis* (*8*, *9*). Vitamin K is also used for osteoporosis medication in Japan (Glakay™, menaquinone-4 [MK-4], 45 mg/d, Eisai, Tokyo, Japan) (*10*, *11*). These studies collectively suggested that daily vitamin K intake improves bone metabolism, bone mineral density (BMD), and bone strength, consequently decreasing fracture risk. However, the effects of low-dose vitamin K intake in Japanese subjects remain unclear.

Therefore, we investigated the effective minimum dose of MK-7 for improving osteocalcin γ-carboxylation as an index of bone health. This is the first study investigating osteocalcin γ-carboxylation by MK-7 supplementation as part of a controlled diet in Japanese subjects. The effective dose was determined for postmenopausal women, who are at high-risk of osteoporosis. Further-

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Abbreviations: BMD, bone mineral density; BMI, body mass index; cOC, carboxylated osteocalcin; Gla, γ-carboxy glutamate; INR, international normalized ratio; MHLW, Ministry of Health, Labor, and Welfare; MK, menaquinone; MK-4, menaquinone-4; MK-7, menaquinone-7; PT, prothrombin time; ucOC, undercarboxylated osteocalcin.

more, the efficacy of daily MK-7 intake was evaluated in healthy adults.

MATERIALS AND METHODS

Study design.

Study 1: Effective minimum dose of dietary MK-7 to affect carboxylated osteocalcin/undercarboxylated osteocalcin ratio in blood: This double-blind, randomized, parallel-group comparison study was conducted in Fukuhara Clinic, Hokkaido, Japan. Healthy, postmenopausal women aged 50–69 y who were not receiving medical treatment were recruited. The exclusion criteria were: ≤2 y since menopause; food allergies; irregular meals; excessive smoking or alcohol intake; routine medication; dietary supplement use; hepatic or renal diseases; chronic diseases; history of gastrointestinal surgery; lactose intolerance; night/irregular shift work; excessive physical activity; blood donation within 12 wk; and those who were judged inappropriate to include in the study by the principal investigator. Sixty women were included (n=30, 50–59 y; n=30, 60–69 y). The wash-out and intake periods were 14 and 28 d, respectively. Based on the order of the carboxylated osteocalcin (cOC)/undercarboxylated osteocalcin (ucOC) ratio in each age group, the women were allocated to one of four dosage groups—0, 50, 100, or 200 μg MK-7 (n=15 each), with stratified randomization in each age group. Each dose of MK-7 was dissolved in 14 g oil (J-Oil Mills, Tokyo, Japan), and the dose was consumed daily with dinner during the 4-wk intake period.

Table 1. Average daily nutrition over 2 wk in study 1.

	Content	Set value[1]	Sufficiency rate (%)[2]
Energy (kcal)	1,981	1,950	102
Protein (g)	67	50	134
Lipid (g)	57.9	54	107
Carbohydrate (g)	284.1	50–70%[3]	57%[3]
K (mg)	1,856	2,000	93
Ca (mg)	626	650	96
P (mg)	971	900	108
Fe (mg)	4.4	6.5	68
Vitamin K (μg)	72	65	111
Dietary fiber (g)	8.6	17	51
Salt (g)	7.2	7.5	96

[1] Recommended daily intake, Ministry of Health, Labor, and Welfare of Japan set in 2010.
[2] Fulfillment rate vs. set value.
[3] Recommended daily carbohydrate intake in Japan was determined as the energy ratio of daily total energy.

Subjects' meals were controlled throughout the study (Table 1). Subjects were provided with all daily meals (mandatory intake), and beverages and snacks (voluntary intake); foods and drinks besides these were prohibited. The nutrient contents of meals were based on the recommended daily intakes determined by the Ministry of Health, Labor, and Welfare (MHLW) of Japan in 2010. The recommended daily intake of 65 μg vitamin K for Japanese adult women was made up with vitamin K_1 and MK-4; MK-7-containing foods were excluded from the meal design. The meals were designed for a 2-wk period, and these were provided three times during the 6-wk study.

Study 2: MK-7 intake at 100 μg and improvement of osteocalcin γ-carboxylation: This double-blind, placebo-controlled, randomized, parallel-group comparison study was conducted at Yaesu Sakura-dori Clinic, Tokyo, Japan. Healthy men and women aged 20–69 y who were not receiving medical treatment and with a body mass index (BMI) of 18.5–28 kg/m^2 were recruited. The exclusion criteria were: food allergies; irregular meals; excessive smoking or alcohol intake; routine medication; dietary supplement use; hepatic or renal diseases; chronic diseases; history of gastrointestinal surgery; lactose intolerance; positivity for hepatitis B antigen, anti-hepatitis C virus antibody, human immunodeficiency virus (HIV) antigen and antibody, or syphilis serodiagnosis; night/irregular shift work; excessive physical activity; blood donation within 12 wk; habitual intake of high vitamin K-containing foods or supplements ≥3 times per week; as well as these who were judged inappropriate to include in the study by the principal investigator. In accordance with these criteria, after an interview and questionnaires about the habitual consumption of vitamin K-containing foods, 150 subjects were screened.

The wash-out/screening, intake, and follow-up periods were approximately 5, 12, and 4 wk, respectively (Fig. 1). The dosage of vitamin K and sample size were determined on the basis of the results of Study 1. Of the 150 subjects who were screened, those whose cOC or ucOC values or cOC/ucOC ratio were abnormal or whose cOC/ucOC ratio differed substantially between blood samplings (approximately 5 wk [S1] and 1 wk [S2] before the start of intake) were excluded to minimize scattering. The remaining 120 subjects included 50

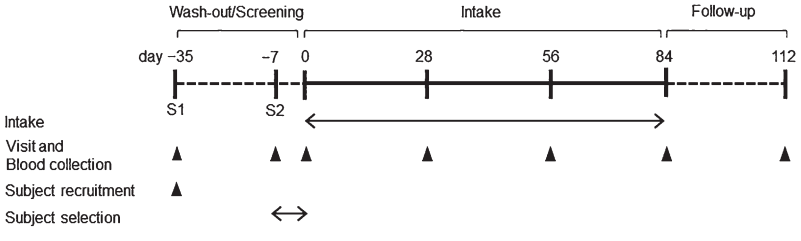

Fig. 1. Study 2 protocol.

Table 2. Study 1: Baseline characteristics.

Parameter	0 μg MK-7	50 μg MK-7	100 μg MK-7	200 μg MK-7
n	15	15	15	15
Age (y)	60.3±4.0	61.7±4.0	60.5±3.5	59.7±3.9
Weight (kg)	55.7±9.7	48.5±5.1*	55.5±7.4	50.5±5.1
Height (cm)	155.8±5.9	152.1±3.1	154.9±4.5	154.3±3.4
BMI (kg/m^2)	22.9±3.5	20.9±2.0	23.2±3.1	21.2±1.9
n	15	14	14	14
cOC (ng/mL)	17.73±4.76	19.47±4.17	19.85±10.28	18.48±3.64
ucOC (ng/mL)	5.34±2.38	6.98±4.07	5.83±2.88	5.61±2.09
cOC/ucOC	3.87±1.55	3.43±1.58	3.86±1.97	3.68±1.43

Data are mean±SD.
MK-7, menaquinone-7; BMI, body mass index; cOC, carboxylated osteocalcin; ucOC, undercarboxylated osteocalcin.
* $p<0.05$ vs. 0 μg MK-7 group (ANOVA with Dunnett's test).

men ($n=10$ each, 20–29 y, 30–39 y, 40–49 y, 50–59 y, and 60–69 y) and 70 women ($n=10$ each, 20–29 y, 30–39 y, 40–49 y; $n=20$ each, 50–59 y, 60–69 y). The subjects were arranged by cOC/ucOC ratio for each age group and each sex, and were allocated to the placebo or MK-7 groups ($n=60$ each) with stratified randomization for each age group and sex. Subjects consumed 11 g oil containing 0 or 100 μg MK-7 daily at an arbitrary time during the 12-wk intake period.

Throughout the study, natto and vitamin K_1-rich foods (e.g., chlorella tablets, green leafy vegetable juice, and *mulūkhīya* [*Corchorus olitorius*]) were prohibited. In addition, other vitamin K_1- or MK-4 rich foods (e.g., dark green leafy vegetables, tea leaves, *foie gras*, pickles, and cheese) were restricted to <300 g per day and <100 g per meal.

In both studies, the intake of study products, wake/sleep times, noticeable changes in health, physical activity, dietary patterns, defecation status, menstruation (for women), smoking, alcohol consumption, and medication use were recorded in a diary, and checked at every visit.

Subject assignment was implemented by third-party doctors. Subjects and investigators were blinded to the assignments until study completion.

Both studies were conducted in accordance with the guidelines of the Declaration of Helsinki, and the protocols were approved by the ethics committees of J-Oil Mills Inc. (for both studies), Miyawaki Clinic (for Study 1), and Yaesu Sakura-dori Clinic (for Study 2). Written informed consent was obtained from all subjects before participation.

Study product. In both studies, each oil was processed into 20 g (Study 1) or 15 g (Study 2) of mayonnaise (Knorr Foods, Kanagawa, Japan) for subjects to take easily. All mayonnaise products containing or not containing MK-7 were identical in appearance, texture, and taste. The daily doses of each mayonnaise were individually packed in polyethylene packages, and stored in shaded zippered polyethylene terephthalate/aluminum/polyethylene bags. The MK-7 content remained at >95% of the initial level during storage.

Blood sampling. In Study 1, blood was collected after overnight fasting at the start of the wash-out period and on days 0, 14, and 28. All blood samples were drawn between 10:00 AM and 12:00 PM. In Study 2, blood was collected more than 12 h after intake of study products and vitamin K_1- and MK-4 rich foods, whose consumption was restricted at S1 and S2; days 0, 28, 56, and 84; and at the end of follow-up (day 112). For plasma preparation, blood was collected in heparinized tubes, centrifuged for 10 min at 1,500 ×*g*, aliquoted into shaded tubes, and stored at −80°C until analysis. Serum was prepared similarly, except blood was allowed to clot at room temperature.

Circulating markers. Serum cOC was analyzed by ELISA (Takara Shuzo, Shiga, Japan). Serum ucOC was analyzed using an electrochemiluminescence immunoassay (Eidia, Tokyo, Japan). The cOC/ucOC ratio was subsequently calculated. Plasma vitamin K_1, MK-4, and MK-7 concentrations were analyzed as described previously (*12*). Briefly, plasma was extracted with hexane, after prepurification on Sep-Pak silica (Waters, Milford, MA), and analyzed by LC-APCI-MS/MS. Deuterium-containing vitamin K_1 (vitamin K_1-d_7) was used as an internal standard. Prothrombin time (PT) was analyzed by SRL (Tokyo, Japan) using a Quick's One-stage Test, which measures the time to fibrin clot formation using Thromborel S (Siemens, Tokyo, Japan) in a coagulometer (CA-7000; Sysmex, Hyogo, Japan) at S1 and S2, and days 0, 84 and 112 in Study 2. The PT-international normalized ratio (PT-INR) was also calculated. ucOC was analyzed by Daiichi-Kishimoto-Rinsho Kensa Center (Hokkaido, Japan) and SRL in Studies 1 and 2, respectively; cOC and vitamin K levels were analyzed by Shimadzu Techno-Research (Kyoto, Japan).

Statistical analyses. In Study 1, changes from baseline (day 0) were analyzed using the paired Student's *t*-test. Inter-group differences were evaluated using ANOVA with Dunnett's test.

In Study 2, because all data were non-normally distributed, the baseline characteristics were compared

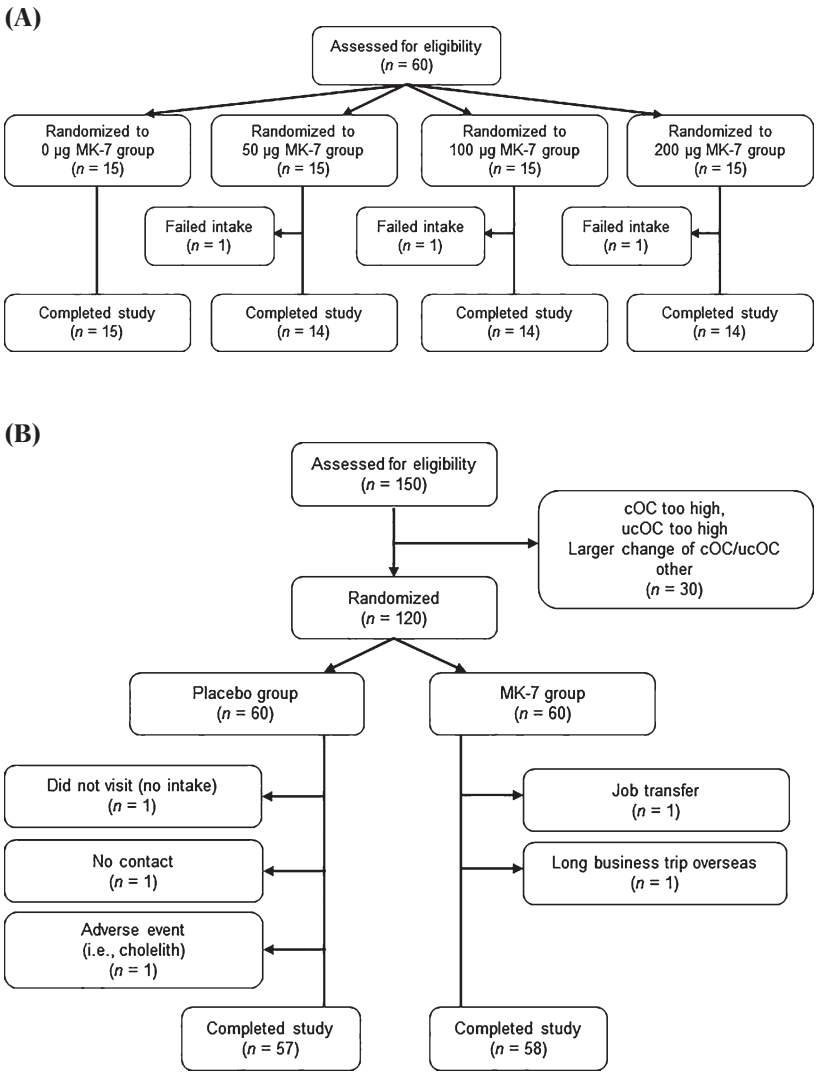

Fig. 2. Flow diagrams of Study 1 (A) and Study 2 (B). MK-7, menaquinone-7; cOC, carboxylated osteocalcin; ucOC, undercarboxylated osteocalcin.

using the Wilcoxon rank-sum test. Changes in bone turnover markers from baseline (day 0) were analyzed using the Wilcoxon signed-rank test. Inter-group differences were evaluated using the Wilcoxon rank-sum test.

The levels of significance were set at $p<0.05$ and 0.01. Statistical analyses for changes in cOC/ucOC ratio from baseline and between group differences in changes in cOC/ucOC ratio in Study 1 were performed using JSTAT v13.0 and for baseline characteristics using SAS v9.1.3 (SAS Institute Japan, Tokyo, Japan). All statistical analyses in Study 2 were performed using SAS v8.02 (SAS Institute Japan). The sample size required for Study 2 was estimated from the results of Study 1 using R v2.14.0. (R Foundation, http://www.r-project.org/), with a level of significance of $p<0.05$ and statistical power of 0.8.

RESULTS

Effective minimum dose of dietary MK-7 to affect carboxylated osteocalcin/undercarboxylated osteocalcin ratio in blood

The baseline characteristics of subjects in Study 1 are summarized in Table 2. A flow diagram of Study 1 is shown in Fig. 2A. One subject each in the 50, 100, and 200 μg MK-7 groups dropped out because of errors in study product intake; therefore, 57 subjects were analyzed. Subject recruitment commenced in August 2011, and the study was completed in December 2011. The intake rates of study products in each group exceeded 99%. There were no differences among groups in the number of dropouts, noticeable changes in health or adverse effects. No adverse effects associated with the study products were observed in any subjects.

There were no significant differences among groups in either the cOC or ucOC concentration, and no dose dependency was observed. The ucOC concentration increased significantly from baseline in the 0 μg MK-7 group ($p<0.05$ on day 28), and decreased significantly from baseline in the 200 μg MK-7 group ($p<0.05$ on day 28). The cOC concentration decreased significantly in the 0 μg MK-7 group ($p<0.01$ on day 28).

The cOC/ucOC ratio in the 0 μg MK-7 group decreased significantly by 1.55 ng/mL from baseline. In other groups, the cOC/ucOC ratios changed slightly from baseline, but the difference was not significant. In

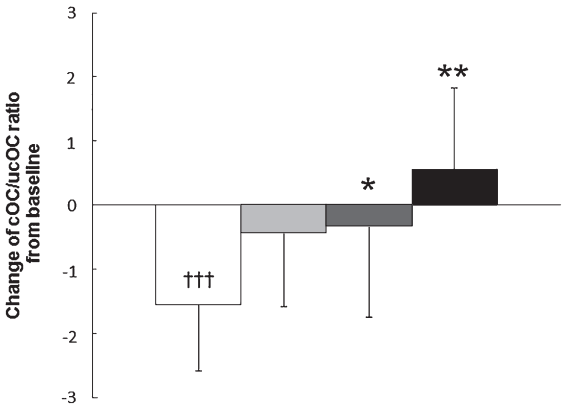

Fig. 3. Change in carboxylated osteocalcin (cOC)/undercarboxylated osteocalcin (ucOC) ratio from baseline in Study 1. Data are mean±SD. White, light grey, dark grey, and black bars indicate the 0, 50, 100, and 200 μg MK-7 groups, respectively. * $p<0.05$, ** $p<0.01$ vs. 0 μg MK-7 group (ANOVA with Tukey-Kramer test). ††† $p<0.0001$ vs. baseline (Student's paired t-test). $n=14$ for 50, 100, and 200 μg MK-7 groups, $n=15$ for 0 μg MK-7 group.

Table 3. Study 2: Baseline characteristics.

Parameter	Placebo	MK-7
n	60	60
Age (y)	47±14	47±14
Weight (kg)	57.4±9.3	58.7±7.9
Height (cm)	162.6±8.8	163.4±8.2
BMI (kg/m^2)	21.6±2.3	22±2.1
cOC (ng/mL)	25.79±7.76	23.55±5.88
ucOC (ng/mL)	5.86±3.32	5.26±2.67
cOC/ucOC	5.38±2.47	5.37±2.57

Data are mean±SD.
MK-7, menaquinone-7; BMI, body mass index; cOC, carboxylated osteocalcin; ucOC, undercarboxylated osteocalcin.

the 100 and 200 μg MK-7 groups, the changes from baseline in the cOC/ucOC ratio were significantly higher than those in the 0 μg MK-7 group (Fig. 3).

MK-7 intake at 100 μg and improvement of osteocalcin γ-carboxylation

In Study 2, there were no significant differences in the baseline characteristics between groups (Table 3). Figure 2B shows a flow diagram of this study. During the intake period, in the placebo group, 2 men dropped out because of a lack of contact, and 1 woman withdrew at 4 wk after the start of intake because of a diagnosis of cholecystitis. In the MK-7 group, 2 men dropped out owing to work circumstances. Therefore, 115 subjects were analyzed. Subject recruitment commenced in May 2013, and the study was completed in December 2013. The intake rates of study products in both groups exceeded 99%. There were no significant differences between groups in the number of dropouts, noticeable

Table 4. Study 2: Circulating vitamin K_1, MK-4, MK-7, cOC concentrations, ΔcOC and PT-INR.

Parameter	Day 0		Day 28		Day 56		Day 84		Day 112	
	Placebo	MK-7	Placebo	MK-7	Placebo	MK-7	Placebo	MK-7	Placebo	MK-7
n	57	58	57	58	57	58	57	57	57	58
Vitamin K_1 (ng/mL)	0.53±0.62	0.42±0.51	0.55±0.46	0.47±0.63	0.44±0.55	0.34±0.53	0.81±0.71††	0.54±0.68*	0.63±0.75	0.39±0.5
MK-4 (ng/mL)	0.00	0.00	0.00	0.02±0.14	0.00	0.00	0.00	0.01±0.07	0.00	0.00
MK-7 (ng/mL)	0.75±1.22	0.95±2.35	0.85±2.12	2.69±2.35**††	0.52±1.57††	3.09±1.91**††	0.7±1.46	4.29±5.34**††	0.95±2.46	1.4±3.37
cOC (ng/mL)	26.08±7.74	23.29±5.79*	24.29±8.1††	22.75±5.75	24.64±8.31†	24.22±6.75	24.98±7.85	24.47±6.19	27±7.344	24.47±7.54
ΔcOC (ng/mL)	0	0	−1.78±5.21††	−0.54±4.12	−1.44±5.3†	0.93±4.61*	−1.1±5.21	1.2±5.05*	0.92±4.03	1.18±5.00
PT-INR	0.98±0.04	0.99±0.05	—	—	—	—	0.99±0.05	1.00±0.04	0.98±0.05	0.99±0.05

Data are mean±SD.
MK-4, menaquinone-4; MK-7, menaquinone-7; cOC, carboxylated osteocalcin; PT-INR, prothrombin time-international normalized ratio.
Placebo: 0 μg MK-7/d, MK-7: 100 μg MK-7/d.
* $p<0.05$, ** $p<0.01$ vs. placebo (Wilcoxon rank sum test).
† $p<0.05$, †† $p<0.01$ vs. baseline (Wilcoxon signed-rank test).

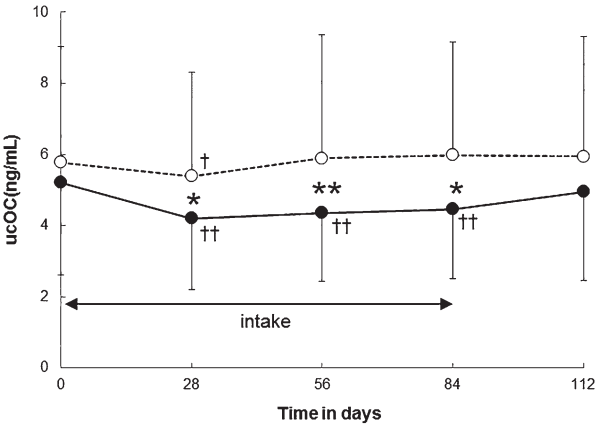

Fig. 4. Circulating undercarboxylated osteocalcin (ng/mL) in Study 2. Data are mean±SD. ○: Placebo, ●: MK-7. $^{*}p<0.05$, $^{**}p<0.01$ vs. placebo (Wilcoxon rank-sum test). $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. baseline (Wilcoxon signed-rank test). $n=57$ for the placebo group, and $n=58$ (day 0, 28, 56 and 112) or $n=57$ (day 84) for the MK-7 groups.

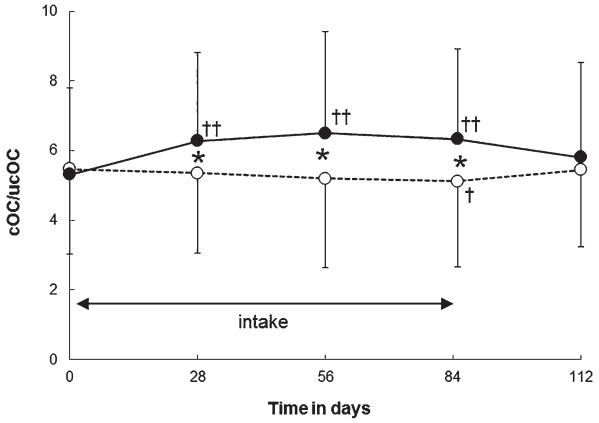

Fig. 5. Carboxylated osteocalcin (cOC)/undercarboxylated osteocalcin (ucOC) ratio in Study 2. Data are mean±SD. ○: Placebo, ●: MK-7. $^{*}p<0.05$ vs. placebo (Wilcoxon rank-sum test). $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. baseline (Wilcoxon signed-rank test). $n=57$ for the placebo group, and $n=58$ (day 0, 28, 56 and 112) or $n=57$ (day 84) for the MK-7 group.

changes in health or adverse effects. No adverse effects associated with study products were observed in any subjects.

In the MK-7 group, plasma MK-7 concentrations increased at day 28, plateaued at ~3 ng/mL during intake, and subsequently returned to baseline values at the end of follow-up. Plasma vitamin K_1 concentrations were considerably lower, and MK-4 concentrations were around the detection limit (Table 4).

For blood coagulation measurements of PT-INR, there were neither significant differences between groups nor clinically relevant changes in either group (Table 4).

No effects were observed regarding circulating cOC concentration, but the change from baseline in cOC was significantly higher in the MK-7 group than in the placebo group on days 56 and 84 (Table 4). The circulating ucOC concentration in the MK-7 group decreased at day 28 and remained unchanged thereafter (Fig. 4). The ucOC concentrations in both groups were not significantly different at baseline, but those in the MK-7 group were significantly below baseline during the intake period. The ucOC concentrations in the MK-7 group were significantly lower than those in the placebo group during the intake period. The cOC/ucOC ratio increased by day 28, plateaued during intake, and returned to baseline values after the end of intake in the MK-7 group; it did not change significantly during the study in the placebo group. The cOC/ucOC ratio in the MK-7 group was significantly higher in the MK-7 group than in the placebo group throughout the intake period (Fig. 5).

DISCUSSION

Vitamin K is involved in the γ-carboxylation of osteocalcin, and cOC functions in the deposition of calcium in bones, leading to bone formation. The ratio between cOC and ucOC is widely used as a bone metabolism marker, and circulating ucOC is utilized as a clinical marker of vitamin K deficiency for osteoporosis treatment in Japan (*10*). The ucOC concentration is higher in osteoporosis patients than in non-osteoporotic individuals. Shiraki et al. indicated that vitamin K insufficiency in bone occurs at a ucOC level ≥4.5 ng/mL, and early and frequent occurrence of fractures is observed in individuals with a ucOC level ≥5.5 ng/mL because of the correlation between the occurrence of fracture and baseline ucOC level (*13*). Circulating ucOC is a predictor of fracture risks independent of BMD (*14–16*). Thus, we considered that a reduction in ucOC concentration is important for bone health.

The recommended daily intake of vitamin K in Japan was set at 60–75 μg in 2010 (*17*); this value was based on the amount required to maintain normal coagulation function. It was not determined based on osteocalcin γ-carboxylation. Fracture incidence was inversely correlated with the intake of natto, suggesting that MK-7 mainly contributes to fracture prevention. In this study, the effective minimum dose of MK-7 required to improve osteocalcin activation was investigated with the use of a controlled diet in Japanese subjects.

This study demonstrated that low-dose MK-7 (50–200 μg/d) dose-dependently improved osteocalcin γ-carboxylation, and more than 100 μg MK-7 in addition to ordinary meals increased the cOC/ucOC ratio and decreased ucOC concentration.

ucOC concentration correlates with age and time since menopause in women, and a high vitamin K_1 and MK-7 concentration is required in the circulation to minimize the ucOC concentration in older women (*18*). The minimum dose of vitamin K for osteocalcin γ-carboxylation was investigated in postmenopausal women (Study 1). MK-7 doses were set at 50, 100, and 200 μg/d. Uematsu et al. reported that simultaneous intake of fat improved vitamin K_2 absorption (*19*). Each dose

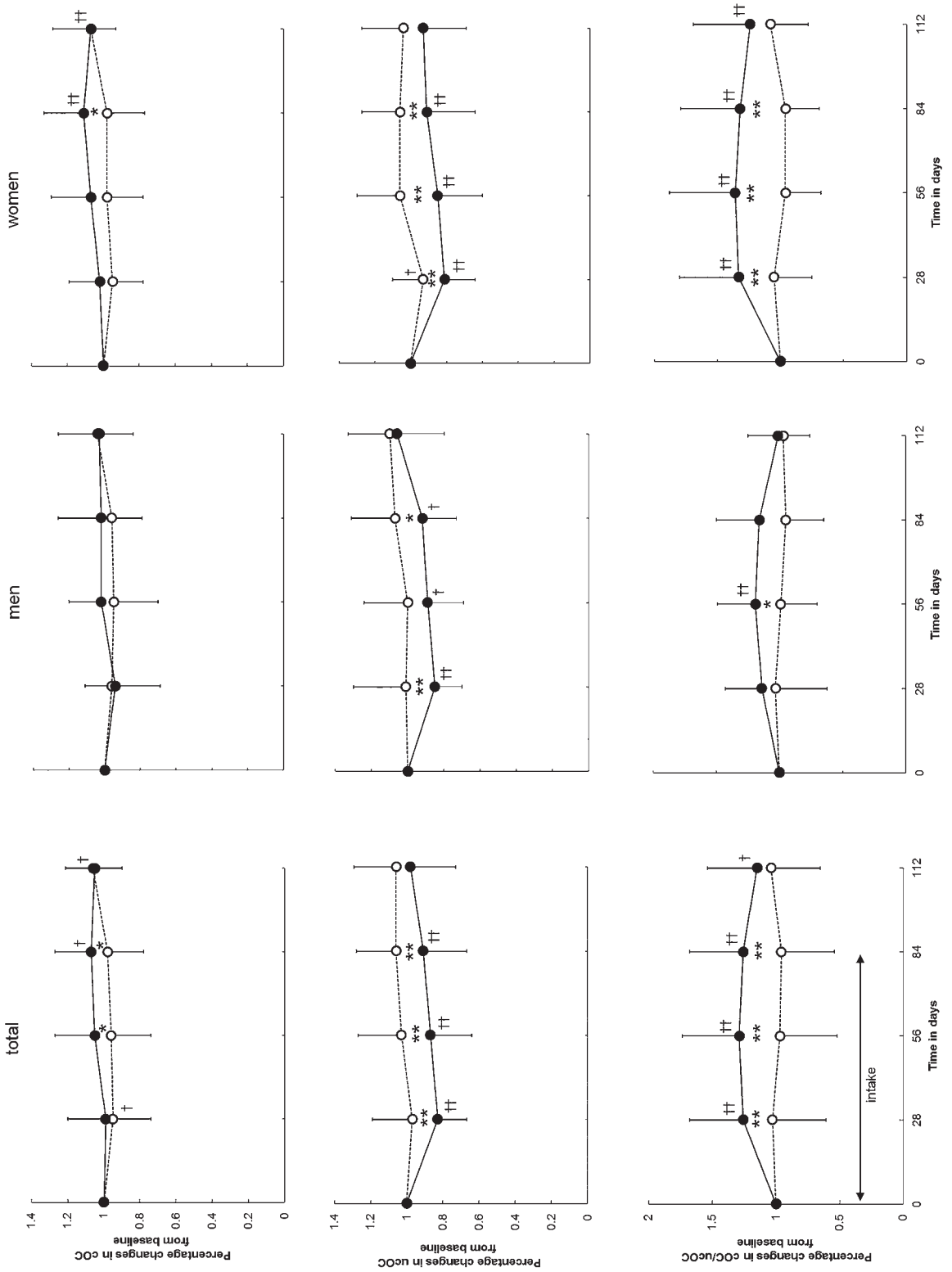

Fig. 6. Comparison of the effects of vitamin K between men and women in Study 2. Percentage changes in carboxylated osteocalcin (cOC) (A), in undercarboxylated osteocalcin (ucOC) (B) and cOC/ucOC ratio (C). Data are mean±SD. ○: Placebo, ●: MK-7. $^{*}p<0.05$, $^{**}p<0.01$ vs. placebo (Wilcoxon rank-sum test). $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. baseline (Wilcoxon signed-rank test). $n=23$ for men and $n=34$ for women in the placebo group, and $n=23$ (day 0, 28, 56 and 112) or $n=22$ (day 84) for men and $n=35$ for women in the MK-7 group.

of MK-7 was dissolved in oil and then incorporated into mayonnaise. Dietary vitamin K intake was completely controlled (mean: 72 μg/d) with mainly vitamin K_1 and small amounts of MK-4 in accordance with the recommended daily intake determined by the MHLW of Japan in 2010 (60–75 μg/d).

In the 0 μg MK-7 group, in which subjects consumed just the amounts in accordance with the recommended daily intake of vitamin K, the cOC/ucOC ratio decreased continuously throughout the intake period. Furthermore, cOC concentrations tended to decrease and ucOC increased. In the 50 μg MK-7 group, the cOC/ucOC ratio at day 28 remained unchanged from baseline. However, MK-7 intake $\geq$100 μg attenuated the decrease in the cOC/ucOC ratio compared with the placebo. In addition, the dose-dependent effect of MK-7 confirmed the effects of low MK-7 intake (50–200 μg) on osteocalcin γ-carboxylation. It was suggested that the minimum dose of MK-7 for improving bone metabolism is $\geq$100 μg.

A placebo-controlled test was performed to evaluate the effect of 100 μg MK-7 for a wide-range of age groups of both sexes (Study 2). MK-7 effects with realistic amounts of vitamin K intake in Japan were investigated in Study 2, in which daily meals were not controlled, but natto and vitamin K_1-rich foods (chlorella tablets, green leafy vegetable juice, and *mulūkhīya*) were prohibited and vitamin K_1 and MK-4 rich foods were restricted to $<$300 g per day. The number of subjects was 60 in each group, because the required sample size was estimated to be more than 54 from the results of Study 1. As expected, 100 μg MK-7 improved the cOC/ucOC ratio throughout the intake period. In addition, the ucOC concentration decreased even with a low MK-7 intake of 100 μg over a short period of 12 wk. During the intake period, the ucOC concentration in the MK-7 group fell below 4.5 ng/mL (standard for vitamin K deficiency) indicating that 100 μg/d MK-7 can improve vitamin K deficiency (*13*). Coagulation markers were not affected during the study period, suggesting that 100 μg MK-7 may be used for bone health, after meeting coagulation requirements.

As for differences in vitamin K effects in each sex, significant differences between groups in percentage changes in cOC from baseline were observed (day 84) (Fig. 6A), and those in ucOC and cOC/ucOC ratio were observed throughout the intake period in women (Fig. 6B and C). In men in the MK-7 group, the percentage change in ucOC was below that in the placebo group (days 28 and 84), and the percentage change in cOC/ucOC was above that in the placebo group (day 56) (Fig. 6B and C). The number of female subjects was larger than for males, and the proportion of women aged over 50 y who would mostly be post-menopausal was more than half of the total number of women in this study. Although relatively larger effects were observed in women, which included postmenopausal women who would be at higher risk of osteoporosis than men, the effects on osteocalcin γ-carboxylation were observed in both sexes.

Previous intervention studies demonstrated that high doses (1–45 mg/d) of vitamin K_1 or MK-4 affected osteocalcin γ-carboxylation or other bone indices (*10*, *11*, *20*, *21*), whereas low doses of MK-7 (90–360 μg/d) can affect osteocalcin concentration (*22*, *23*). MK-4 intake (1.5 mg/d for 12 wk) showed a 30% increase in cOC and a 40% decrease in ucOC (*24*). MK-7 intake at 650 μg/d for 2 wk demonstrated a 60% increase in cOC (*25*). These studies demonstrated larger effects than the present study, in which approximately a 7% increase in cOC and 20% decrease in ucOC were observed (Fig. 6A and B). These studies suggested that larger amounts of vitamin K are required for an increase in cOC than for a decrease in ucOC. In these studies, vitamin K intake was considerably larger than in the present study, or the proportion of perimenopausal women to whole subjects was larger than in the present study, suggesting that the sensitivity of osteocalcin to vitamin K was higher than in the present study. Additionally, it is difficult to compare the effects observed in this study with those in previous studies, because this study investigated the minimum dose of MK-7 for improving osteocalcin γ-carboxylation, but most of these other previous studies assessed the dose required to achieve maximal osteocalcin γ-carboxylation.

Shurgers et al. (*23*) reported that MK-7 had a longer half-life than vitamin K_1; it accumulates to higher concentrations in serum and has stronger effects on osteocalcin γ-carboxylation than vitamin K_1 when equimolar amounts of MK-7 and vitamin K_1 are taken. MK-7 was also reported to have a longer half-life than MK-4 (*26*). Therefore, MK-7 is considered to have higher bioavailability than other K vitamins. Shiraki et al. (*10*) demonstrated that MK-4 intake (45 mg/d for 2 y) decreased serum ucOC concentration, improved BMD, and decreased fracture incidence. In this study, we demonstrated that daily intake of MK-7 decreased serum ucOC. Although bone strength or mass were not evaluated, continuous intake of 100 μg MK-7 was expected to decrease future fracture risk. Moreover, this study targeted healthy adults. Thus, the doses of MK-7 required for young children, who are undergoing skeletal formation, and pregnant women, remain to be determined. The precise amounts required for each age group are also unknown, because the sample sizes were insufficient. Therefore, it is necessary to determine how the dose in this study affects each age group.

Vitamin D deficiency or abnormal calcium homeostasis causes secondary hyperparathyroidism by stimulating parathyroid hormone (PTH) production (*27*). Vitamin D and PTH play important roles in mobilizing calcium from bone (*28*). Lian et al. reported that calcium deficiency increased the serum osteocalcin level (*29*). Pietschmann et al. indicated that serum calcium concentration negatively correlates with serum levels of PTH and osteocalcin (*30*). Therefore serum levels of calcium and vitamin D are important for improvement of bone metabolism by vitamin K.

The recommended daily intake of vitamin K in Japan was revised to 150 μg/d from 60–75 μg/d in April 2015. Considering the result of this dose-finding study,

the newly recommended value, which was also determined based on coagulation requirements, is suggested to maintain osteocalcin γ-carboxylation status, assuming that the increment (of 75–90 μg) was made up with MK-7.

Japanese practice guidelines recommended vitamin K intake of 250–300 μg/d for the prevention and treatment of osteoporosis (*31*). Kamao et al. estimated the average dietary intake of vitamin K at 154.1 μg/d in Japanese who do not eat natto habitually (*32*). Vitamin K intake in the MK-7 group of Study 2 could be assumed to meet the recommended value in the guidelines.

In conclusion, the present study demonstrated that ≥100 μg MK-7 in addition to ordinary meals could improve osteocalcin γ-carboxylation status. Furthermore, osteocalcin γ-carboxylation decreased ucOC concentration even over a short period of intake. A longer time period of this level of MK-7 would be expected to maintain γ-carboxylation status and bone metabolism, leading to improved bone health.

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Conflict of interest

No authors declare a conflict of interest.

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