Vitamin K supplementation reduces serum concentrations of under-γ-carboxylated osteocalcin in healthy young and elderly adults[1–3]

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ABSTRACT

Background: Subclinical vitamin K insufficiency, manifested by under-γ-carboxylation of the bone matrix protein osteocalcin, may be common.

Objective: Our objective was to delineate the prevalence of submaximal γ-carboxylation as assessed by response to phylloquinone supplementation and to evaluate the effect of this intervention on skeletal turnover in healthy North American adults.

Design: Healthy subjects (n = 219), approximately equally distributed by sex and age (18–30 y and ≥65 y), received daily phylloquinone (1000 μg) or placebo for 2 wk. Serum undercarboxylated osteocalcin (ucOC) and total osteocalcin, *N*-telopeptides of type I collagen (NTx), bone-specific alkaline phosphatase (BSAP), and phylloquinone concentrations were measured at baseline and after weeks 1 and 2.

Results: At baseline, the mean serum phylloquinone concentration was lower in the young than in the old group; there was no effect of sex. Concomitantly, baseline %ucOC was highest in the young and lowest in the old men ($P < 0.0001$) but did not differ significantly by age in women. After supplementation, serum phylloquinone concentration increased ≈10-fold ($P < 0.0001$) at week 1 (from 0.93 ± 0.08 to 8.86 ± 0.70 nmol/L, $\bar{x}$ ± SEM); this was sustained through week 2. Among all supplemented groups, mean %ucOC decreased from 7.6% to 3.4% without significant differences by age or sex; 102 of 112 subjects had a >1% decrease. Phylloquinone supplementation reduced serum osteocalcin but did not alter NTx or BSAP concentration.

Conclusions: Usual dietary practices in this population did not provide adequate vitamin K for maximal osteocalcin carboxylation. Phylloquinone supplementation reduced serum osteocalcin concentration but did not alter other markers of serum bone turnover. *Am J Clin Nutr* 2000;72:1523–8.

KEY WORDS Vitamin K, phylloquinone, osteocalcin, undercarboxylated osteocalcin, ucOC, bone turnover, osteoporosis, elderly

INTRODUCTION

Undercarboxylation of the bone matrix protein osteocalcin appears to be a sensitive measure of vitamin K status (1–3). When defined as elevated concentrations of undercarboxylated osteocalcin (ucOC), vitamin K insufficiency appears to be common in postmenopausal women (4, 5). Whether this insufficiency has clinical relevance is unclear. However, a high serum ucOC concentration has been associated with skeletal turnover (6), low bone mineral density (7), and increased risk of osteoporotic fracture (8–10). Additionally, clinical use of vitamin K antagonists as anticoagulants has been related to low bone mineral density (11–15) and increased risk of fracture (16). These observations imply that vitamin K insufficiency contributes to osteoporosis development. If these associations are causally related, ie, if undercarboxylation of osteocalcin produces adverse skeletal consequences, increased vitamin K intake is indicated. However, such a recommendation is premature because other reports showed no effect of warfarin-induced vitamin K insufficiency on bone density (17–19) or fracture (20). As such, the role of vitamin K insufficiency in skeletal health remains unclear. The purpose of this study was to further delineate the prevalence of submaximal γ-carboxylation, as assessed by response to vitamin K supplementation, and to evaluate the effect of this intervention on skeletal turnover in young and old individuals of both sexes.

SUBJECTS AND METHODS

Subjects

This study was approved by the University of Wisconsin Health Sciences institutional review board and informed consent was obtained from all volunteers. The study population consisted of 219 healthy subjects recruited from central and southern Wisconsin (**Table 1**). Age groups were arbitrarily defined as young (18–30 y of age) and old (≥65 y of age). Approximately equal numbers of men and women were enrolled in both age groups. Screening laboratory values, including prothrombin time (PT), complete blood count, and a serum chemistry panel, were

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TABLE 1
Demographic data[1]

Group	Age	BMI	Compliance	Withdrew from study
	y	*kg/m²*	%	*n*
Young men (*n* = 55)	24.8 ± 0.4	25.4 ± 0.4	89 ± 1.5[2]	2
Phylloquinone (*n* = 28)	25.3 ± 0.6	25.5 ± 0.7	90 ± 2.1	0
Control (*n* = 27)	24.2 ± 0.6	25.4 ± 0.6	88 ± 2.2	2
Young women (*n* = 55)	24.2 ± 0.5	23.0 ± 0.5[2]	93 ± 1.3	5
Phylloquinone (*n* = 28)	24.4 ± 0.7	22.9 ± 0.7	94 ± 1.7	2
Control (*n* = 27)	24.1 ± 0.6	23.2 ± 0.8	92 ± 2.1	3
Old men (*n* = 53)	75.8 ± 0.7	26.9 ± 0.7	96 ± 1.1	2
Phylloquinone (*n* = 27)	75.3 ± 1.1	26.7 ± 0.7	97 ± 1.3	1
Control (*n* = 26)	76.3 ± 1.1	27.1 ± 1.3	94 ± 1.9	1
Old women (*n* = 56)	75.4 ± 1.0	25.4 ± 0.6	96 ± 1.7	4
Phylloquinone (*n* = 29)	74.9 ± 1.4	25.4 ± 0.9	99 ± 0.7	1
Control (*n* = 27)	75.9 ± 1.5	25.6 ± 0.9	94 ± 3.3	3

[1] $\bar{x}$ ± SEM.
[2] Significantly different from all other groups, $P < 0.05$.

required to be normal or without clinically significant abnormalities for enrollment. Volunteers with a medical history of renal or hepatic disease or malabsorption or who were receiving current warfarin therapy were excluded from participation.

Study design

In this single-blind, placebo-controlled trial, subjects were unaware of their random assignment to receive either two 500-μg tablets of phylloquinone (vitamin K1; Roche Vitamins Inc, Parsippany, NJ) or a matching placebo with the evening meal for 14 d. Compliance with study preparation was evaluated by tablet counts after 1 and 2 wk. Concomitant use of medication and nutritional supplements was documented before and during the study.

Serum and plasma were obtained at baseline and at the end of both treatment weeks. Blood samples were obtained by routine venipuncture between 0800 and 1100 after subjects fasted for ≥8 h. Specimens were shielded from light and allowed to clot at room temperature for 30 min before centrifugation a 750 × *g* for 15 min at room temperature. Aliquots were quick-frozen in liquid nitrogen and stored at −80°C until thawed for analysis.

Assays

Serum phylloquinone, osteocalcin, ucOC, bone-specific alkaline phosphatase (BSAP), and *N*-telopeptides of type I collagen (NTx) concentrations were measured for all time points. PT and decarboxyprothrombin, ie, the protein induced by phylloquinone absence or antagonist-II (PIVKA-II) were evaluated at the baseline and week 2 visits.

PT was determined by adding 2 parts Simplastin (Organon Teknika Corporation, Durham, NC) to 1 part freshly thawed (at 37°C) citrated plasma in a fibrometer (Becton Dickinson, Franklin Lakes, NJ). PIVKA-II values were measured at baseline and at week 2 by enzyme-linked immunosorbent assay (ELISA, Asserachrom; Diagnostica Stago, Asnieres-sur-Seine, France). Serum phylloquinone concentrations were determined by HPLC separation with fluorescence detection (21). BSAP was measured by enzyme immunoassay (Alkphase-B; Metra Biosystems, Mountain View, CA). Serum NTx was measured by using a competitive-inhibition ELISA (Osteomark, Ostex International, Inc, Seattle). Osteocalcin was determined by immunoradiometric assay (ELSA-OSTEO; CisBio International, Gif-sur-Yvette, France). The concentration of ucOC was determined by modification of the hydroxyapatite binding assay (22). Briefly, 500 mL serum was treated with 25 mg hydroxyapatite (no. 4280; Mallinkrodt, Inc, Paris, KY) and rotated end-over-end for 30 min at 4°C. The samples were then centrifuged at 16 000 × *g* for 5 min. The supernate was removed and analyzed for osteocalcin by immunoradiometric assay. Percentage ucOC was calculated as the ratio of unadsorbed, ie, remaining in the supernate, to total osteocalcin, multiplied by 100.

Statistical analysis

Study groups were defined by sex, treatment (phylloquinone or control), and age (young and old). Baseline comparisons of variables between groups were performed by using Student's *t* test. Spearman's rank correlation coefficient (*r*) was used to evaluate the relation of baseline serum phylloquinone concentration with osteocalcin and %ucOC. Change over time in serum phylloquinone, %ucOC, and osteocalcin was evaluated by repeated-measures analysis of variance (ANOVA) with full interaction. All analyses were conducted by using STATVIEW software (version 4.5; Abacus Concepts, Berkeley, CA).

RESULTS

Subjects

The demographic characteristics of the young and old groups were not significantly different by age or sex, with the exception of a lower body mass index ($P < 0.01$) in the young female group (Table 1). Screening laboratory values for all groups were within the normal range (data not shown). Within the age and sex categories, there were no significant differences in demographics or baseline laboratory results between the phylloquinone and control groups (Table 1). Compliance and adherence were excellent but were lower in the young male group than in any other group; no other compliance differences were observed. Some 94% of volunteers enrolled completed the study; withdrawals were evenly distributed across groups (Table 1). No study participants used nutritional supplements other than calcium or vitamins (most frequently one multiple vitamin daily). Supplement use did not differ significantly between the control and phylloquinone-treated groups.

TABLE 2
Baseline laboratory values[1]

Group	Phylloquinone	ucOC	Osteocalcin	NTx	BSAP
	nmol/L	*%*	*μg/L*	*BCE*	*U/L*
Young men (n = 55)	0.68 ± 0.06	9.0 ± 0.3	31.1 ± 1.4	18.9 ± 1.1	24.5 ± 1.4
Phylloquinone (n = 28)	0.71 ± 0.08	8.8 ± 0.5	29.2 ± 1.7	19.3 ± 1.6	26.2 ± 2.2
Control (n = 27)	0.66 ± 0.08	9.3 ± 0.4	33.0 ± 2.1	18.4 ± 1.5	22.6 ± 1.7
Young women (n = 55)	0.72 ± 0.09	7.7 ± 0.3[2]	25.3 ± 1.0[2]	13.3 ± 0.7[2]	22.5 ± 1.5
Phylloquinone (n = 28)	0.63 ± 0.07	8.0 ± 0.5	25.3 ± 1.5	12.9 ± 1.0	22.6 ± 2.3
Control (n = 27)	0.81 ± 0.16	7.4 ± 0.4	25.2 ± 1.5	13.7 ± 0.9	22.4 ± 1.8
Old men (n = 53)	1.03 ± 0.14[3]	6.3 ± 0.3[2]	22.2 ± 1.3[2]	12.3 ± 0.8[2]	23.7 ± 1.5
Phylloquinone (n = 27)	1.06 ± 0.19	6.3 ± 0.4	20.7 ± 1.9	11.1 ± 1.0	22.8 ± 2.1
Control (n = 26)	1.00 ± 0.22	6.3 ± 0.4	23.8 ± 1.6	13.5 ± 1.2	24.6 ± 2.1
Old women (n = 56)	1.16 ± 0.12[3]	7.4 ± 0.3[2]	23.1 ± 1.7[2]	13.4 ± 1.0[2]	23.2 ± 1.3
Phylloquinone (n = 29)	1.26 ± 0.20	7.6 ± 0.5	24.1 ± 2.6	15.0 ± 1.5	21.7 ± 1.8
Control (n = 27)	1.06 ± 0.12	7.2 ± 0.5	22.1 ± 2.1	11.4 ± 0.9	25.2 ± 1.9

[1] $\bar{x}$ ± SEM. ucOC, undercarboxylated osteocalcin; NTx, *N*-telopeptides of type I collagen; BSAP, bone-specific alkaline phosphatase; BCE, bone collagen equivalents.
[2] Significantly different from young men, $P < 0.05$.
[3] Significantly different from young men and young women, $P < 0.05$.

Baseline comparisons

Mean serum phylloquinone values ranged from 0.68 to 1.16 nmol/L and were lower in young individuals of both sexes (**Table 2**). These baseline values correlated negatively with %ucOC and were unrelated to total osteocalcin concentration (**Figure 1**). Individual serum %ucOC values ranged from 2.2% to 15.3%; group means were 6.3–9.0%, being highest in young and lowest in old men ($P < 0.05$); no significant age difference was found in women. Serum osteocalcin and NTx concentrations were highest in young men ($P < 0.05$); no other significant age or sex differences were observed. Within age and sex categories at baseline, no significant differences in %ucOC, osteocalcin, phylloquinone, NTx, or BSAP were present between the phylloquinone-treated and control groups (Table 2). PT and all but 2 PIVKA-II values were within normal ranges and not significantly different by age or sex (data not shown).

Phylloquinone supplementation effects

Supplementation led to an ≈10-fold increase ($P < 0.001$) in serum phylloquinone by week 1, and this effect persisted through week 2. The increase was greater in the old than in the young subjects ($P < 0.001$; **Figure 2**), with no observed sex difference (data not shown). In the supplemented group, %ucOC decreased to ≈3% after 1 wk ($P < 0.001$); this effect was sustained through week 2, with no significant age (**Figure 3**) or sex differences (data not shown). Furthermore, %ucOC was reduced by more than one percentage point in 102 of 112 individuals who received phylloquinone for 1 wk (data not shown).

Serum osteocalcin decreased ($P < 0.001$) by the end of week 1; this effect was maintained at the end of week 2. No significant age or sex difference was observed (**Figure 4**; age and sex data not shown). To further assess skeletal turnover, NTx and BSAP concentrations were measured and showed no change with supplementation (data not shown). Additionally, when PT and PIVKA-II values were evaluated to determine the effect of phylloquinone on coagulation parameters, no change was observed (data not shown).

DISCUSSION

In this study, supplementation promptly increased serum phylloquinone concentration and reduced %ucOC in almost all supplemented individuals. This occurred in healthy subjects who had normal coagulation variables and whose baseline phylloquinone concentrations were similar to those reported previously in healthy adults (1, 2, 23), showing that usual dietary practices

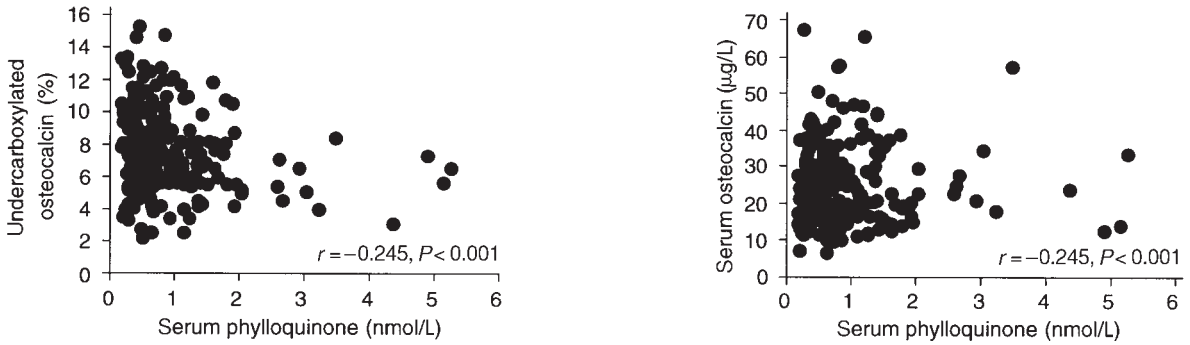

FIGURE 1. Baseline serum phylloquinone concentrations. At baseline, serum phylloquinone concentration (n = 219) was negatively correlated with the percentage undercarboxylated osteocalcin (%ucOC). Serum phylloquinone and total osteocalcin concentrations were not significantly correlated.

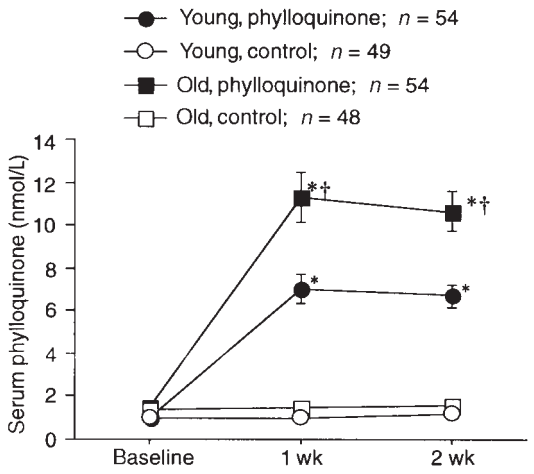

FIGURE 2. Effect of supplementation on serum phylloquinone concentration. Serum phylloquinone increased significantly in all supplemented groups compared with their respective control groups ($P < 0.0001$). The rise in serum phylloquinone concentration was significantly greater in the older group and did not differ by sex ($P < 0.0001$). $\bar{x} \pm$ SEM. *Significantly different from respective control group, $P < 0.0001$. †Significantly different from the young, phylloquinone-supplemented group, $P < 0.0001$.

provide inadequate phylloquinone to allow maximal osteocalcin γ-carboxylation. Less than maximal γ-carboxylation of vitamin K–dependent proteins has been suggested to be a more sensitive definition of vitamin K deficiency than are coagulation measures (2, 24–26). However, whether the high prevalence of vitamin K insufficiency observed in this study has physiologic relevance is unclear.

In contrast with some prior reports (4, 5), %ucOC was not highest in postmenopausal women. In fact, %ucOC was comparably elevated in young women and highest in young men. This may reflect low vitamin K intake in the young group, which comprised primarily college students. If maximal osteocalcin γ-carboxylation is important for peak bone mass accrual, the vitamin K insufficiency observed in young men and women might be of physiologic importance.

Accumulating evidence suggests that vitamin K insufficiency contributes to the development of osteoporosis (27). However, much of this evidence is based on submaximal osteocalcin γ-carboxylation, ie, elevated ucOC was associated with low bone mass (7) and increased risk of osteoporotic fracture (9, 10). Additionally, individuals with vitamin K intakes in the lowest quintile were observed recently to be at increased risk of hip fracture (28). Although these findings implicate vitamin K in skeletal health, the observed associations do not establish that vitamin K insufficiency causes osteoporosis (29). Furthermore, a mechanism by which impaired osteocalcin γ-carboxylation could contribute to bone loss has not been defined. In this regard, the results of our study suggest that submaximal γ-carboxylation might lead to a state of high skeletal turnover because phylloquinone supplementation reduced serum osteocalcin, an accepted marker of bone formation (30). This observation is to some extent congruent with the recent finding that 15 d of dietary vitamin K depletion led to increased bone turnover as measured by serum osteocalcin and urinary NTx concentration; these markers were subsequently normalized by 10 d of phylloquinone repletion (≈200 μg/d) (31). On the basis of these observations, we speculated (32) that vitamin K insufficiency impairs the function of the calcium homeostatic system (33, 34), thereby requiring increased skeletal turnover. Because elevated skeletal turnover is associated with rapid bone loss (35), if vitamin K insufficiency accelerates bone turnover, then vitamin K insufficiency would be anticipated to contribute to the development of osteoporosis.

This speculation requires further study because other studies showed no change (2) or an increase in serum markers of bone turnover after phylloquinone supplementation (6, 36). Furthermore, anticoagulant-induced vitamin K insufficiency has been associated with unchanged (11) or decreased (14, 19, 37) serum osteocalcin concentration. Additionally, our study showed no effect of vitamin K supplementation on other biochemical measures of bone turnover (NTx and BSAP) despite the study's power to detect changes of 5% and 9%, respectively. This may suggest that osteocalcin is the first biochemical marker of bone turnover affected by phylloquinone supplementation. Had the duration of supplementation been extended, perhaps a change in NTx and BSAP would have been observed. Alternatively, one may speculate that the antibody used in our osteocalcin assay might have a lower affinity for carboxylated osteocalcin, leading to a reduction in measured serum osteocalcin. Thus, the effect, if any, of phylloquinone supplementation on bone turnover remains to be clarified. Phylloquinone supplementation studies using longer observation periods and markers of bone turnover that are not γ-carboxylated (eg, NTx and BSAP) should clarify this issue. Given the high prevalence of both osteoporosis and submaximal γ-carboxylation of osteocalcin, elucidation of the effect of phylloquinone on bone turnover is required.

Note that the phylloquinone supplement dose (1000 μg/d) chosen was empirical and based on previous reports. Because pharmaceutical supplements provide greater vitamin K bioavailability than does food, achievement of a comparable intake from the diet would require consumption of ≈2000–5000 μg vitamin K/d (2, 38).

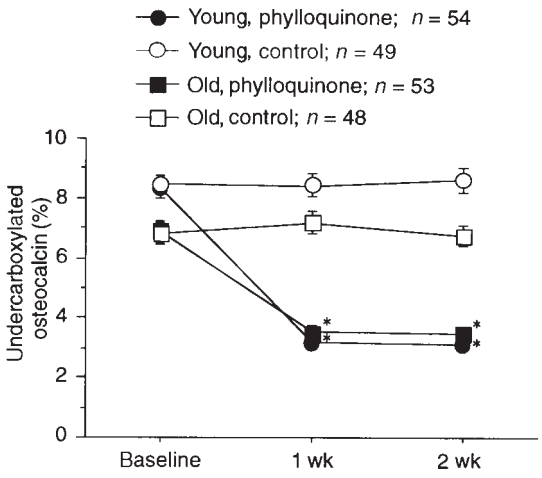

FIGURE 3. Effect of phylloquinone supplementation on serum undercarboxylated osteocalcin (ucOC) compared with a placebo control in young and old subjects. Supplementation significantly decreased %ucOC to ≈3% ($P < 0.0001$); this effect did not differ by age or sex. $\bar{x} \pm$ SEM. *Significantly different from respective control group, $P < 0.0001$.

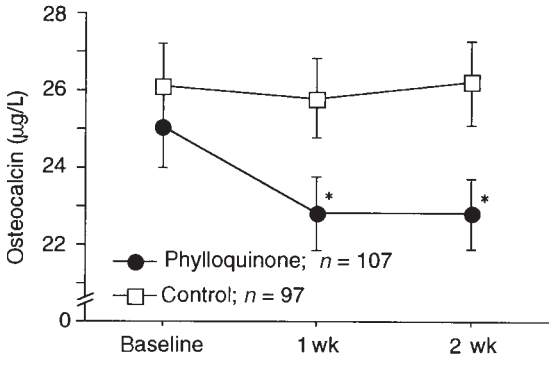

FIGURE 4. Effect of phylloquinone supplementation on serum osteocalcin. Supplementation significantly decreased total osteocalcin ≈9% (from 25.0 ± 1.1 μg/L at baseline to 22.8 ± 1.0 μg/L at 1 wk; $P < 0.005$). No change was observed in the control group. $\bar{x}$ ± SEM. *Significantly different from respective control, $P < 0.005$.

This intake is markedly higher than current recommendations (39), which were based on coagulation parameter measurements and would be essentially impossible to obtain from food alone. Although the phylloquinone dose required to maximize osteocalcin γ-carboxylation is unknown, it appears likely that dietary supplementation will be required if elevated ucOC is determined to produce adverse skeletal effects because many individuals do not consume even the currently recommended amounts (23).

Older individuals were observed to have higher serum phylloquinone concentrations both at baseline and after supplementation than did younger individuals. Because triacylglycerol-rich lipoproteins are carriers of phylloquinone (40), plasma concentrations of phylloquinone and triacylglycerol are positively correlated (41), and triacylglycerol concentration increases with advancing age (42, 43), it is probable that the phylloquinone concentrations reflect higher triacylglycerol with advancing age. Serum triacylglycerol concentrations were not measured in this study.

In conclusion, usual dietary vitamin K intake of both young and old individuals is inadequate to allow maximal osteocalcin γ-carboxylation. Thus, if submaximal γ-carboxylation is used to define vitamin K status, insufficiency is widespread. Determination of the physiologic relevance of this observation is essential before widespread phylloquinone supplementation is recommended.

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