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ORIGINAL CONTRIBUTION

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Six weeks phylloquinone supplementation produces undesirable effects on blood lipids with no changes in inflammatory and fibrinolytic markers in postmenopausal women

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Abstract *Background* Cardiovascular disease is the major cause of death in the Western world, but some recent studies indicate that vitamin K may play a role in atherosclerosis protection. *Aim of study* The aim of this study was to evaluate the effect of phylloquinone supplementation on blood lipids, inflammatory markers and fibrinolytic activity in postmenopausal women. *Methods* Thirty-one postmenopausal women completed this placebo-controlled, randomized crossover study and received 500 μg phylloquinone or placebo in addition to their habitual diet during two periods of 6 weeks' duration. Blood concentration of lipids, inflammatory markers and fibrinolytic parameters were measured after each period. *Results* Inflammatory markers, fibrinolytic parameters, total cholesterol and LDL-C were unaffected by the supplementation, whereas a 15% increase was seen in triacylglycerols ($P = 0.015$) and a 5% decrease in HDL-C ($P = 0.06$). *Conclusions* Six weeks supplementation with a dose of phylloquinone similar to that obtainable from the diet induced a deterioration of the lipid profile with no improvement in any of the other risk markers analysed. Thus, these results do not support a cardioprotective effect of vitamin K as has been suggested by others.

Key words vitamin K – lipids – inflammation – cardiovascular disease – fibrinolytic activity

Introduction

Cardiovascular disease (CVD) is the leading cause of death throughout the Western world and atherosclerosis is recognized as the main cause of CVD. Recent research has led to a better understanding of the role of inflammation in atherogenesis. Increased cholesterol concentration, in particular LDL-cholesterol (LDL-C), is a traditional risk factor, but inflammatory markers have emerged, the most prominent being high sensitivity C-reactive protein (CRP). Interleukin-6 (IL-6) induces CRP synthesis, thus the combined measure of IL-6 and CRP may provide a better indication of inflammation, and both elevated IL-6 and CRP has been associated with increased mortality and risk of CVD [7].

Vitamin K is a cofactor in the post-translational γ-carboxylation of glutamate (Glu) residues of vitamin K-dependent proteins. The major naturally occurring forms of vitamin K are phylloquinone and the menaquinones varying in side-chain length. This vitamin is well known for its role in the synthesis of a number of coagulation factors, but in recent years, a possible role in cardioprotection has been suggested. In one study, supplementation with phylloquinone for 3 years improved the elastic properties of the arterial wall in postmenopausal women [2], and anti-athero-

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sclerotic effects of pharmacological doses of menaquinone-4 has been demonstrated in rats with experimental atherosclerosis [15].

Two prospective studies have investigated the association between vitamin K intake and aortic and coronary calcification, mortality and CVD incidence [6], where menaquinone but not phylloquinone intake was found to be inversely associated to incidence of CVD. However, plasma phylloquinone concentrations and phylloquinone intake was found to be inversely associated with concentrations of inflammatory markers in the Framingham Offspring cohort [16].

Here, we investigate the potential cardio-protective properties of phylloquinone on cardiovascular risk markers in a study, which was originally designed to study the effect of phylloquinone on bone metabolism in postmenopausal women.

Subjects and methods

Study design and subjects

As previously described [3], 31 apparently healthy postmenopausal women (>5 years postmenopausal) completed this study for which a total of 48 women were recruited. The 17 that dropped out did so for personal reasons not related to the study. It was originally designed as a double-blinded study arranged in a randomized crossover design consisting of three six-week experimental periods each separated by wash out periods of 3 weeks duration, in which the subjects consumed either a placebo tablet or a tablet containing 200 or 500 μg of phylloquinone (kindly provided by F. Hoffman La-Roche Ltd, Switzerland). In addition, all subjects received a daily vitamin D_3 supplement of 10 μg throughout the whole study period as well as 2 months prior to the study. Otherwise the subjects consumed their habitual diet. The research protocol was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg (KF no. 01-104/00), and written consent was obtained from each subject after they received both written and oral information about the study.

Blood samples obtained from the periods where the subjects received placebo and 500 μg phylloquinone were analysed for the purpose of this investigation. Serum and plasma samples were obtained from blood drawn under fasting conditions at baseline and on the last day of the six-week periods. Subjects were asked to fast for 12 h, and to avoid severe physical activity and any kind of drugs for 48 h, and alcohol consumption 24 h before blood sampling. Inter-assay variation was avoided by analyzing all samples in the same run.

Experimental procedures

High sensitivity CRP was measured in serum by a solid-phase chemiluminescent immunometric assay using an IMMULITE® 1000 Automated Immunuassay Analyzer (Diagnostic Products Corporation, Los Angeles, USA), with an intra-assay precision of 3.4%. IL-6 was measured by sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Abingdon, UK); intra-assay variation was 5.5%. Plasma concentrations of soluble intracellular and vascular adhesion molecules 1 (sICAM-1and sVCAM-1) were determined by quantitative sandwich ELISA kits (R&D Systems Inc., Abingdon, UK). Intra-assay precisions were 6.7 and 13.9%, respectively.

Serum TG and total cholesterol (TC) were measured using enzymatic colorimetric test kits (Roche CHOL and TG, Roche Diagnostics GmbH, Mannheim, Germany). The intra-assay precisions were 0.6 and 0.9%, respectively. HDL-cholesterol (HDL-C) and LDL-C were measured in serum using a homogeneous enzymatic colorimetric test kits (Roche HDL-C plus 2nd generation and LDL-C plus 2nd generation, Roche Diagnostics GmbH, Mannheim, Germany). Both intra-assay precisions were 1.8%. All lipid analyses were performed on a COBAS MIRA Plus (Roche Diagnostic Systems Inc., Mannheim, Germany).

Plasminogen activator inhibitor type I (PAI-1) was determined by ELISA and intra-assay variation was 6% (TintElize PAI-1; Biopool, Umeå, Sweden). Percentage of plasma factor VIIc was assessed using a one-step clotting assay, in which clotting time is recorded on a coagulometer and expressed relative to an internal standard (Schnittger-gross, Amelung, Germany) after incubation with human fVII-deficient plasma (Biopool, Umeå, Sweden), and initiation of the clotting process by addition of CaCl and human thromboplastin; intra-assay precision was measured to be 5%. Plasma concentration of clottable fibrinogen (fibrinogen-c) was determined by a modified Clauss assay; intra-assay variation was 2.3%.

Statistical analysis

All statistical analyses were performed using the Statistical Analysis System software package, version 9.1 (SAS Institute inc., Cary, NC, USA). Biochemical markers were evaluated as the dependent variable in a mixed linear model analysis (proc mixed). Denoting the *i*'th observation (1, 2…, n), the effect of treatment (placebo or phylloquinone) and subject was modeled as a class variable. BMI, age and baseline plasma phylloquinone concentration were included in the model as covariates as well as the interactions be-

Table 1 Baseline characteristics of the 31 postmenopausal women given as mean ± SD

Variable	Mean ± SD
Age (years)	62.5 ± 4.0
Height (m)	1.65 ± 0.06
Weight (kg)	71.3 ± 9.7
BMI (kg/m^2)	26.1 ± 3.0

tween treatment and all covariates when $P < 0.10$. Homogeneity of variance and normal distribution among random effects were investigated by plots of residuals, which showed it necessary to log-transform CRP, sVCAM-1 and sICAM-1, which were back-transformed before presentation. Data are presented as means and SD or means and SEM where appropriate and effects are considered significant when $P < 0.05$.

Results

The characteristics of the 31 postmenopausal women are presented in Table 1; age ranged from 53 to 69 years, and BMI from 20.2 to 31.4 kg/m^2. Compared to placebo, daily supplementation with 500 μg of phylloquinone did not affect inflammatory markers (CRP, IL-6, sVCAM-1 and sICAM-1), fibrinolytic activity (factor VIIc, fibrinogen-c and PAI-1), TC or LDL-C (Table 2). The supplementation did however result in a 15% increase in TG ($P = 0.015$) and a near-significant 5% decrease in HDL-C ($P = 0.06$). As could be expected, the supplementation resulted in a significant dose-dependent increase in plasma phylloquinone and the degree of carboxylation of osteocalcin, which is often used as a measure of vitamin K status [3].

Because high levels of CRP are indicative of an ongoing infectious or inflammatory disease, a cut-off value of 10 mg/l based on two measurements made two weeks apart is normally used when predicting CVD risk [19]. Although only one measurement of CRP was made here, we decided also to apply this cut-off value. This resulted in the exclusion of three subjects, for whom blood levels of CRP were >10 mg/l, all three measured after the placebo treatment (13, 14 and 17 mg/l, respectively). The difference in CRP concentration between placebo and phylloquinone was −32% ($P < 0.001$) when all subjects were included in the statistical analysis, but only −2% and non-significant when the cut-off value was applied.

Discussion

This study showed that supplementation with a low dose of phylloquinone induced a 15% increase in TG ($P = 0.015$) with a concomitant 5% decrease in HDL-C ($P = 0.06$). Phylloquinone supplementation did not result in an over-stimulation of the coagulation system, as neither fVIIc, fibrinogen-c or PAI-1 were affected, so the supplementation can be considered safe in this respect and also, no effect on a range of inflammatory markers was observed.

Hypertriglyceridemia is a risk factor for CVD independent of both LDL- and HDL-C [8], but the mechanisms by which TG is related to atherosclerosis is not yet fully understood. The mechanism respon-

Table 2 Biochemical parameters after 6 weeks of daily placebo or 500 μg phylloquinone supplementation in postmenopausal women presented as means (SEM) ($n = 31$)

Variable	Placebo	Phylloquinone	Difference (%)	*P*-value*
Phylloquinone (nmol/l)	2.42 (0.40)	4.70 (0.40)	2.28 (94%)	<0.001
Blood lipids				
TG (mmol/l)	1.00 (0.08)	1.15 (0.08)	0.15 (15%)	0.015
TC (mmol/l)	6.06 (0.16)	6.10 (0.16)	0.04 (0.7%)	0.75
HDL-C (mmol/l)	1.50 (0.07)	1.42 (0.07)	−0.08 (−5%)	0.060
LDL-C (mmol/l)	4.48 (0.17)	4.46 (0.17)	−0.02 (−0.4%)	0.83
Inflammatory markers				
CRP (mg/l)				
All CRP ($n = 31$)	3.21 (0.56)	2.17 (0.56)	−2.04 (−32%)	<0.01
CRP ≤ 10 mg/l$^{\#}$ ($n = 28$)	2.16 (0.28)	2.15 (0.28)	−0.01 (−2%)	0.35
IL-6 (ng/l)	1.99 (0.32)	1.63 (0.32)	−0.36 (−18%)	0.18
sICAM-1 (μg/l)	213.9 (8.3)	207.7 (8.3)	−6.2 (−3%)	0.56
sVCAM-1 (μg/l)	503.1 (41.0)	477.3 (41.0)	−25.8 (−5%)	0.67
Coagulation factors				
Factor VIIc (U/l)	1.13 (0.03)	1.13 (0.03)	–	0.80
Fibrinogen-c (g/l)	3.48 (0.10)	3.46 (0.10)	–	0.83
PAI-1 (μg/l)	15.87 (1.37)	16.15 (1.37)	0.28 (1.8%)	0.78

*Level of significance for treatment effect
$^{\#}n = 28$ due to application of a cut-off value for CRP of 10 mg/l

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sible for the increase in TG seen here is unknown, but may possibly be related to the fact that phylloquinone is transported in TG-rich fractions. However, to the authors' knowledge, no previous records of an increase in TG due to increased intake of phylloquinone exist. HDL-C has several potentially anti-atherogenic properties, including a role in reverse cholesterol transport, but the decline in HDL-C observed was small, and it is not likely that a change of this magnitude is of clinical significance. Importantly, no change was observed in LDL-C, which is one of the major risk factors for CVD.

In contrast to our findings, a cholesterol-lowering effect of vitamin K has been observed in animals and humans with a different form of vitamin K [10]. A decrease in TC was obtained by a daily pharmacological dose (45 mg) of menaquinone-4 in 17 dialysis patients [11]. However, the 0.25 mmol/l decrease was not observed until the seventh month of the study; HDL-C and TG concentrations were unchanged throughout the study, and no control group was included in the study. TC-lowering following menaquinone-4 administration has also been seen in hypercholesterolemic rabbits [10], but again HDL-C and TG were unaffected. The authors suggested that the side chain of menaquinone-4 might competitively inhibit cholesterol synthesis as a potential mechanism, and if valid, a lowering of TC would not be anticipated following phylloquinone supplementation.

An 18% decrease in IL-6 from 1.99 to 1.63 pg/ml following phylloquinone supplementation compared to placebo treatment was observed but did not reach statistical significance due to large variations in IL-6 ($P = 0.18$). An effect of this magnitude may however be of clinical significance, in that similar differences were seen in a nested case-control study with postmenopausal women [13] and a prospective male cohort [14]. In the present study, none of the other inflammatory markers showed similar trends pointing towards anti-inflammatory properties of phylloquinone. Even though a significant lower mean CRP concentration was seen after the phylloquinone period compared to placebo when the all subjects were included in the analysis, but this difference was absent when the cut-off of 10 mg/l was applied. It should be noted that all three elevated measures of CRP were observed after the placebo period. The present findings is thus not in concordance with previously published results, where one in vitro study [12] and one rat study [4] report anti-inflammatory effects of various vitamin K forms, and an association between high vitamin K status and lower concentrations of a range of inflammatory markers was observed in the Framingham Off Spring cohort [16].

There appear to be functional differences between phylloquinone and the menaquinones. In addition to the differences between the different vitamin K forms observed in the prospective studies [6, 16, 18], menaquinone-4, but not phylloquinone was capable of preventing arterial calcification in warfarin-treated rats [17], emphasizing the potentially important differences in the tissue-specific utilization of the different vitamin K forms. It has been proposed that these effects are related to an increased γ-carboxylation of matrix Gla protein thought to play a role in the inhibition of vascular calcification [2], but other functional characteristics of the different vitamin K forms, including redox properties and specific functions of the side chains, have been proposed [17].

The existing evidence concerning vitamin Ks role in cardiovascular disease is limited, but all previously published works indicate either a beneficial or no effect on cardiovascular risk markers. Also, the vast majority of the published work is observational data, where a high intake of phylloquinone may be an indicator of an overall healthier life style pattern as phylloquinone is mainly found in green vegetables. The present study is the first to indicate potential harmful effects on cardiovascular risk markers, and thus it is important to repeat the findings in larger, long-term studies also using higher doses. It emphasizes the importance of measuring blood lipids in future intervention studies investigating potential beneficial effects of supplemental phylloquinone on cardiovascular risk markers.

The work presented here has some limitations. The study was originally designed to evaluate the effect of phylloquinone on bone markers, thus it was underpowered to detect small differences in some of the measurements reported here. Also, no records of the subjects' habitual phylloquinone intake exist, as the food frequency questionnaire used in the study did not contain sufficient information to estimate this. However, we expect their daily phylloquinone intake to be similar to that estimated in a similar population of Danish women, which was 189 ± 182 µg/d based on a 2 × 3 days weighed food recording from another study also conducted in Danish postmenopausal women ($n = 28$) (ISOHEART) [Bügel, unpublished results]. This is similar to the phylloquinone intake among postmenopausal women reported elsewhere [1]. The design does not allow for control of similar baseline values at the beginning of each period, and finally, the number of drop outs during the intervention was considerable.

In conclusion, this study showed that supplementation with a low dose of phylloquinone for 6 weeks does not affect the inflammatory response, fibrinolytic activity, but adversely affects the lipid profile of apparently healthy postmenopausal women. Thus, a

beneficial role of vitamin K, which has been suggested by others, is not supported by the work presented here. It is possible that a larger dose than the one used in this study is required, a longer period of supplementation and perhaps the use of a different form of the vitamin.

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