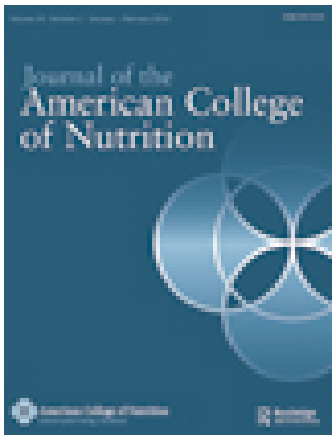

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Effects of Vitamin K on Matrix Metalloproteinase-3 and Rheumatoid Factor in Women with Rheumatoid Arthritis: A Randomized, Double-Blind, Placebo-Controlled Trial

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Original Research

Effects of Vitamin K on Matrix Metalloproteinase-3 and Rheumatoid Factor in Women with Rheumatoid Arthritis: A Randomized, Double-Blind, Placebo-Controlled Trial

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Key words: vitamin K, phylloquinone, matrix metalloproteinase-3, rheumatoid factor, rheumatoid arthritis

Objectives: Rheumatoid arthritis (RA) is an autoimmune disease characterized by an increase in some autoantibodies and proteolytic enzymes, leading to joint destruction. Although recent investigations have considered vitamin K as an anti-inflammatory nutrient with an important role in bone metabolism, there is currently limited information on its efficacy in RA. We aimed to examine the effects of vitamin K_1 (phylloquinone) on the biomarker of joint destruction and autoantibody in patients with RA.

Materials and Methods: This was a randomized clinical trial in which 64 women with RA who fulfilled the eligibility criteria were randomly allocated to an intervention or a control group. Vitamin K_1 or placebo was administered to the participants for 8 weeks. Baseline characteristics and anthropometric measures were obtained. Clinical status using disease activity score in 28 joints (DAS-28), serum levels of matrix metalloproteinase-3 (MMP-3), and rheumatoid factor (RF) were assessed before and after the intervention.

Results: The serum level of MMP-3 compared with the baseline values did not change significantly in the groups. However, the serum concentration of RF decreased significantly in the vitamin K_1 group ($p = 0.041$). Intergroup comparison showed no significant change in RF serum level after adjusting for relevant confounders ($p > 0.05$).

Conclusions: Vitamin K_1 supplementation at 10 mg/day for 8 weeks did not alter joint destruction and immune status in the patients with RA compared with the controls.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterized by cartilage and bone destruction [1,2]. Inflammation processes in RA are triggered by antigenic insults mainly involved in the pathogenesis of the disease [3]. First, the balance between anti- and pro-inflammatory chemokines in favor of pro-inflammatory mediators, such as tumor necrosis factor (TNF-α) and interleukin (IL-1), is impaired [3,4]. Next, an inflammatory cascade up-regulates the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$_K$B), which is a key agent in exacerbating inflammation and apoptosis inhibition [5,6]. In this condition, expression of some proteolytic enzymes, i.e., matrix metalloproteinases (MMPs) including MMP-3, increases and the expression of tissue inhibitors of MMPs (TIMPs) decreases and leads to cartilage destruction and extracellular matrix degeneration [2,5,7]. In addition, RA is typically associated with serological evidence of systemic autoimmunity, as indicated by the presence of serum and synovial fluid autoantibodies such as rheumatoid factor (RF)

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[8,9]. Regarding RA pathology, it appears that treatment with anti-inflammatory agents may have high clinical efficacy and delay joint destruction [1].

The vitamin K family which has been recently considered as an anti-inflammatory nutrient [10–13], mainly includes three vitamins: vitamin K_1 (phylloquinone), found in green leafy vegetables; vitamin K_2 (menaquinone), produced by intestinal bacteria; and vitamin K_3 (menadion), a synthetic form of vitamin K [10,14]. All these vitamins contain a functional naphthoquinone ring.

There are a number of studies showing that vitamin K inhibits the expression of $NF_{-K}B$ and consequently suppresses inflammation [15]. In addition to its anti-inflammatory role, vitamin K is necessary for gamma-carboxylation of some proteins that are involved in coagulation and bone metabolism [16,17]. Overall, it is postulated that women with RA may benefit from an adjuvant therapy using vitamin K [14,18].

Recent developments in the study of the anti-inflammatory action of vitamin K have led to a renewed interest in investigating the effects of vitamin K on other mediators such as MMPs. However, to date, very little attention has been given to vitamin K. In addition, most of these experiments were conducted on cell lines or animal models. Thus, owing to limited information in this field, the present paper is designed to focus on the effects of phylloquinone supplementation (10 mg/day) for 8 weeks on serum levels of MMP-3 and RF.

MATERIALS AND METHODS

Subjects

A randomized double-blind placebo-controlled clinical trial was conducted on 64 women with RA who were recruited from the outpatient rheumatology clinic of Imam Reza Hospital, Tabriz, Iran. The inclusion criteria for the trial were as follows: age range of 20 to 50 years and documentation of RA diagnosed by a rheumatology specialist according to the American College of Rheumatology (ACR, 2010) classification for RA [6].

The exclusion criteria included smoking; pregnancy and lactation; being on hormonal therapy; receiving contraceptive pills, antibiotics, coumarins, or antiepileptic drugs; postmenopausal status; being in a severe stage of RA (28-joint disease activity score (DAS28) $>$ 5.1); using antioxidants, anti-inflammatory, or other dietary supplements during the past 3 months; history of chronic diseases, e.g., diabetes, liver and kidney disorders, malabsorption and cardiovascular diseases, thyroid disorders, coagulation disorders, hyperprolactinemia, Cushing syndrome, superobesity (body mass index (BMI) $>$ 40), cancer, or high blood pressure; changing treatment protocol and lifestyle during the study; and unwillingness to continue the study.

This trial was conducted in accordance with the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Medical Ethics Committee of Tabriz University of Medical Sciences and registered in the Iranian Registry of Clinical Trials (http://www.irct.ir) under the number IRCT201205203140N4.

Study Design

Sample size (with power = 95%, $\alpha = 0.05$), was calculated on the basis of the results (mean $\pm$ standard deviation (SD)) for C-reactive protein (CRP) as reported in a similar study [19]. This was found to be 25 patients; considering a 30% loss rate, 32 subjects were assigned to each group.

After explanation about the study purpose and protocol, all of the eligible subjects were asked to complete a consent form. Then, the participants were interviewed for demographic characteristics including age, disease history, education, marital status, medication, etc. Patients were advised not to change aspects of their usual lifestyle, such as dietary intake, physical activity, and medication, during the intervention period.

Weight and height were measured in the standard position before and after the trial, and BMI was calculated as weight in Kg divided by squared meters of height. Dietary intakes also were assayed using a 3-day dietary record before and after this period. After entering grams of all food and beverages they consumed during 2 weekdays and 1 weekend, data were analyzed using Nutritionist IV software (First Databank, San Bruno, CA) modified for Iranian foods.

DAS28 was calculated on the basis of the tender and swollen joint count and the serum levels of CRP at baseline and after the intervention. The following formula was applied [20]:

$$\begin{aligned}\text{DAS28} = {} & 0.56\ (\text{tender joint count})^2 \\ & + 0.28\ (\text{swollen joint count})^2 \\ & + 0.36^{*}\ \text{Ln}\ (\text{CRP} + 1)\ ^{*}\ 1.10 + 1.15\end{aligned}$$

During the trial, patients were monitored by weekly phone calls for any possible adverse events.

Finally, subjects were randomly allocated to two groups (with allocation ratio 1:1) matched for age and disease severity using 4-factor randomized block design (RBD in sequentially numbered containers). The participants in each group were supposed to take 1 pill daily after lunch. The vitamin K_1 used for this intervention was a chewable pill that contained 10 mg phylloquinone (manufactured by Minoo Company, Iran). A placebo was especially designed for this study, which was exactly similar to vitamin K_1 in appearance (prepared by the Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran).

Given the various dosages of vitamin K (from microgram to milligram) used in other clinical trials, it appears that an optimal dose of vitamin K has not yet been defined. In addition to different high doses of vitamin K used in past studies [21–23], safety issues relating to those [24], and given that the only

available dosage in Iran was the 10 mg pill, we decided to use 10 mg/day pills of vitamin K_1.

Blood Sampling and Biochemical Assays

Seven ml of blood was taken from the forearm vein after an overnight fast (8–12 h) at the beginning and at the end of study. After centrifugation for 15 minutes at 3000 rpm, serum samples were stored at −70°C until biochemical analysis. Serum concentration of MMP-3 was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit (eBioscience Inc., Vienna, Austria) according to the manufacturer's instructions and the outputs expressed as nanograms per milliliter (ng/ml). In addition, serum concentration of RF was measured by immunoturbidimetric assay using Pars Azmun kit (Pars Azmun., Karaj, Iran) that was expressed as international units per milliliter (IU/ml). In order to screen patients with coagulation abnormalities and to assess vitamin K status, the prothrombin time test (PTT) was conducted on the day of sampling and the results were reported in seconds. The laboratory reference range value was considered 10–13.5 seconds.

Statistical Analysis

Statistical analyses were performed using SPSS (version 11.5) for Windows (SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to determine normality of data distribution and the data were expressed as mean ± standard deviation (SD) for normally distributed data, median ± interquartile range for non-normally distributed data, and frequency (percentage) for qualitative data. The Mann-Whitney U Test and independent sample *t*-tests were used to compare mean values between groups at baseline. Mean values before and after the study period were compared within groups using the paired *t*-test or Wilcoxon test. In order to compare mean values between groups after the intervention adjusting for basic measurements, the analysis of covariance (ANCOVA) test was used. In all analyses, *p*-values less than 0.05 were considered statistically significant.

RESULTS

Baseline Characteristics

Of 64 patients assigned to the trial, 58 participants completed the study [phylloquinone group ($n = 30$) versus placebo group ($n = 28$)]. Overall 6.25% of the vitamin K group and 12.5% of the placebo group that dropped out did so for personal reasons not related to the study, such as having surgery or medication changes during the 8-week intervention period. However, one subject refused to consume the vitamin K pills because of slight heartburn experienced at the beginning of the trial. No other serious adverse effects of the treatment were reported (Fig. 1). Baseline patient characteristics including age, history of the disease, BMI, PTT, and medications are presented in Table 1.

Based on Student's *t*-test, age, height, weight, BMI, and prothrombin time had no statistically significant difference between the two groups before the intervention ($p > 0.05$).

Mann-Whitney test showed that there were no significant differences between the two groups at the beginning of the study regards to doses and types of drugs ($p > 0.05$). Also, drugs maintained as the same dosage over the course of this study ($p > 0.05$, data not shown). Statistically significant differences were observed regarding duration of the disease and folic acid supplementation between the two groups. Thus, these parameters were considered as covariates in analyses of efficacy outcomes.

In addition, the results of PTT were in normal range for all of the subjects which partially show the vitamin K sufficiency status. According to Table 2, dietary intakes including energy and some nutrients showed no significant inter- and intragroup changes using the Student's *t*-test and paired-*t* test, respectively ($p > 0.05$).

Biochemical Data

Table 3 indicates serum levels of MMP-3, RF in each group throughout the intervention. On the basis of the results from Student's t-test and Mann-Whitney U test, the baseline values of these markers were not statistically significant between groups ($p > 0.05$).

Inter- and intragroup comparisons of MMP-3 serum levels after 8 weeks of intervention using ANCOVA and paired *t*-test, respectively, revealed no significant differences ($p > 0.05$). However, a nonsignificant decrease in MMP-3 level following vitamin K supplementation (−4.62%) and a nonsignificant increase in placebo group (+2.88%) were observed. Meantime, in the vitamin K group, serum levels of RF showed a statistically significant reduction using Wilcoxon signed ranks (10.94%, $P = 0.041$), while in the placebo group, they remained unchanged.

Similarly, RF, DAS-28 also decreased significantly in the vitamin K group (12.56%, $P = 0.041$) with no significant change between the two groups (data not shown).

DISCUSSION

Our study showed a great reduction in RF serum levels in the vitamin K_1 group. However, non-significant changes were noted in the serum level of this marker between both intervention and placebo groups, after taking into account the duration of RA, energy intake, weight, folic acid supplementation, and baseline values as covariates. The serum concentration of MMP-3 had no significant change after 8 weeks of intervention within and between the two groups.

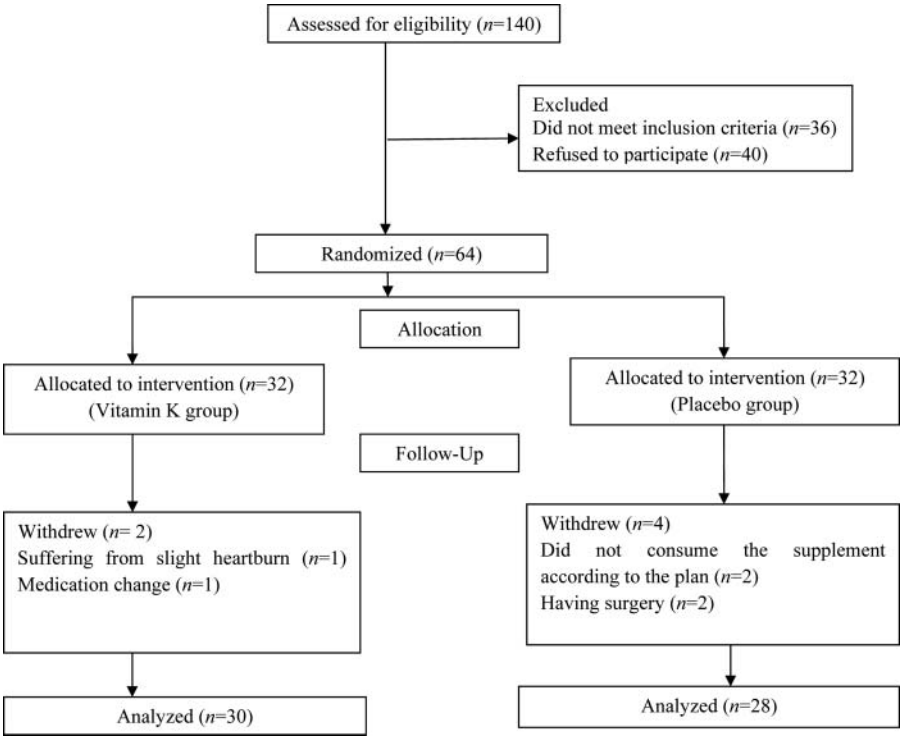

Fig. 1. Flow chart of the study.

The present study was mainly designed to examine the effects of vitamin K on inflammation, which significantly decreased in the vitamin K group. However, no significant change was observed in comparing the two groups. These results are consistent with findings regarding joint destruction markers and serum levels of auto-antibody examined in the present study.

Although it appears that every agent with anti-inflammatory effects may prevent joint destruction [25], the role of vitamin K on inflammatory cascades, and consequently on serum levels of proteolytic enzymes such as MMP-3, is subject to debate. Earlier experiments using cell lines or animals [11,15] and other investigation in humans [21] confirmed the protective function of vitamin K in controlling inflammation. On the other hand, several studies conducted in healthy subjects do not support this hypothesis [16,19]. Kristensen et al. [19] did not find any beneficial role of vitamin K on inflammation in

Table 1. Demographic Data in Women with RA at Baseline in Both Groups

Variables	Intervention group ($n = 30$)	Placebo group ($n = 28$)	P_{value}
Age (y)	37.97 ± 8.22	39.46 ± 8.22	0.491
Weight (Kg)	71.63 ± 13.79	70.39 ± 13.58	0.731
Height (cm)	156.05 ± 7.163	155.64 ± 7.09	0.829
BMI (Kg/m^2)	29.44 ± 5.27	29.26 ± 6.24	0.91
Duration of the disease (y) †	3 (1.5,8.5)	7 (4, 10.57)	**0.006***
Conventional therapy†			
Prednisolone	30 (100%)	27 (96.4%)	0.483
Methotrexate	26 (86.7%)	27 (96.4%)	0.354
Chloroquine	23 (76.7%)	19 (67.9%)	0.561
Sulfasalazine	3 (10%)	2 (7.1%)	1
Folic acid	25 (83.3%)	16 (57.1%)	**0.043***
Ca-vitamin D	22 (73.3%)	22 (78.6%)	0.762
Prothrombin time (second)	12.76 ± 0.67	12.89 ± 0.94	0.56

BMI: body mass index.

Data are presented as mean ± SD

†median (percentile 25, percentile 75) and ‡frequency (percent).

P_{value} for comparisons of baseline characteristics between groups.

*Significant differences between two groups at baseline ($P < 0.05$).

Table 2. Daily Dietary Intakes in Women with RA at Baseline and after 8 Weeks in Both Groups

Variables (daily)	Intervention group ($n = 30$)	Placebo group ($n = 28$)	P_{value}
Energy (kcal)			
Baseline	2062.7 ± 476.65	2135.36 ± 556.92	0.595[a]
After 8 weeks	2056.93 ± 609.71	2086.92 ± 569.92	0.848[b]
P[c]	0.956	0.563	
Carbohydrate (g)			
Baseline	288.88 ± 89.9	283.01 ± 82.67	0.808[a]
After 8 weeks	291.55 ± 107.64	276.03 ± 96.52	0.889[b]
P[c]	0.905	0.603	
Total fat (g)			
Baseline	77.8 ± 27.14	86.1 ± 31.85	0.289[a]
After 8 weeks	73.12 ± 36.81	85.56 ± 32.34	0.351[b]
P[c]	0.578	0.908	
Protein (g)			
Baseline	58.40 ± 16.38	64.2 ± 31.88	0.382[a]
After 8 weeks	57.16 ± 15.23	60.14 ± 17.33	0.417[b]
P[c]	0.682	0.436	

Data are presented as mean ± SD for normally distributed variables.

[a]P to compare values between-group differences at baseline.

[b]P to compare values between-group differences after 8 weeks.

[c]P to compare values within group.

postmenopausal women. There were high rates of drop-outs through the intervention period, and baseline measurements were not conducted [19]. This null finding in the study by Shea et al. was attributed to the healthy status of participants [16].

The only study conducted on the effects of vitamin K on serum metalloproteinase in patients with RA noted a significant reduction in serum levels of MMP-3 in the intervention group following vitamin K_2 supplementation, compared with the control group [21]. No other study has yet been conducted to test this hypothesis. Despite these divergent findings in prior studies, the function of vitamin K and its role in inflammation and joint and bone health has recently attracted more attention. Some mechanisms have been suggested in this regard. Vitamin K is involved in gamma-carboxylation of certain proteins such as osteocalcin, which is important in bone health. Vitamin K-dependent carboxylation of osteocalcin allows the binding to calcium and its influx to bone [14]. The role of vitamin K in bone and cartilage health is not confined to its role in carboxylation of proteins. It appears that vitamin K has some other potential functions through contributions in the inflammatory cascade.

NF-κB, a transcription factor mainly implicated in the control of certain cellular processes, such as inflammation, triggers inflammatory mediators and is further upregulated by these mediators through a vicious cycle in inflammatory diseases [26]. Vitamin K inhibits NF-κB activation and TNF-α production in micee [12]. Joint damage is one of the early symptoms of RA as a result of increased activity in the proteolytic system following inflammation. Among several enzymes involved in this process, metalloproteinases, especially MMP-3, play an important role in tissue and cartilage destruction [2]. MMP-3

Table 3. Biochemical Markers and Disease Activity Score-28 in Both Groups at Baseline and after 8 Weeks

Variables	Intervention group ($n = 30$)	Placebo group ($n = 28$)	P_{value}
MMP-3 (ng/ml)			
Baseline	34.93 ± 9.94	31.3 ± 11.23	0.198[a]
After 8 weeks	33.3 ± 11.71	32.2 ± 12.61	0.415[b]
P[c]	0.257	0.548	
RF (IU/ml)			
Baseline	19.0 (10.5,60.5)	47.0 (13,75)	0.215[a]
After 8 weeks	16.0 (9.0,57.5)	41.0 (13,70)	0.829[b]
P[c]	**0.041**[*]	0.229	

Data are presented as mean ± SD and median (percentiles 25, 75)[†].

[a]P_{value} to compare values between-group differences at baseline.

[b]P_{value} to compare values between-group differences after 8 weeks.

[c]P_{value} to compare values within group.

*Significant differences between two groups at baseline ($P < 0.05$).

causes degradation of the extracellular matrix and acts as a precursor of other metalloproteinases, including MMP-1 and MMP-9. Patients with RA and other connective tissue diseases such as lupus have a high serum level of MMP-3. Previous studies have shown a significant association between serum levels of MMP-3 and other inflammatory markers such as ESR, CRP, and IL-6 [7]. Given RA pathogenesis, the expression of metalloproteinase, which is partially regulated by signaling through NF-κB and AP-1 (activating protein-1) pathways, increases in response to inflammatory cytokines [27–29]. Thus, it is hypothesized that vitamin K may be a promising agent in control of joint damage in RA patients.

In the present study, these nonsignificant changes in serum levels of MMP-3 and RF between the two groups can be explained. First, the target population in the present study had the same hormonal status, physical activity, and daily dietary intake. This uniformity not only allows us to interpret the data by rolling out the confounders, but it can also result in such outcomes. The findings would be more reliable with having patients at a more severe stage of the disease, postmenopausal women, men, and subjects with vitamin K deficiency into account.

It is postulated that the trial, which involves a large population with different groups of subjects, may help to elucidate the role of vitamin K. These diverse findings may be explained by different doses and various forms of vitamin K used in other studies. Further studies considering all these limitations are required. In addition, measurement of plasma levels of vitamin K in subjects before and after the intervention would be helpful, not only in determining the patients' baseline nutritional status assessments, but also for the effects of vitamin K supplementation on its plasma levels. Due to financial limitation, instead of plasma levels of vitamin K measurement, PTT was conducted for all of the subjects at baseline. Furthermore, owing to an association between periodontal disease (PD) and RA severity [30–31], and given the high concentrations of oral bacteria that occurs in RA patients [32], it appears that patients with PD are more susceptible to developing RA through the release of inflammatory cytokines (IL-1b, TNF–α, and IL-6) in response to bacterial dental plaque [33]. Therefore, it would be more valuable to consider the effects of intervention on the growth of mouth bacteria in patients with RA.

Despite these limitations, the double-blind design of the study, high compliance with supplementation in both groups, and assessment of dietary intake at baseline and throughout the intervention period, were not considered in previous studies and are strengths of the present study.

CONCLUSION

Our study showed no beneficial effects of vitamin K on serum levels of MMP-3 and RF in patients with RA compared with the control group. More studies with a longer intervention period and that consider molecular and synovial assessments are needed to clarify the exact effects of vitamin K in different clinical forms of RA, including remission, mild, moderate, and severe spectrums.

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