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Does vitamin A supplementation affect GATA3 and IL-4 genes expression in TCD4+ cell culture? A double blind randomized clinical trial on MS patients

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A B S T R A C T

Objective: Multiple sclerosis (MS) is an inflammatory central nervous system disease. It has been shown that Th2 cells can induce anti-inflammatory properties that can have a role in treatment of inflammatory disease through overexpression of GATA3 and IL-4 genes. The aim of this study was to determine the effect of vitamin A supplementation on GATA3 and IL-4 genes expression in TCD4+ cell culture in MS patients.

Methods: This study was a double-blind placebo-controlled randomized clinical trial of a 6-month duration. Thirty-six MS patients were enrolled and randomly divided into a vitamin A group (n=19, receiving daily 25000IU vitamin A in the form of retinyl palmitate) and a placebo group (n=17). After the intervention the gene expression pattern of Th2-related cytokines was determined by real-time PCR.

Results: There was no significant difference in vitamin A intake, age or BMI of the participants at the baseline. Vitamin A supplementation significantly increased GATA3 and IL-4 gene expression in cell cultures treated with MOG ($P<0.001$ and $P=0.004$, respectively) and non-stimulated cells as compared with placebo group ($P<0.001$ and $P=0.001$, respectively).

Conclusion: Supplementation with vitamin A can be beneficial in slowing disease progression through overexpression of anti-inflammatory cytokines. It is recommended to investigate RXRs and RARs genes expression and their polymorphisms in future studies.

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Introduction

GATA-binding protein 3 (GATA3) is a transcription factor which is expressed in T cells and is necessary for Th2 differentiation [3]. IL-4 produced IL-4 by Th2 cells will affect GATA3 and induce its expression through the STAT6

transcription factor [4]. This process causes overexpression of GATA3 and leads to increased differentiation of CD4+ cells into Th2 cells [5]. Also IL-4 has a remarkable regulatory effect on the balance between Th1 and Th2 cells and inhibits interferon-γ production, which is a pro-inflammatory cytokine produced by Th1 cells [6]. On the other hand, the T-bet transcription factor helps greatly the differentiation of CD4+ cells into Th1 cells. Therefore, these transcription factors regulate cytokine production from Th1 and Th2 cells [7].

The role of vitamin A, and mostly retinoic acid (RA), in the improvement of the immune system has been shown [8]. RA plays a fundamental role in cell-mediated and humoral immunity and amplifies the Th2-related anti-inflammatory cytokines profile [9]. It has been shown that RA leads to an increase in Th2 cytokine profile by overexpression of GATA3 and suppression of the T-bet transcription factor. The metabolites of vitamin A such as 9-cis RA and all-trance retinoic acid (ATRA) are responsible for GATA3 expression [10]. Moreover, supplementation with ATRA decreases INF-γ and increases IL-4 production [11]. The aim of the present study was to determine the effects of vitamin A supplementation on GATA3 gene expression in cell cultures of MS patients stimulated by MOG and PAH compared with a non-stimulated group.

Subjects and methods

Patients and enrollment

This was a double-blind randomized placebo-controlled clinical trial study approved by the Ethics Committee of Tehran University of Medical Sciences (TUMS) and registered at a trial registration center (www.clinicaltrials.gov, Reference code: NCT01225289). Thirty-nine patients with MS, referred to Imam Khomeini Hospital Tehran, Iran, were randomly divided into an intervention (n=20) and a placebo (n=19) group. All the subjects were informed about the aim and the procedure of the study and signed the TUMS written consent form. The inclusion criteria were as follows: 1. No relapsing-remitting MS (RRMS); 2. Age between 20 and 45 years (both sexes); 3. A 0-5 score in the Expanded Disability Status Scale (EDSS); and 4. No vitamin supplements consumption during the previous 3 months. On the other hand, the exclusion criteria were 1. Any of the following disorders/diseases/conditions: lupus, asthma, food allergies, major comorbidity (especially hepatic and pancreatic diseases), type-1 diabetes, inflammatory bowel diseases, pregnancy, alcoholism, malnutrition (body mass index (BMI) <18.5); and 2. Any changes in medication during the study. All the patients received interferon beta-1a (Avonex®) as medication. The design of the study was block randomization by age and sex. The patients in the intervention and placebo groups received, daily for 6 months, 25000 IU vitamin A supplements in the form of retinyl palmitate and edible paraffin oil, respectively. The researcher and patients were blinded for group assignment at all the stages of the study; a third person marked vitamin A and placebo capsules as A and B, respectively. General characteristics of the patients such as age, sex, height, weight and BMI were recorded before and after the intervention. A 24-hour recall and a food frequency questionnaire (FFQ) were completed by all the participants at the beginning of the study. The patients were contacted and followed up during the intervention. Three patients (one from the intervention and two from the placebo group) withdrew from the study due to changes in their medication during the study; 36 patients completed the study.

The primary outcome of the study was the effect of vitamin A supplementation on gene expression of cytokines produced from CD4+ T-cells in patients suffering from MS, the secondary outcome being comparison of the initial and final anthropometric measurements between the vitamin A and placebo groups.

Gene expression procedure

Blood samples were taken from the patients before and after the intervention for analysis in the laboratory of the School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences. PBMC isolation, RNA extractions, cDNA synthesis and real time-PCR were performed as described by Mottaghi et al. [12]. The cell samples were cultivated in growth culture media to determine the proliferation response and measure cytokines in the supernatant and divided into three groups: one was treated with human MOG (1mg MOG in 1ml sterilized distilled water), one with PAH (1mg PAH in 1ml growth medium without phosphate buffer saline), and one with no treatment (that is, with no stimulation). MOG and PAH powder were purchased from Anaspec, Inc. CA, USA and Sigma, USA, respectively.

Statistical analysis

SPSS 18.0 (SPSS Inc. Released 2009, PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.) was used for statistical analysis of the data as described in a previous study (Honarvar et al.) [13]. For compression between before and after variables and within groups, two related sample tests (Wilcoxon) and two independent sample tests (Mann–Whitney U) were used. For normal distributed data, the independent sample test and paired t test were used for comparison between before and after variables and within groups, respectively. A P-value <0.05 was considered as significance level.

Results

General information

The general characteristics of the participants and the flow diagram of the study are shown in Tables 1 and Figure 1, respectively. There were no statistically significant differences between the intervention and placebo groups as regards age, sex, BMI and total vitamin A intake. Neither were any harmful effects observed in the intervention or placebo patients.

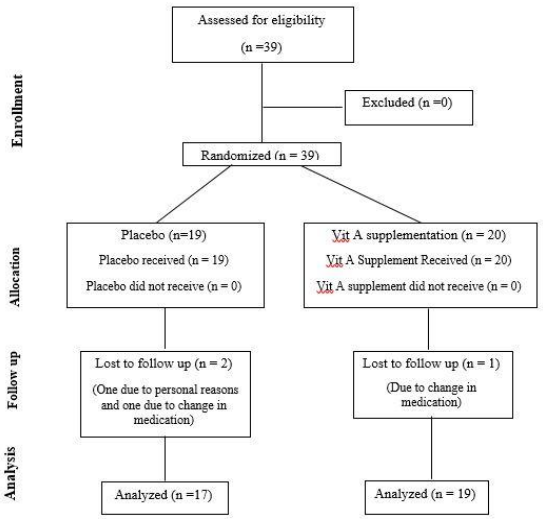

Figure 1. Flow diagram of participants

GATA3 and IL-4 gene expressions in cells stimulated by MOG

There were significant differences in GATA3 and IL-4 genes expression changes in the MOG-stimulated cells between the vitamin A and placebo groups (P = 0.0001, P = 0.004, respectively) (Table 2). Vitamin A supplementation led an increase of 2.5- and 1.99-fold in GATA3 and IL-4 gene expressions, respectively, while in the placebo group a decrease of 0.82- and-0.88 fold were observed **(P** = 0.0001 and P = 0.007, respectively) (Figure 2).

Table 1. Characteristics of participants in the intervention and placebo groups

	Vitamin A	Placebo	P-value
Age (year)	30.9±7.23	33.3±5.56	0.27[a]
Gender (M/F)(n)	6/13	4/13	0.71[b]
Body mass index	18.91±2.50	20.41±3.00	0.11[a]
Total vitamin A intake (RE/day)	400.2±132.4	358.0±144.3	0.37[c]

All values are expressed as means ± SD or numbers
RE; retinol equivalent
[a] Independent sample t-test
[b] Chi-squared test
[c] Two independent sample tests (Mann–Whitney U)

GATA3 and IL-4 gene expressions in cells stimulated by PAH

The mean GATA3 and IL-4 gene expressions in the PAH-stimulated cells were not different between the vitamin A and placebo groups (P = 0.924 and P = 0.964) (Table 3). The extent of expression increased in the intervention and placebo groups as regards GATA3 (1.9- and 1.65-fold, respectively) and IL-4 (3.79- and 3.57-fold, respectively), but the changes were not statistically significant (P = 0.506 and P = 0.778, respectively).

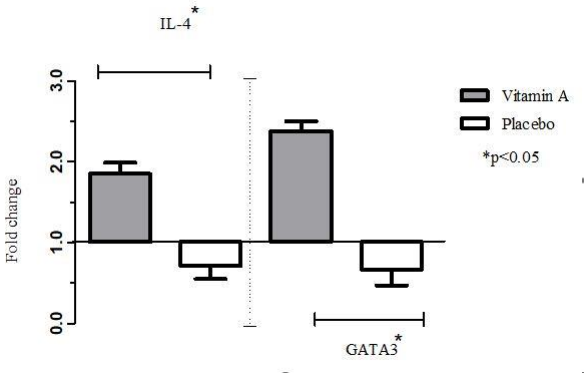

Figure 2. Fold changes in GATA3 and IL-4 genes expressions in MOG-stimulated cells in the vitamin A and placebo groups

GATA3 and IL-4 gene expressions in cells with no stimulation

Supplementation with vitamin A had a significant effect on GATA3 and IL-4 gene expression changes in the non-stimulated cells (P = 0.0001 and P = 0.001, respectively) (Table 4). There was an increase of 2.01- and 1.13-fold in the GATA3 and IL-4 gene expressions, respectively, in the vitamin A group; however,

there were decreases of 0.64- and 0.68-fold in the placebo groups, respectively (P = 0.0001 and P = 0.030, respectively) (Figure 3).

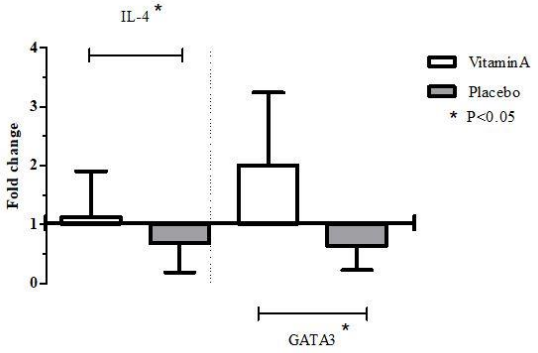

Figure 3. Fold changes in the GATA3 and IL-4 genes expressions in the non-stimulated cells in the vitamin A and placebo groups

Discussion

Our previous studies investigated the effects of vitamin A supplementation on the expression of genes involved in producing anti- and pro-inflammatory cytokines in intact cells such as IFN-γ, T-bet, FoxP3, TGF-β and RORγt [14-16]. The objective of the present randomized clinical trial, which included 39 patients with MS, was to further investigate the effects of vitamin A supplementation on GATA3 gene expression in samples of cell culture stimulated by MOG, PAH and a sample with no MOG stimulation.

The results showed that supplementation with vitamin A significantly increased GATA3 and IL-4 genes expressions as compared with the placebo group. In patients receiving placebo, expressions of GATA3 and IL-4 decreased except in cells stimulated with PAH. However, the differences between the two groups were statistically significant as regards both genes.

In-vitro and in-vivo studies have shown the effect of vitamin A on the immune system [17]. Vitamin A acts to establish the balance between the Th1 and Th2 cells and helps in the differentiation of Treg and Th17 cells through conversion to RA, an active metabolite. In fact, RA increases Th2 differentiation through inducing the expression of IL-4 gens [18]. Our results generally confirm those reported in some previous studies showing that high doses of vitamin A can lead to increases in the production of Th2 cytokines and decreases in the production of Th1 cytokines [19]. The results of a study by Kang and et al. have also revealed that RA-treated macrophages could increase IL-4 production in antigen-primed CD4+ T cells in mice [20]. Furthermore, Hoag and et al. showed that ATRA could, by influencing the function of antigen-representing cells (APC) in the presence of exogenous IL-4, increase the development of Th2 cells [21]. In addition, Iwata and et al. (23) showed that all-trance RA, 9-cis RA and RAR agonists would increase the development of Th2 cells; moreover, GATA3 and IL4Rα gene expressions could increase in the presence of 10nM all-trance RA [22]. In another study reported by Dawson and et al. it was shown that PBMC stimulation with ATRA and 9-cis RA could increase IL-4 production [23]. Moreover, Yu and et al. demonstrated that increases in the mRNA of IL-4 were due to the intake of ATRA in mice [10]. In another study by Stephensen and et al. the effect of 9-cis RA on the Th1 and Th2 cells through transcription of RAR and RXR was investigated. The results showed that selective agonists of RXR led to the development Th2 cells more efficiently than the RAR agonist, and RXR agonist increased IL-4 and IL-5 production and decreased IFN-γ; furthermore, it increased mRNA of the genes involved in Th2 development (GATA3, IL-4, C-maf) and decreased mRNA of the genes involved in Th1 differentiation (IL-

Table 2. GATA3 and IL-4 gene expressions in the MOG-stimulated cells before and after the intervention

		Vitamin A (n=19)	Placebo (n=17)	P-value[a]
Δ-CT of GATA3 gene expression	Before	8.76 ± 0.89	8.82 ± 0.91	0.844
	After	7.89 ± 0.99	9.28 ± 1.06	0.0001
	Change	-0.86 ± 1.21	0.46 ± 0.76	0.0001[c]
	P-value[b]	0.006	0.023	
Fold change in GATA3 gene expression		2.5 ± 2.26	0.82 ± 0.47	0.0001[c]
Δ-CT of IL-4 gene expression	Before	12.75 ± 0.95	12.71 ± 1.11	0.898
	After	12.01 ± 0.82	13.13 ± 0.93	0.002
	Change	-0.58 ± 1.08	0.42 ± 0.87	0.004[c]
	P-value[b]	0.031	0.062	
Fold change in IL-4 gene expression		1.99 ± 1.71	0.88 ± 0.55	0.007[c]

Data are reported as mean ± SD
Δ-CT CT of target gene-CT of β-actin
[a] Independent samples t-test
[b] Paired sample t-test
[c] Mann-Whitney test

Table 3. GATA3 and IL-4 gene expressions in the PAH-stimulated cells before and after the intervention

		Vitamin A (n=19)	Placebo (n=17)	P-value[a]
Δ-CT of GATA3 gene expression	Before	10.03 ± 1.16	10.21 ± 0.75	0.615
	After	9.53 ± 0.93	9.67 ± 0.59	0.529
	Change	-0.5 ± 1.3	0.53 ± 0.84	0.924[c]
	P-value[b]	0.111	0.012	
Fold changes of GATA3 gene expression		1.9 ± 1.34	1.65 ± 0.73	0.506[c]
Δ-CT of IL-4 gene expression	Before	11.53 ± 1.09	11.72 ± 0.65	0.537
	After	10.15 ± 0.92	10.35 ± 1.24	0.567
	Change	-1.38 ± 1.41	-1.36 ± 1.19	0.964[c]
	P-value[b]	0.001	0.001	
Fold changes of IL-4 gene expression		3.79 ± 3.19	3.57 ± 3.2	0.778[c]

Data are reported as mean ± SD, Δ-CT CT of target gene-CT of β-actin
[a] Independent samples t-test; [b] Paired sample t-test
[c] Mann-Whitney test

12R, T-bet, IFN-γ) [24]. Nevertheless, in the Nozaki et al. study, ATRA intake caused no significant changes in IL-4 production in patients with rheumatoid arthritis in either the placebo or intervention group [25].

In our study, decreases in GATA3 and IL-4 genes expressions in the placebo group, observed in most treatments except in cells stimulated with PAH, indicate a progressive process of inflammation compared with the intervention group. Considering that vitamin A could reduce the proliferation of cells treated with and exclusive stimulant, it can be concluded that vitamin A supplementation, in addition to its therapeutic effects, can lead to increases in anti-inflammatory cytokine gene expressions and decreases in pro-inflammatory ones. This combination slows down the progressive attenuation of the disease observed in the placebo group. Royal and et al. reported, based on two MS patients, that not only RXR-α but also RXR-β and RXR-γ genes are expressed in patients treated by IFN-β1a (Avonex) [26]. It seems that vitamin A enhances GATA3 and IL-4 gene expressions and the Th2-cell development by increasing the expression of the RXR-related genes. However, in the presence of a general stimulant (PAH), cell proliferation is more pronounced than increases in GATA3 and IL-4 gene expressions. Therefore, in order to be able to draw more definite conclusions, investigating gene expression of receptors and measuring vitamin A metabolites are essential. In addition, the fact that there was no statistically significant difference between the two groups in the PAH-treated cells as regards the GATA3 and IL-4 expression would imply a strong effect of this general stimulant on the Th2-related gene expression. It is to be noted that similar effects of PAH have been observed in Th1-related genes. Based on the findings of the present study and previous investigations, it can be concluded that vitamin A might have a key role in establishing the balance between Th1 and Th2 cells ─ an increase in Th2 cells differentiation and a decrease in pro-inflammatory cytokines.

Our study had some limitations. It was unethical to change dosages of the medications of the patients in order to eliminate their effect. Also, we were unable to assess clinical manifestations and MRI changes in the subjects due to budget

Table 4. GATA3 and IL-4 gene expressions in the non-stimulated cells before and after the intervention

		Vitamin A (n=19)	Placebo (n=17)	P-value[a]
Δ-CT of GATA3 gene expression	Before	11.68 ± 0.8	11.26 ± 0.63	0.096
	After	10.96 ± 1.12	12.13 ± 0.92	0.002
	Change	-0.71 ± 0.83	0.87 ± 0.83	0.0001[c]
	P-value[b]	0.006	0.001	
Fold changes in GATA3 gene expression		2.01 ± 1.23	0.64 ± 0.41	0.0001[c]
Δ-CT of IL-4 gene expression	Before	12.46 ± 0.64	11.63 ± 0.69	0.001
	After	12.6 ± 1.15	12.47± 1.46	0.725
	Change	0.13 ± 0.96	0.83 ± 0.89	0.001[c]
	P-value[b]	0.551	0.001	
Fold changes in IL-4 gene expression		1.13 ± 0.78	0.68 ± 0.49	0.03[c]

Data are reported as mean ± SD
Δ-CT CT of target gene-CT of β-actin
[a] Independent samples t-test
[b] Paired sample t-test
[c] Mann-Whitney test

constraints.

Conclusion

To our knowledge, the present study is the first study investigating the effect of retinyl palmitate supplementation on transcription factor expression and cytokine production of the Th2 cells. The results have revealed that GATA3 and IL-4 genes expressions increase in patients receiving vitamin A, suggesting a protective role of vitamin A in multiple sclerosis. It can be concluded that vitamin A could be beneficial in downregulating the inflammation progressive process and increasing the remission duration of the disease. However, further studies are needed to be able to draw more definite conclusions. It is recommended to investigate the RXRs and RARs genes expression and polymorphisms in future studies.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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