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The effect of retinyl-palmitate on the level of pro and anti-inflammatory cytokines in multiple sclerosis patients: A randomized double blind clinical trial

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ABSTRACT

Objective: Multiple sclerosis (MS) is an inflammatory and autoimmune disease associated with the imbalance of cytokines secreted from $CD4^+$ T cells. Studies have shown that vitamin A and its active derivatives are able to modulate the immune system in MS patients. The aim of the present study was to investigate the effect of supplementation of retinyl palmitate (RP), the dietary form of vitamin A, on pro- and anti-inflammatory cytokines in the plasma and supernatants of cultured peripheral blood mononuclear cells (PBMCs) of MS patients.
Patients and methods: Thirty-six relapsing-remitting MS patients were enrolled in this double-blind randomized clinical trial. Participants received one capsule of 25,000 IU RP or a placebo per day for six months. Blood samples were taken before and after intervention. After intervention, the PBMCs were isolated and cultured. The levels of pro- and anti-inflammatory cytokines in the plasma and supernatant of cells stimulated with myelin oligodendrocyte glycoprotein, phytohemagglutinin or vehicle (media) were determined. The sample *t*-test and Mann Whitney U test were used to compare data between groups.
Results: The changes in pro-inflammatory cytokine levels (IL-1β, TNF-α, IFN- γ, IL-2, IL-6, and IL-17) in the serum and supernatant of MS patients were not significant ($p > 0.05$). There were also no significant changes in the levels of anti-inflammatory cytokines (IL-10, IL-13, IL-4, and TGF-β) ($p > 0.05$).
Conclusion: Unexpectedly, this study found no significant changes in cytokine levels after six months of RP supplementation in MS patients. The results of other studies by our team have shown significant changes in the gene expression of the cytokines in response to RP supplements. Therefore, we recommend that periodic follow-up of RP supplementation may be needed to reveal changes in the level of the cytokines in the plasma and PBMCs and to clarify the real effect of RP on the immune factor levels in the serum of MS patients.

1. Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune T cell-mediated disease that affects the central nervous system [1]. The etiology of MS is not clearly known, but it is believed that the imbalance between T helper (Th)2/Th1 and also the Th17/T-regulatory (T-reg) populations leads to the production of inflammatory cytokines that contribute to nerve injury [2]. The distinct function of each $CD4^+$ T helper cell subset is related to the particular cytokine [3,4].

Vitamin A and its active derivatives are essential for immune functions such as innate, cell mediated and humoral immunity [5,6]. Vitamin A acts as a cofactor in immune responses through its regulatory effect on $CD4^+$T helper cells. Many studies have investigated its effects on MS [7]. These studies have used different forms of vitamin A and its derivatives to investigate their immune regulatory functions in MS. [8–10]. Retinyl palmitate (RP) is the common form of vitamin A in

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food. It can be converted to retinoic acid (RA) in the liver in a safe process [11].

Few studies have investigated the effect of RP in patients with MS [12,13]. The present study investigated the effects of RP on the pro- and anti-inflammatory cytokines in the plasma and supernatant of cultured peripheral blood mononuclear cells (PBMCs) of MS patients. To the best of our knowledge, the current study is the only one in this regard.

2. Patients and methods

2.1. Participants and intervention

The present study was a randomized double-blind, placebo-controlled phase III clinical trial. All participants were randomly distributed into intervention and control groups by randomized block design according to sex. The age of the patients was 18–50 years. Thirty-six relapsing remitting MS (RRMS) patients participated. The patients in the intervention group received one capsule of vitamin A containing 25,000 IU RP (Zahravi; Iran) and the placebo group received one capsule filled with edible paraffin daily for six months. The vitamin A and placebo capsules were similarly shaped and coded as A and B by an independent person. Both patients and researchers were completely blind to group assignment during the study and analyses. To monitor a patient's adherence to the schedule of medication, pill counting and telephone reminders were employed. Patients were called every two weeks to remind them to take their medication. Patients delivered to the researcher previous drug packs for counting the remainder of the supplements at the end of the third and sixth months of supplementation. If the patient took more than 90% of the pills (vitamin A or placebo), it was considered to be adherence to the schedule of medication.

Exclusion criteria included a diagnosis of liver, biliary or pancreatic disease, malnourishment (BMI under 18.5), impaired Th1/Th2 balance (such as asthma, rheumatoid arthritis and type 1 diabetes mellitus), granulomatous disease, abnormal biochemical tests and history of stroke or myocardial infarction. Only one patient in the placebo group was excluded due to irregular intake of supplements. The data was analyzed for 35 patients. All patients were under the guidance of two neurologists and used interferon beta-1a (IFN β-1a) (Avonex) as treatment.

At the end of three months, all participants were checked for liver function and clinical conditions to be approved for additional three months of intervention (data not shown).

The intake of dietary vitamin A and β-carotene was assessed at the onset and end of the intervention using a food frequency questionnaire. All participants provided signed informed consent forms. The Ethics Committee of the Tehran University of Medical Sciences approved the study (ID: 9567, 10,033).

2.2. Blood samples and cell culture

Blood samples were taken at the onset and the last day of intervention and were stored in heparinized sterile tubes as an anticoagulant factor. The plasma was then isolated (gradient method), divided into micro tubes and stored at −70 °C.

PBMCs were isolated from the blood on Ficoll gradients (Ficoll-Histoprep; BAG Health Care; Germany) and diluted to 1×10^6 cells per milliliter of RPMI 1640 (PAA Laboratories; Austria) with 10% fetal calf serum (Gibco; Invitrogen; UK). The cells were seeded at 1×10^6 cells/well and each well was stimulated with 5% phytohemagglutinin (PHA; Sigma; USA) or myelin oligodendrocyte glycoprotein (MOG; Anaspec; USA). MOG is an auto-antigen on the surface of the myelin sheath responsible for pathogenesis of MS and PHA is a common polyclonal stimulus. The control wells had no mitogen (untreated cells). The supernatant was collected after 96 h of incubation (at 37 °C, 5% CO_2). After the incubation period, cell supernatants were collected for cytokine analysis and stored at −70 °C until their assay for cytokines. The cytokine levels of the specimens were measured over a few months after the sampling phase using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience; USA).

2.3. Statistical analysis

The data is presented in the mean ± SE form. The analyses were carried out in SPSS version 17. Sample *t*-tests and Mann–Whitney U tests were used to compare changes in the level of cytokines in the RP and placebo groups at baseline and after intervention. A p-value was considered significant at less than 0.05.

3. Results

The pro- and anti-inflammatory cytokines levels in the plasma and PBMC supernatant were measured at the onset and after six months of RP supplementation by MS patients. The supernatant on PBMCs was stimulated by MOG, PHA or vehicle (media).

Of thirty-five participants, 18 were in the intervention group and 17 were in the placebo group. There were no significant differences in female-to-male ratio, mean age or BMI of patients between the intervention and placebo groups ($p > 0.05$). The data also showed no significant differences in dietary intake of vitamin A and β carotene between the two groups (Table 1).

There were no significant differences between the groups for pro-inflammatory cytokine concentrations of interleukin (IL) -1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN- γ), IL-2, IL-6 and IL-17 in the plasma or supernatant before and after treatment with vitamin A in comparison with the placebo group ($p > 0.05$) (Table 2). This means that none of the differences in pro-inflammatory cytokine levels in the supernatant and plasma was significant in response to RP supplementation ($p > 0.05$) (Table 4).

The baseline and post-supplementation level of anti-inflammatory cytokines (IL-10, IL-13, IL-4, and TGF-β) in the supernatant and plasma were not significantly different ($p > 0.05$) (Table 3). This means that none of the changes in the anti-inflammatory cytokine levels in the supernatant and plasma between groups was significant in response to RP supplementation ($p > 0.05$) (Table 5).

4. Discussion

This study was part of a large clinical trial project (ID: NCT01225289) which explored the immunomodulatory effects of RP supplementation in RRMS patients. No side effects or biochemical complications were observed after six months of supplementation (data not reported). In other studies by the authors, it has been shown that RP regulate gene expression of T helper-related transcription factors [14–16] and clinical symptoms [17,18] in MS patients.

In the current study, there were no significant changes in serum and

Table 1
Participants general information.

characteristic		Vitamin A (n: 18)	Placebo (n:17)	*P* Value
Age (year)		33.22 ± 7.26	31.6 ± 5.90	**.341**[€]
Sex	Female	13 (37.1%)	13 (37.1%)	**.774**[¥]
	Male	5 (14.3%)	4 (11.5%)	
Body Mass Index	Before	23.7 ± 0.9	24.8 ± 1.1	**.477**[*]
(Kg/m^2)	After	24.8 ± 1.0	25.6 ± 1.1	**.578**[*]
Vitamin A intake	Before	667.5 ± 155.5	718.1 ± 217.1	**.121**[*]
(RE)	After	411.3 ± 58.1	412.3 ± 67.7	**.187**[*]
β carotene intake	Before	223.4 ± 90.2	142.9 ± 43.1	**.656**[*]
(μg)	After	244.8 ± 71.8	202.7 ± 75.3	**.306**[*]

Data is reported in mean ± SEM or n (%).
[€] Independent sample Test.
[¥] Chi-square Test.
[*] Mann_Whitney Test.

Table 2
Pro-inflammatory cytokines in plasma and PBMCS supernatant untreated and stimulated with MOG and PHA, before and after supplementation period.

Pro-inflammatory Cytokines (pg/ml)		PHA (Mean ± SEM)			MOG (Mean ± SEM)			Un-treated (Mean ± SEM)			Plasma (Mean ± SEM)		
		Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value
IL-1β	Before	66.03 ± 19.93	50.87 ± 12.97	**.391**	39.93 ± 12.46	44.39 ± 8.90	**.276**	10.5 ± 4.1	12.0 ± 2.5	**.204**	6.36 ± 2.36	4.78 ± 1.42	**.921**
	After	151.34 ± 50.82	98.98 ± 26.93	**.882**	90.61 ± 26.55	85.74 ± 23.37	**.947**	36 ± 11.5	37.8 ± 10.8	**.895**	5.72 ± 1.91	4.69 ± 127	**.156**
IL-2	Before	13.87 ± 4.30	13.62 ± 3.16	**.655**	29.78 ± 4.91	29.64 ± 5.00	**.974**	36.5 ± 9.2	27.5 ± 5.8	**.679**	50.15 ± 11.73	31.60 ± 5.96	**.283**
	After	28.82 ± 8.32	63.03 ± 45.64	**.597**	29.42 ± 4.71	33.33 ± 7.09	**.754**	31.3 ± 5.1	32.8 ± 3.7	**.844***	21.41 ± 4.29	24.60 ± 3.85	**.262**
IL-6	Before	2744.29 ± 737.65	3187.85 ± 683.95	**.428**	2681.29 ± 664.77	3806.96 ± 932.14	**.355**	817.5 ± 214.3	915.7 ± 215.4	**.448**	21.92 ± 10.83	25.95 ± 9.90	**.235**
	After	6768.35 ± 1646.05	6522.56 ± 1390.96	**.717**	8162.02 ± 2447.33	6867.67 ± 1791.43	**.766**	2062.3 ± 515.4	2250.18 ± 592.7	**.692**	30.78 ± 10.46	30.98 ± 6.65	**.478**
TNF-α	Before	420.27 ± 104.48	400.54 ± 83.12	**.792**	66.70 ± 17.60	51.25 ± 12.13	**.563**	400.5 ± 83.1	77.9 ± 29.8	**.585**	139.64 ± 98.74	98.05 ± 48.90	**.987**
	After	518.58 ± 132.47	555.43 ± 118.11	**.620**	70.66 ± 18.20	38.59 ± 15.54	**.438**	555.4 ± 118.1	32.2 ± 9.35	**.374**	200.81 ± 105.15	208.64 ± 88.27	**.303**
IFN-γ	Before	9570.43 ± 2636.29	12609.10 ± 3766.21	**.869**	199.10 ± 107.40	246.88 ± 89.89	**.974**	12609 ± 3766.2	187 ± 76.2	**.741**	117.25 ± 42.82	122.45 ± 32.73	**.291**
	After	6884.05 ± 2075.57	7193.69 ± 2384.48	**.792**	67.35 ± 17.19	53.83 ± 12.14	**.779**	7193.7 ± 2384.5	50.7 ± 8.1	**.753**	112.96 ± 40.19	93.82 ± 32.48	**.804**
IL-17	Before	129.03 ± 35.07	127.80 ± 24.68	**.338**	15.16 ± 2.97	19.42 ± 4.70	**.668**	15.26 ± 2.84	14.82 ± 2.91	**.934**	9.43 ± 2.62	17.14 ± 6.27	**.487**
	After	238.96 ± 39.51	176.65 ± 27.79	**.409**	26.12 ± 5.51	28.91 ± 13.56	**.499**	± 21.01 ± 4.81	± 17.01 ± 3.86	**.541**	10.73 ± 4.03	9.74 ± 2.98	**.947**

Not-normal data was analyzed by Mann_Whitney Test.
* Normal data was analyzed by Independent sample *t*-test.

Table 3
Anti-inflammatory cytokines in plasma and PBMCs supernatant untreated and stimulated with MOG and PHA, before and after supplementation period.

Anti-inflammatory Cytokines (pg/ml)		PHA (Mean ± SEM)			MOG (Mean ± SEM)			Un Treated (Mean ± SEM)			Plasma (Mean ± SEM)		
		Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	P Value	Vitamin A	Placebo	*P* Value
IL-10	Before	273.37 ± 59.67	295.24 ± 56.66	**.717**	58.51 ± 12.6	57.47 ± 12.16	**.960**	39.02 ± 7.77	35.6 ± 7.74	**.717**	13.48 ± 6.78	4.30 ± 2.66	**.684**
	After	419.60 ± 100.67	396.25 ± 93.24	**.934**	80.04 ± 28.34	92.20 ± 36.20	**.908**	63.5 ± 24.2	92.1 ± 36.5	**.974**	2.78 ± 1.25	7.06 ± 3.67	**.779**
IL-13	Before	166.69 ± 38.31	150.45 ± 30.54	**.843**	23.50 ± 3.81	16.44 ± 2.95	**.248**	19.0 ± 3.77	12.6 ± 3	**.096**	24.90 ± 5.04	16.92 ± 3.18	**.448**
	After	371.40 ± 68.71	353.09 ± 61.84	**.869**	23.83 ± 3.63	19.13 ± 3.45	**.586**	20.9 ± 3.78	32.1 ± 18.04	**.531**	21.45 ± 3.52	14.82 ± 3.02	**.075**
TGF-β	Before	456.77 ± 56.64	478.36 ± 87.2	**.792**	403.51 ± 67.81	923.79 ± 472.34	**.171**	377.6 ± 49.4	362.5 ± 34.5	**.908**	3266.54 ± 1056.37	2697.6 ± 463.33	**.255**
	After	571.10 ± 95.55	754.52 ± 170.70	**.468**	679.61 ± 165.61	769.66 ± 181.94	**.382**	720.5 ± 163.4	704.8 ± 225.4	**.882**	2421.62 ± 619.53	3142.50 ± 673.09	**.262**
IL-4	Before	1.82 ± 0.35	1.90 ± 0.29	**.656**	1.73 ± 1.90	1.29 ± 0.82	**.934**	0.75 ± 0.31	0.4935 ± 0.12438	**791***	1.95 ± 0.76	1.93 ± 0.53	**.531**
	After	4.09 ± 1.19	3.75 ± 1.10	**.921**	1.89 ± 2.01	1.73 ± 1.28	**.692**	1.51 ± 0.60	1.16 ± 0.26	**.817***	1.01 ± 0.25	1.23 ± 0.41	**.766**

Not-normal data was analyzed by Mann_Whitney Test.
* Normal data was analyzed by Independent sample *t*-test.

supernatant cytokine levels after six months of RP supplementation in MS patients. Our hypothesis was that vitamin A exerts an anti-inflammatory response through the induction of anti-inflammatory cytokines and inhibition of pro-inflammatory cytokines following regulation of related gene expression.

The likely underlying mechanism of the effect of vitamin A was determined to be the conversion of RP to the active metabolite, RA, and its interaction with RA receptors [19]. This leads to the inhibition of RA-related orphan receptor$_{\gamma t}$ (ROR$_{\gamma t}$) and the induction of differentiated naive CD4$^+$ T cells into Treg cells, followed by suppression of the pathogenic functions of Th17 cells [20,21].

These findings are not in agreement with the results of some studies evaluating cytokine levels in MS patients in response to vitamin A. Xu et al. used 9-cis RA and observed lower production of Th1-related cytokines in lipopolysaccharide-stimulated microgelia [9]. The results of this study also were not in agreement with those of Mathew et al., who used all-trans RA and observed a decrease in Th1- related cytokine levels [22].

This contradiction may be due to the form of vitamin A supplementation, retinyl-palmitate, in the current study [9,22]. Other studies that reported significant changes in the production of anti- and pro-inflammatory cytokines in cell cultures or animal models in response to vitamin A have used RA, the active form of vitamin A [9,22,23]. Because of the possible harmful effects of RA on humans [24,25], the RP form of vitamin A was used in this study. When RP is converted to RA in the body, many regulatory mechanisms are involved that inhibit the peak of RA concentration in the circulatory system. So, The effect of RP cannot be as was hypothesized for RA [19,26]. It is likely that a different dose or period from that used in this study is needed to obtain results that are similar to direct intervention with RA.

Because the same dose and duration of RP supplementation has been shown to be sufficient to regulate the expression of cytokine genes [14,27], another reason for the lack of significant changes in the serum level of the cytokines may be that six months of supplementation is an insufficient time for cytokine production. Therefore, it would be interesting if the cytokine levels periodically checked after the end of supplementation in next studies.

5. Conclusion

Other our studies have shown significant changes in the gene expression of the cytokines involved in the pathogenesis of MS in response to six months of RP supplementation. [15,16]. It was also expected in the current study the serum and supernatant levels of these genes products would significantly change. However, no significant changes in cytokines were detected after supplementation by RP for six months.

Table 4
Pro-inflammatory cytokine changes in plasma and PBMCs supernatant untreated and stimulated with MOG and PHA, before and after supplementation period.

Pro-inflammatory Cytokines (pg/ml)	PHA Change (Mean ± SEM)			MOG Change (Mean ± SEM)			Un Treated Change (Mean ± SEM)			Plasma Change (Mean ± SEM)		
	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value
IL-1β	85.3 ± 54.7	48.1 ± 19.4	**.856**	50.7 ± 28.1	41.3 ± 21.8	**.563**	25.5 ± 12.3	25.8 ± 9.8	**.741**	−0.7 ± 1.8	−0.1 ± 0.47	**.574**
IL-2	14.9 ± 9.9	27.4 ± 25.9	**.586**	−0.4 ± 5.7	3.8 ± 5.7	**.620**	−5.2 ± 8.3	5.3 ± 5.3	**.934**	−28.8 ± 12.1	−7 ± 6.6	**.146**
IL-6	4024 ± 1967.7	3334.7 ± 1650.8	**.490***	5480.7 ± 2534.5	3060.8 ± 2074	**.575**	1244.8 ± 596.8	1334.5 ± 651.6	**.920***	8.9 ± 3.5	5.1 ± 4.92	**.921**
TNF-α	98.3 ± 179.3	154.9 ± 102.9	**.644**	4 ± 20.1	−12.6 ± 15.5	**.921**	−45.7 ± 30	−11.8 ± 10	**.596**	61.2 ± 37.7	110.6 ± 70.1	**.921**
IFN-γ	−2686.3 ± 3464.5	−5415.3 ± 2935.3	**.843**	−131.8 ± 108	−193.1 ± 87.2	**.552**	−136.3 ± 71.5	20.7 ± 157.7	**.921**	−4.3 ± 20.7	−28.6 ± 11.4	**.228**
IL-17	109.93 ± 31.07	48.84 ± 37.56	**.217***	10.96 ± 4.38	9.49 ± 10.96	**.409**	5.75 ± 3.20	2.19 ± 4.32	**.283**	1.30 ± 2.10	−7.40 ± 6.54	**.355**

Not-normal data was analyzed by Mann_Whitney Test.
* Normal data was analyzed by Independent sample *t*-test.

Table 5
Anti-inflammatory cytokine changes in plasma and PBMCS supernatant untreated and stimulated with MOG and PHA, before and after supplementation period.

Anti-inflammatory Cytokines (pg/ml)	PHA Change (Mean ± SEM)			MOG Change (Mean ± SEM)			Un Treated Change (Mean ± SEM)			Plasma Change (Mean ± SEM)		
	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value	Vitamin A	Placebo	*P* Value
IL-10	142.2 ± 88.9	101 ± 72.77	**.692**	21.53 ± 28.8	34.72 ± 35.16	**.843**	24.48 ± 24.7	56.5 ± 35.2	**.644**	−10.7 ± 6.16	2.74 ± 2.92	**.592**
IL-13	204.7 ± 73.78	202.6 ± 57.9	**.982***	0.3 ± 0.97	2.7 ± 1.74	**.276**	1.9 ± 2.23	19.5 ± 18.1	**.276**	−3.45 ± 2.55	−2.1 ± 1.3	**.804**
TGF-β	115.2 ± 73.7	276.2 ± 130.4	**.330**	276.1 ± 130.7	−154.2 ± 308.6	**.766**	342.8 ± 170.1	342.3 ± 219.9	**.717**	−844.9 ± 928.7	444.83 ± 458.3	**.400**
IL-4	2.26 ± 1.19	1.8 ± 10	**.597**	0.154 ± 0.33	0.43 ± 0.20	**.974**	0.76 ± 0.32	0.67 ± 0.19	**.704**	−0.94 ± 0.68	−0.70 ± 0.18	**.419**

Not-normal data was analyzed by Mann_Whitney Test.
* Normal data was analyzed by Independent sample *t*-test.

Because protein biosynthesis is a time-consuming process, it is possible that the measurement of cytokines a few months after the end of supplementation will show significant changes in the levels of the target cytokines. Measurement of the serum and supernatant levels of the cytokines immediately after RP supplementation was probably one of the weaker points of the present study; therefore, follow-up studies are recommended. In addition, low sample size can be a weaker point of this study. Supplementation with higher doses of vitamin A for a longer duration may alter the insignificance of the current results but it is a limitation of our study because of the prevention of hypervitaminosis A [28–30].

Conflict of interest

There was no conflict of interest.

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