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Research Article

The effect of Vitamin a Supplementation on IL-4 and GATA-3 Gene Expression in Atherosclerotic Patients

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Abstract: Since T helper cells type 2 (Th2) are considered as atheroprotective agents, this study was designed to investigate the possible role of vitamin A as a regulator of immune function on gene expression of IL-4 and GATA-3 in atherosclerotic patients. The vitamin A treated groups (patients and healthy controls) received 25,000 IU of retinyl palmitate while the placebo group (control patients) was given one pearl of placebo per day for 4 months. Peripheral blood mononuclear cells were isolated and divided into 3 groups; fresh cells, activated with PHA and activated with ox-LDL. Gene expressions of Th2 cells were studied by real-time PCR. IL-4 gene expression in fresh cells significantly increased in vitamin A treated patients compared with the other two groups. IL-4 gene expression in PHA-activated cells also showed a significant increase in vitamin A treated patients compared with the placebo group (p=0.027), however, there were no significant differences in IL-4 gene expression in ox-LDL activated cells between the 3 groups (p=0.737) although all groups showed an increase in the gene expression levels. There were no significant differences in GATA-3 gene expression in fresh cells, among the 3 groups (p=0.084), while the mean of GATA-3 gene expression in PHA and ox-LDL-activated cells in vitamin A treated patients showed a significant difference in comparison with healthy controls (p=0.016) and placebo group (p=0.019) respectively. It can be concluded that vitamin A supplementation led to increase in the gene expression of IL-4 and GATA-3 which in turn may reduce the complications of atherosclerosis.
Keywords: IL-4, GATA-3, Atherosclerosis, Gene expression

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that involves arterial walls and leads to vascular events like coronary arterial disease, stroke, abdominal aortic aneurysm and ischemic gangrene. T lymphocytes are incorporated in the process of atherosclerosis [1]. The process of atherogenesis is initiated through digestion of oxidized low-density lipoproteins (ox-LDL) by macrophages and presentation to T lymphocytes. During this process, response of T helper cells arises. T helper 2 (Th2) cells and its mediators such as interleukin-4, 5, 10 and 13 inhibit the progression of atherosclerosis [2]. These cytokines exert their effect by decreasing IL-12 and IL-18 production and inhibiting any Th1-based immune responses [3]. It is believed that Th2 cytokines play a prominent role in anti inflammatory process by inhibiting macrophage activity, including the secretion of matrix degrading enzymes [4]. The differentiation of naïve T cells into Th2 version needs specific factors. IL-6, IL-13 and OX40-L (CD-134, OX-40 ligand), may play a role in this differentiation induced by dendritic cells [5]. IL-4 activates the transcription factor of Th2 cells (GATA3) through STAT6 which induces IL-5 and down regulates IFNγ. The balance between Th1 and Th2 cells may be due to the equilibrium between T-bet and GATA-3, suggesting that a trend towards Th2 responses might deactivate the atherogenic effects of Th1 cells [6]. As a matter of fact, some studies including those which demonstrate the role of the humoral immunity in atherosclerosis, suggest that Th2 derived humoral responses may be protective [7]. Any shift towards the Th2 cytokines profile relates to increase in ox-LDL protective antibodies in atherosclerotic mice [7]; hence splenectomy in apoE-/ - mice with low levels of IgM and IgG antibodies fed

with cholesterol, increases incidence of atherosclerosis [8]. Increased production of IgG1 related to Th2 in mice with high expression of IL-10, relates to decreased complications of atherosclerosis [9]; increased Th2 responses in mice with mild hypercholesterolemia, also lead to decreased formation of early fatty streaks [10]. Although some studies report that Th2 responses may be pro-atherogenic, IL-4 deficiency as a Th2 protective cytokine, is considered as an effective agent for decreasing the atherogenic complications [11] specially in advanced stages of disease [12]. Therefore, even if the primary spread of injuries strongly under control of Th1 associated immunity and could be regulated by Th2 responses, the progress of complications in the presence of hypercholesterolemia may lead to the formation of plaques [1].

Vitamin A plays important roles in both cell-mediated and humoral antibody response and supports a Th2-mediated anti-inflammatory cytokine profile. Vitamin A deficiency impairs both innate immunity (mucosal epithelial regeneration) and adaptive immune response, resulting in an impaired ability to counteract extracellular pathogens [13]. Retinoic acid nuclear receptors may play a role in Th2 differentiation and primary T cells changing into Th2 cells. An increase in GATA3 accompanied by a decrease in T-bet gene expression, 4-12 hours post PBMC stimulation with ATRA, provides strong evidence for the specific role of retinoids in the development of Th2 cells [14]. It has been shown that vitamin A treatment reduces serum concentration of IL-1B/IL-4 ratio in obese women [15].

The present study was designed to determine how vitamin A supplementation may affect Th2 gene expression in atherosclerotic patients.

MATERIALS AND METHODS

This study was performed on 31 atherosclerotic patients (mean age 56 years) and 15 healthy controls (mean age 56.5 years). Details of methodology have been illustrated elsewhere [16]; in short, the patients were randomly divided into two groups (vitamin A or placebo) by a double blind pattern. None of the patients were suffering from diseases or taking medications affecting the immune system. Patients in the vitamin A treated groups and healthy controls received 25,000 IU of retinyl palmitate per day for 4 months.

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll–Hypaque gradient centrifugation and PBMCs divided into 3 groups. Group 1 contained fresh cells and mRNA extracted immediately whereas PBMCs in group 2 and 3 cultured for 72 hours at 37°C and 5% CO_2 under stimulation of ox-LDL (Biomedical Technology Inc, Mass., USA), phytohemagglutinin (PHA; Sigma). After this period, all the cultured cells were used for RNA extraction. Cytoplasmic RNA by RNeasy Plus Mini Kit (Qiagen, Valencia, Calif.,USA) was extracted and cDNA using QuantiTect Reverse Transcription Kit (Qiagen) was synthesized.

Primer Express 3 software (Applied Biosystems) was used for designing Polymerase Chain Reaction (PCR) primers for IL-4 and GATA-3 and β-actin as housekeeping gene (Table 1), following which real time PCR was performed by the SYBR green detection method. Gene expression levels were normalized with β-actine gene expression level, and gene expression changes were computed by comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Statistical analyses were performed by SPSS software (SPSS, Inc., Chicago, Ill., USA) and P values <0.5 were considered significant.

Table 1: Primer sequence for real-time PCR

Gene	Sequence	Amplicon size, bp
IL-4	Forward: 5'-CTGCAAATCGACACCTATTAATGG-3' Reverse: 5'-GCACATGCTAGCAGGAAGAACA -3'	76
GATA-3	Forward: 5'-AGATGGCACGGGACACTACCT -3' Reverse: 5'-CCTTCGCTTGGGCTTAATGA -3'	75
B-actin	Forward:5'-CCTGGCACCCAGCACAAT-3' Reverse: 5'-GCCGATCCACACGGAGTACT-3'	70

RESULTS

Basic Characteristics of Patients

Demographic, clinical and biochemical characteristics of patients and healthy controls are illustrated in table 2. As we can see, there were no statistically significant differences in age, sex, body mass index, and total vitamin A intake among the patients and healthy controls. However, abdominal circumference measurement in patients was found to be significantly higher than in healthy controls. There were no significant differences among 3 groups in both SGOT and SGPT enzyme levels after adjusting weight, BMI, waist circumference, waist to hip ratio and total energy intake.

Table 2: Demographic, clinical and biochemical data of patients and healthy controls

Characteristic	Healthy controls n=12	Patients		p- value
		Vitamin A n=16	Placebo n=15	
Age(years)	56±7	56±8	55±7	0.292
Sex(male/female)	5/7	8/8	8/7	
Body mass index	28.6±4.6	29.1±2.3	30.2±5.3	0.568
Waist to hip ratio	0.89±0.1	0.94±0.1	0.93±0.1	0.267
Abdominal circumference(cm)	94.17±8.3	99.2±5.7	103.2±5.3	0.01
Total vitamin A intake(RE/day)	497.7±343.6	813.1±695.0	765.1±918.9	0.559
SGOT	24.9±7.8	28.1±8.5	24.5±4.7	0.321
SGPT	10.2±4.7	13.2±8.7	13.4±6.3	0.442

All values are expressed as means± SD or numbers; RE = Retinol equivalent

IL-4 Gene Expression in Fresh Cells

Results of this study showed statistically significant differences among the three studied groups (p=0.038). Although all groups had increases in IL-4 gene expression after intervention, it was higher in vitamin A treated patients, compared to the other two groups; the vitamin A treated patients, the placebo group and healthy controls showed 12.7- (p=0.0001), 2.9- (p=0.323) and 3.5-fold (p=0.009) increases respectively. Multiple comparison showed a significant difference between the mean gene expression in vitamin A treated patients and placebo group (p=0.017), and also between vitamin A treated patients and healthy controls (p=0.043) (Table 3, Fig. 1).

Table 3: ΔCT and mean of IL-4 gene expression in fresh cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of IL-4 In fresh cells	Before	6.25±1.47	6.33±1.46	6.42±1.40	0.932
	After	4.02±1.65	5.84±1.22	5.15±0.91	0.002
	Difference	-2.50±1.12	-0.49±1.84	-1.27±1.40	0.015[c]
	p-value[b]	0.0001	0.323	0.009	
Mean of IL-4 gene expression in fresh cells		12.71±14.67	2.88±3.65	3.54±3.11	0.038[c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test; c: Kruskal-Wallis test.

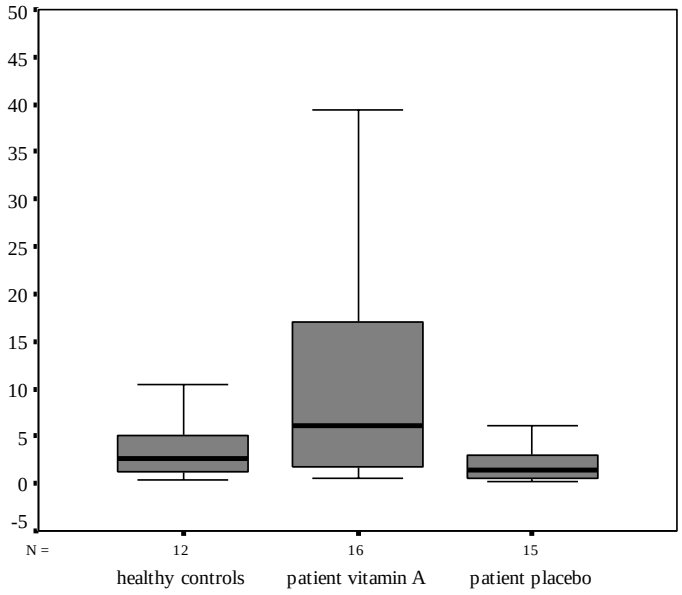

Fig. 1: Fold changes of IL-4 expression in fresh PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box.

IL-4 Gene Expression in PHA-Activated Cells

Significant differences were observed among the groups (p=0.037). The level of IL-4 gene expression in vitamin A treated patients, the placebo group and healthy controls showed 6.3- (p=0.035), 2.0- (p=0.609) and 2.4-fold (p=0.641) increases respectively. Multiple comparison showed a significant difference between the mean gene expression in vitamin A treated patients and the placebo group (p=0.027; Table 4, Fig. 2).

Table 4: ΔCT and mean of IL-4 gene expression in PHA-activated cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of IL-4 In PHA-activated cells	Before	10.25±1.81	8.82±2.14	9.46±1.90	0.138
	After	8.77±2.20	9.17±2.49	9.79±2.59	0.545
	Difference	-1.48±2.55	+0.35±2.57	+0.33±2.41	0.086[c]
	p-value[b]	0.035	0.609	0.641	
Mean of IL-4 gene expression in PHA-activated cells		6.36±5.82	2.04±2.67	2.44±3.76	0.037[c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test; c: Kruskal-Wallis test.

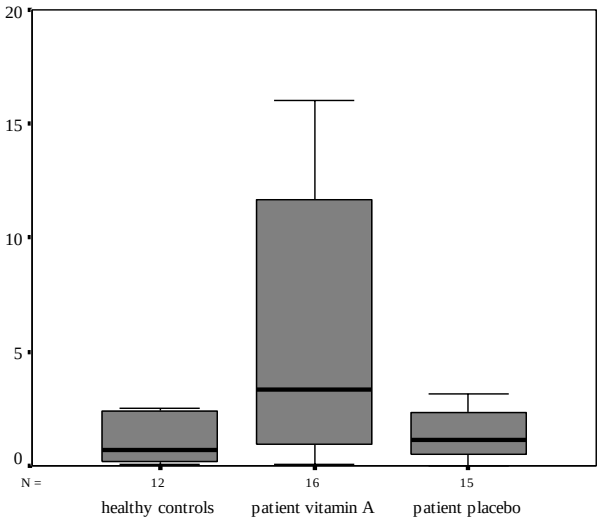

Fig. 2: Fold changes of IL-4 expression in PHA-activated PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box.

IL-4 Gene Expression in ox-LDL-Activated Cells

There were no significant differences in IL-4 gene expression in ox-LDL activated cells between the groups (p=0.737), despite all groups having increases in gene expression level, vitamin A treated patients, the placebo group and healthy controls showed 6.6- (p=0.003), 5.6- (p=0.008) and 3.4-fold (p=0.012) increases respectively (Table 5, Fig. 3).

Table 5: ΔCT and mean of IL-4 gene expression in ox-LDL-activated cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of IL-4 In ox-LDL -activated cells	Before	10.46±1.48	10.52±1.17	1.57±2.09	0.982
	After	8.75±2.03	9.14±1.80	9.35±2.77	0.762
	Difference	-1.71±1.89	-1.39±1.76	1.22±1.40	0.745[c]
	p-value[b]	0.003	0.008	0.012	
Mean of IL-4 gene expression in ox-LDL -activated cells		6.60±8.03	5.65±8.22	3.40±2.89	0.737[c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test; c: Kruskal-Wallis test

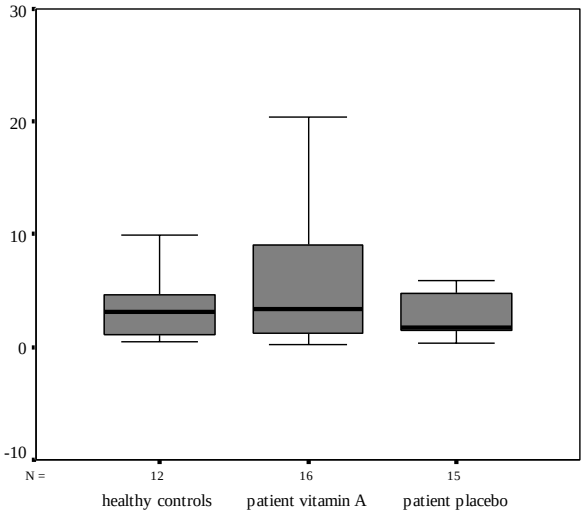

Fig. 3: Fold changes of IL-4 expression in ox-LDL-activated PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box

GATA-3 Gene Expression in Fresh Cells

Results of this study showed no statistically significant differences in GATA-3 gene expression in fresh cells, among the three studied groups (p=0.084). Vitamin A consumption led to 8.9- (p=0.016) and 3.8-fold (p=0.081) increases in gene expression in these patients and in the healthy controls respectively. The placebo groups show a 3.0-fold (p=0.608) increase (Table 6, Fig. 4).

Table 6: ΔCT and mean of GATA-3 gene expression in fresh cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of GATA-3 In fresh cells	Before	7.49±1.39	6.31±1.59	6.60±1.18	0.065
	After	5.81±1.61	6.66±2.00	5.65±1.08	0.225
	Difference	-1.68±2.48	+0.35±2.60	-0.94±1.71	0.063[c]
	p-value[b]	0.016	0.608	0.081	
Mean of GATA-3 gene expression in fresh cells		8.92±11.14	3.03±6.07	3.86±5.43	0.084 [c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test; c: Kruskal-Wallis test.

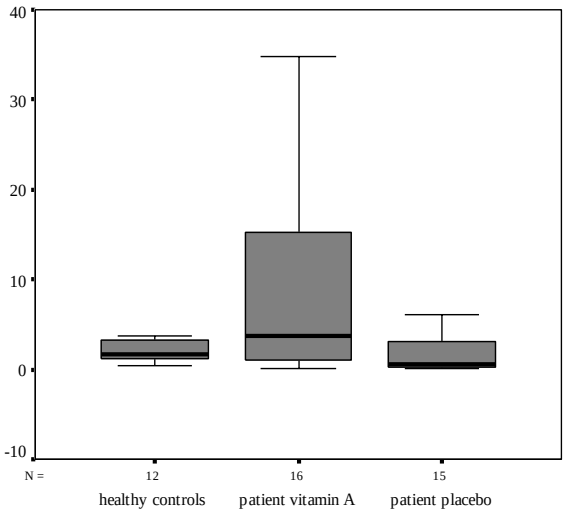

Fig. 4: Fold changes of GATA-3 expression in fresh PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box

GATA-3 Gene Expression in PHA-Activated Cells

Mean GATA-3 gene expression in PHA-activated cells showed statistically significant differences among the three groups studied (p=0.002). Multiple comparison showed there are difference between vitamin A treated patients and healthy controls (p=0.016). All groups had an increase in the gene expression after intervention. Vitamin A treated patients, the placebo group and healthy controls showed 9.2- (p=0.0001), 4.9- (p=0.0001) and 2.0-fold (p=0.208) increases respectively (Table 7, Fig. 5).

Table 7: ΔCT and mean of GATA-3 gene expression in PHA-activated cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of GATA-3 In PHA-activated cells	Before	8.35±1.44	7.01±0.97	6.36±0.76	0.0001
	After	5.67±0.79	5.40±1.60	5.85±1.55	0.678
	Difference	-2.68±1.28	-1.61±1.38	-0.51±1.31	0.0001[c]
	p-value[b]	0.0001	0.0001	0.208	
Mean of GATA-3 gene expression in PHA-activated cells		9.22±8.52	4.90±6.12	2.01±1.59	0.002[c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test; c: Kruskal-Wallis test

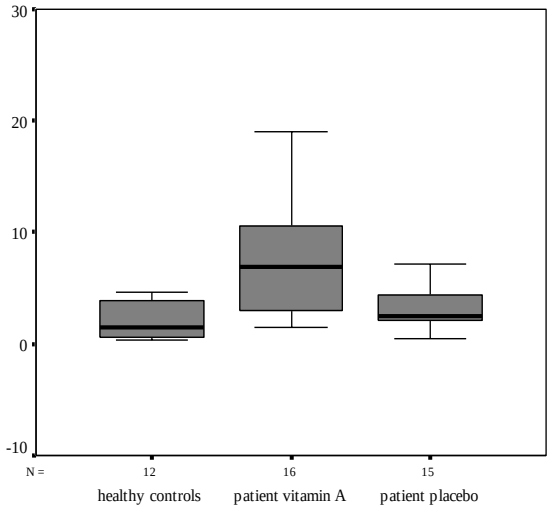

Fig. 5: Fold changes of GATA-3 expression in PHA-activated PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box.

GATA-3 Gene Expression in ox-LDL-Activated Cells

Results of this study showed statistically significant differences among the three studied groups (p=0.0001). The gene expression after intervention showed 4.4- (p=0.191) and 8.1-fold (p=0.001) increases in healthy controls and vitamin A treated patients respectively, but a 0.45-fold (0.0001) decrease in the placebo group. Multiple comparison showed a significant difference between the mean gene expression in vitamin A treated patients and the placebo group (p=0.019; Table 8, Fig. 6).

Table 8: ΔCT and mean of GATA-3 gene expression in ox-LDL-activated cells

		Patients		Control group n=12	p-value[a]
		Vitamin A n=16	Placebo n=15		
ΔCT of GATA-3 In ox- LDL -activated cells	Before	7.60±1.37	4.47±1.91	6.73±1.43	**0.0001**
	After	5.68±1.50	6.72±1.51	5.76±1.71	0.143
	Difference	1.92±1.80	2.26±1.56	0.97±2.41	0.0001[c]
	p-value[b]	0.001	0.0001	0.191	
Mean of GATA-3 gene expression in ox-LDL -activated cells		8.16±11.17	0.46±0.94	4.49±5.48	0.0001[c]

Data are reported as means ± SD; ΔCT = CT of target gene – CT of β-actin; a: One-way ANOVA; b: Paired-sample t test, c: Kruskal-Wallis test

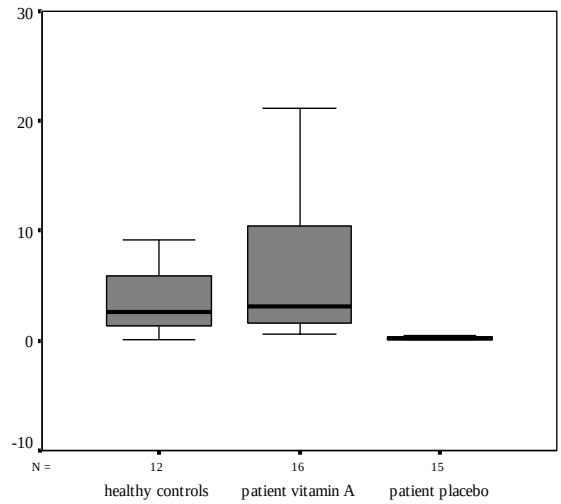

Fig. 6: Fold changes of GATA-3 expression in ox-LDL-activated PBMCs after supplementation. Box plot with whiskers displays the interquartile range. The bottom box (lower quartile) and top box (upper quartile) represent the 25th and 75th percentile, respectively. The median value is represented by the horizontal line in the box.

DISCUSSION

This study was designed to determine the influence of vitamin A supplementation on the gene expression of IL-4, and GATA-3, in 31 patients and 12 healthy controls during a 4 month consumption of vitamin A.

In the current study no significant differences were observed, among the 3 studied groups, in both SGOT and SGPT enzyme levels, similar to findings of Cartmel *et al*. [17] study. Other studies have also shown the same results in SGOT and SGPT levels in patients and control groups [18-19]. However contrary to the findings mentioned, consumption of 25000 IU vitamin A, led to liver injury and increased of its enzyme levels in the Geubel *et al*. [20] study.

The results of this study showed that the intervention caused an increase in Th2 cells gene expression in all groups except for expressing GATA-3 gene in ox-LDL-activated cells in the placebo group in which a decrease of gene expression occurred. The expression of Th2 related genes was more remarkable in vitamin A treated patients, compared with other groups.

Our results showed a significant increase in IL-4 gene expression in fresh and PHA-activated PBMCs obtained from vitamin A treated patients, compared with the placebo group. Also GATA-3 gene expression in ox-LDL-activated cells showed an increase in both groups of vitamin A consumers, while it decreased in the placebo group.

High levels of oral vitamin A supplement are reported to lead increased production of Th2 cytokines and Immunoglobulin A responses but also decreased Th1 cytokines [21]. Kang *et al*. [22] showed that treating mice microphages with retinoic acid caused an increase of IL-4 production in antigen-primed $CD4^+$ T cells. Although Abbasalizadeh *et al*. [23] indicated that vitamin A treatment significantly reduced serum concentration of IL-1b and IL-4 in obese women, this reduction was more remarkable in IL-1b. On the other hand, in another study [24], 8 weeks treatment with ATRA caused a significant decrease in inflammatory cytokines (IL-6 and TNF-α), plasma lipids and improvement in aortic lesions in atherosclerotic rabbits. In the Hoag *et al*. [25] study it was seen that ATRA by affecting function of antigen cells (APC) in the presence of exogenous IL-4, led to development of Th2 cells. Although Stephensen *et al*. [26] reported that 9-cis RA and RXR agonist, AGN194204, caused the development of Th2 cells in $CD4^+$ T-cells and clonotypic TCR cells stimulated by APC from mice DO-11.10 TCR-trans-genic and also development of Th2 within lymph node $CD4^+CD62L^{high}$ cells derived from normal mice C57BL/6 under stimulation of CD28 and CD3 antibodies. The Stephensen study shows the probable role of RXR in the development of Th2 cells. In line with our results, the study done by Iwata *et al*.[27], demonstrated the role of ATRA, 9-cis RA and RAR agonists but not that of RXR agonists, in the development of Th2 cells, although the time of adding of RA into the culture media also affects the process. Also it was seen that RAR antagonists and not RXR antagonist inhibit development of Th1 and Th2 cells. Meanwhile it was observed that GATA3 and IL4Rα genes in the presence of 10 nM concentration of ATRA were expressed. In the study of Nozaki *et al*. [28] no changes were seen in the production of IL-4 due to ATRA, in patients with rheumatoid arthritis or in the control group. The Dawson *et al*. study [14] demonstrated that stimulation of PBMC cells by ATRA and 9-cis RA cause increase in IL-4 levels. In a study conducted by YU *et al*. [29] on vaccine receiving mice, showed the increased level of mRNA IL-4 following treatment by ATRA.

Results of studies on the role of IL-4 in atherosclerosis show contradictions. Some studies [11-12, 26] have found that increased IL-4 can worsen atherosclerosis, and the deficiency of this cytokine can even reduces the size of the lesions. However, Huber *et al*. [10] study report the protective effect of Th2 cells on the development of primary fatty streaks in BALB/c mice with mild hypercholesterolemia. Data revealed that reinforcement of Th2 cells responses neutralized pro-atherogenic effects of Th1 cells, confirming the protective role of Th2 cells [30]. In the Schulte et al. study [31], the formation of primary fatty streaks was delayed in mice that had active Th2 cells responses. Among the anti-atherogenic effects of IL-4, prevention of the proliferation of muscle cells and macrophage adhesion should be pointed out [32]. In the Engelbetsen *et al*. [33] Study, high numbers of Th2 cells and IL-4 were independently associated with a decreased risk of acute myocardial infarction and CVD, Tracy *et al*. [34] also observed a significant negative association of Th2 effect with common carotid IMT.

This study had several limitations. One of the limitations was inability to stop taking all medications used by patient in order to exclude possible effects due to ethical considerations. Another limitation was budget constraints that would foreclose the possibility of further investigation on role of specific antigen, HSP60 and more Th2-drived cytokines. Finally, in this study there was no possibility of separating patients according to disease stage such as stable angina, unstable angina and myocardial infarction.

CONCLUSION

The present study is the first study done on humans with the aim of understanding the role of vitamin A in the process of atherosclerosis is the view of Th2 cells. It seems that given the protective role of regulatory T cells in the progression of atherosclerosis, and increase in population of these cells following to vitamin A supplementation in most cases in patients with atherosclerosis, we can concluded that increased expression of Th2 cells even though these cells considered as pro-atherogenic, cannot be caused adverse effects in patients with atherosclerosis.

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