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The Impact of Vitamin D Supplementation on the IFNγ-IP10 Axis in Women with Hashimoto's Thyroiditis Treated with Levothyroxine: A Double-blind Randomized Placebo-controlled Trial

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ABSTRACT

Hashimoto's thyroiditis (HT) results from chemoattraction of inflammatory cells toward the thyroid gland by inducing the production of interferon-gamma (IFNγ)-induced protein 10 (IP10) by T helper (Th) 1 cells. Vitamin D may suppress the IFNγ-IP10 axis, but this new function of vitamin D has not yet been investigated in HT patients.

In an intervention and control group, patients received 50000 IU cholecalciferol or placebo every week for three months, respectively. The CD4+ T cells of 40 patients were isolated, and the mRNA expression levels of vitamin D receptor (VDR), peroxisome proliferator-activated receptors (PPAR)-α, and PPAR-γ genes were determined by real-time PCR. ELISA method was used to determine serum levels of vitamin D, tumor necrosis factor-alpha (TNF-α), IFN-γ, and IP10.

Vitamin D levels in the intervention group were significantly higher than in the placebo group after supplementation. PPAR-α and PPAR-γ gene expression levels did not differ significantly between the two groups. The serum levels of IP10, IFNγ, and TNF-α decreased significantly in the vitamin D group, as well as in the placebo group.

During this study, vitamin D levels significantly increased in the intervention group and inflammatory factors decreased. Based on the similar results obtained in the placebo group, further studies with larger sample sizes and longer intervention times are recommended.

Keywords: CD4-positive T-lymphocytes; Hashimoto disease; Interferon-gamma; Peroxisome proliferator-activated receptors; Th1 cells; Vitamin D

INTRODUCTION

Hashimoto's thyroiditis (HT) is an autoimmune thyroid disease (AITD) identified by inflammatory cells' migration into the thyroid gland, biosynthesis of antibodies against thyroglobulin (TG), and

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thyroperoxidase (TPO). It is associated with hypothyroidism resulting from the destruction and fibrosis of thyroid follicular cells.[1,2] Autoimmune thyroiditis occurs due to immune system dysregulation and invasion of immune cells to the thyroid gland.[3] A series of statistical data, modeling, and twin studies have suggested the role of genetic factors in about 80% of AITD cases.[4] The remaining 20% is related to environmental factors such as stress, smoking, level of dietary iodine, selenium absorption, infections, and vitamin D deficiency.[5] In general, up to 5% of the general population with a female to male ratio of 4 to 1 suffer from AITD disease.[6] T helper (Th)1 cell together with interferon-gamma (IFNγ) and its associated chemokines Chemokine C-X-C motif ligand (CXCL) 9, 10, and 11 play a significant role in the infiltration of leukocytes into the thyroid gland. The destruction of thyroid cells by cellular and humoral mechanisms starts following the recruitment of the autoimmune lymphocytes in the thyroid gland.[7,8] CXCL10, also known as IFNγ-induced protein 10 (IP10) and its receptor, Chemokine C-X-C motif Receptor (CXCR) 3, play an important role in the recruitment of leukocytes in the inflamed tissue and the process of tissue destruction. Recent studies found an increase of IP10 in serum and/or tissues of autoimmune diseases,[9] cancer,[10,11] and chronic hepatitis C patients .IP10 production is not seen in basal conditions in the primary culture of normal thyroid cells. IFNγ induces the secretion of IP10 in a dose-dependent manner.[12] While tumor necrosis factor-alpha (TNF-α) alone does not affect IP10 secretion, its combination with IFNγ has a synergistic effect on IP10 secretion by thyrocytes compared to IFNγ alone.[13] The cells of the immune system, including activated T cells, dendritic cells, and macrophages, express vitamin D receptors (VDRs) and respond to 1,25 (OH) 2D3, the active form of vitamin D. Vitamin D and its receptor are effective on nuclear factor kappa B (NF-Kb), c-Jun N-terminal kinase (JNK), and signal transducer and activator of transcription (STAT) key pathways that regulate inflammatory responses. The NF-Kb pathway has a central role in inflammatory reactions.[14-18] Mayne et al, found that vitamin D could not suppress experimental autoimmune encephalomyelitis when T cells lacked VDR function.[19] Vitamin D prevents T cell infiltration to the central nervous system (CNS), strengthening this hypothesis.[20] One member of the nuclear receptor superfamily, peroxisome proliferator-activated receptor (PPAR), is involved in fat cell differentiation and lipid metabolism. Studies have shown that PPAR-α and PPAR-γ isoforms are expressed in T lymphocytes and other immune cells.[21-23] In addition to their metabolic roles, PPARs are involved in regulating immune reactions by suppressing the activity of the T-bet transcription factor as well as other transcription factors that are effective in inflammation, like NF-kB.[24-26] On the other hand, there is evidence of the enhancing effect of the VDR on the PPAR-α. Therefore, it can be concluded that vitamin D may have anti-inflammatory properties in the immune system via its impact on the VDR and increased the PPAR-α expression.[27] In this study, the effects of vitamin D supplementation on the IFNγ-IP10 axis in HT women were investigated.

MATERIALS AND METHODS

Study Design

Forty people with HD whose disease was confirmed by a specialist in Erfan and Imam Khomeini hospitals were included in this study. The Ethics Committee of Tehran University of Medical Sciences approved this trial study (Ethics committee approval code: IR.TUMS.SPH.REC1396.3685), and this study was registered in the Iranian Registry of Clinical Trials (IRCT2016110130644N1). Willingness to participate in the study was confirmed by written informed consent. All participants received fixed doses of levothyroxine (LT-4) during the study (Table 1).

Inclusion and Exclusion Criteria for Selecting the Patients

Women with HD, aged 18-48 years, with BMI between 18.5-30, who have been treated with LT-4 for 6 months, and are willing to participate in the study voluntarily, were enrolled.

Patients with severe known hepatic, biliary, pancreatic, and fatty liver disease, malnutrition, and obesity treated with vitamin D supplementation within three months before the study were not registered in this investigation. Patients with diseases affecting the balance of CD4+ T cells such as asthma, active viral diseases, and any autoimmune disease did not enroll in this study. Moreover, patients had no conditions such as pregnancy, lactation, alcoholism, history of stroke, or MI.

During the study, patients who were reluctant to cooperate, whose treatment protocol was changed by a

physician, or who had any sensitivity to vitamin D were excluded from the study.

Sample Size Calculation

The concentration of IP10 was used to calculate the sample size to detect a difference of 35 pg/mL between HT patients and control subjects with a standard deviation of 34 pg/mL,[28] power of 90%, and type 1 error of 5%, 20 subjects were needed in each group according to the following formula:

$$n = \frac{(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta})^2 \times 2\sigma^2}{\Delta^2} = \frac{(1.96+1.28)^2 \times 2 \times 34^2}{35^2} = 20$$

Groups and Interventions

The patients were assigned to two groups of 20 using double-blind randomization. However, 2 patients in the intervention group could not finish the study. The intervention group received 50000 IU vitamin D (cholecalciferol) per week orally for three months, and the control group received a placebo, edible paraffin. A general information questionnaire and a 24-hour diet recall were completed for patients before and after the study. Ten sex-matched subjects who met the criteria were selected as control subjects to measure the serum levels of IFNγ, TNF-α, and IP10 compared with the intervention group.

Separation of Peripheral Blood Mononuclear Cells (PBMC) and CD4⁺ T Lymphocytes

Fasting blood samples (10 mL) were collected from all subjects before and after the intervention, and PBMCs were isolated using the Ficoll method. A negative selection $CD4^+$ T cell isolation kit (Miltenyi Biotec Co.) was used to isolate $CD4^+$ T lymphocytes from PBMCs. A cocktail of biotin-binding antibodies was added to PBMCs and then incubated with magnetic streptavidin nanobeads. Due to its high affinity, magnetic streptavidin nanobeads bind to biotin. This complex then passed through a magnetic separator column which absorbed unwanted cells. The untouched CD4 Naïve T cells are collected by decanting the liquid in a clean tube.[29]

Quantitative Real-time PCR

The RNeasy mini kit (Qiagen) extracts RNA from $CD4^+$ T lymphocytes. After quantitative and qualitative evaluation of the extracted RNA by spectrophotometry (A260/A280 ratio) and agarose gel electrophoresis (1.5% agarose gel), the QuantiNova Reverse Transcription Kit (Qiagen) was used for cDNA synthesis from the extracted RNA. The Beacon Designer, Oligo Analyzer (IDT), and NCBI Primer Blast tools were applied to design primers for the real-time PCR reactions (Supplementary Table).

ELISA

According to the manufacturer's instructions, the enzyme linked immunosorbent assay (ELISA) kits were used to evaluate the levels of IFNγ (Invitrogen 88-7316-22), TNF-α (Invitrogen 88-7346-22), IP10 (R&D DY266-05), and vitamin D (25(OH)D3) (Monobind Inc., Lake Forest, CA, USA). The ELISA kits contained two uncoated microtiter plates, pre-matched antibody pairs, and reagents for performing quantitative ELISA tests. The detection ranges for IFNγ, TNF-α, IP10 and vitamin D were 4-500 pg/mL, 4-500 pg/mL, 31.20-2000 pg/mL and 2-120 ng/mL, respectively.

Table 1. Patients' Characteristics

Characteristics	Vitamin D group	Placebo group	*p*
Age (years)	36.4±5.2	35.9±7.8	0.81
Body Mass Index (kg/m²)	25.65±5.1	27.80±5.74	0.22
Mean LT-4 dose (μg/day)	102±66	113±65	0.63
TSH (μIU/mL)	3.7 (3.3)	4.3 (7.1)	0.75
Anti-TPO (IU/mL)	258.1 (132.9)	312.0 (122.68)	0.2
Anti-TG (IU/mL)	551.1 (1094.11)	395.6 (812.93)	0.61

Mean± SD, *LT-4*: Levothyroxine, *TSH:* Thyroid-stimulating hormone, *TPO:* Thyroid peroxidase, *TG:* Thyroglobulin

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Statistical Analysis

The SPSS software version 24 was used for data analysis. Mean, and standard deviation are used to describe the data. The Kolmogorov-Smirnov and Shapiro-Wilk tests were applied for normality check. If the data were distributed normally, the mean values of the variables were compared before and after the intervention using the paired t-test in each group and independent t-test between the two groups; otherwise, the Wilcoxon signed ranks, and Mann-Whitney U test was used, respectively. Analysis of covariance was administered to control the effect of confounders. *p* values less than 0.05 were considered significant.

RESULTS

The Serum Level of Vitamin D in the Intervention and Placebo Groups

The serum level of vitamin D (25-OH Vit D3) changed from 25.29±11.33 to 50.65±15.31 ng/mL in the vitamin D group and from 19.90±9.03 to 22.19±9.68 ng/mL in the placebo group, indicating the effectiveness of intervention in the vitamin D group compared to the placebo group (Figure 1).

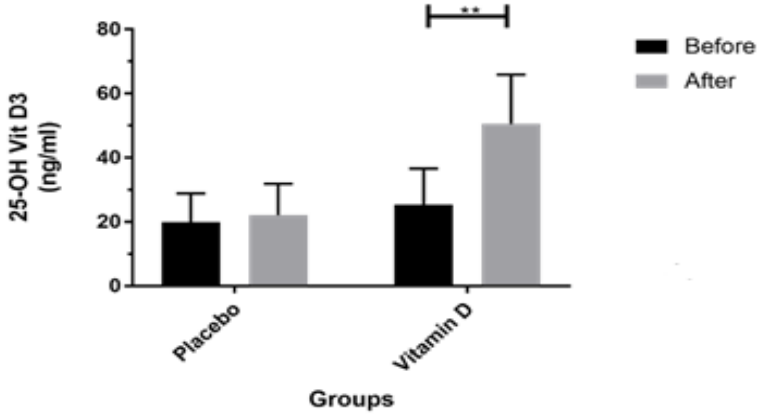

Figure 1. The study groups evaluated the effect of vitamin D before and after supplementation on the serum level of 25-OH Vit D3 (vitamin D). Data are presented as means±SD. *****p*<0.01 (Significant compared with the control group)

Expression of VDR, PPAR-α, and PPAR-γ Genes in Vitamin D and Placebo Groups

The results of VDR gene expression in $CD4^+$ T cells after supplementation indicated a 0.92-fold decrease in vitamin D and a 0.96-fold decrease in the placebo group; however, the statistical comparison was not significant (*p*=0.949) (Figure 2a). After supplementation, the PPAR-α gene expression was increased by 1.82-fold and 1.5-fold in vitamin D and placebo groups, respectively (*p*=0.465) (Figure 2b). The expression level of the PPAR-γ gene decreased by 0.93-fold in the vitamin D group and increased by 1.09-fold in the placebo group after supplementation (*p*=0.600) (Figure 2c).

Serum Levels of Chemokine and Cytokines in Vitamin D and Placebo Groups

IFNγ

After the intervention, the serum concentration of IFNγ significantly decreased from 13.86±7.91 to 8.39±4.76 pg/mL in the vitamin D group (*p*=0.001). Similarly, the serum level of this cytokine decreased from 14.13±8.19 to 8.81±5.55 pg/mL in the placebo group, which was significant (*p*=0.000). The statical comparison of IFNγ concentration in vitamin D and placebo groups showed not significant changes (*P*=0.868). The mean level of IFNγ in healthy control was obtained at 13.59±10.62 pg/mL (Figure 3a).

TNF-α

After supplementation, the serum concentration of TNF-α was decreased significantly from 29.70±18.43 to 15.25±10.84 pg/mL in the vitamin D group (p=0.010) and from 26.66±24.81 to 12.33±11.83 pg/mL in the placebo group (p=0.008). The difference in the TNF-α level showed a decrease of 14.45 and 14.33 pg/mL in the vitamin D and placebo groups, respectively which obtained non-significant (p=0.987). The mean serum level of TNF-α was 5.03±3.99 pg/mL in healthy control (Figure 3b).

IP10

The mean serum concentration of IP10 was decreased significantly from 76.92±34.85 to 22.71±17.91 pg/mL in the vitamin D group (p=0.000) and from 78.10±38.03 to 34.03±23.32 pg/mL in the placebo group (p=0.000). The difference in the IP10 level showed a non-significant decrease of 54.20 and 44.08 pg/mL in the vitamin D and placebo groups, respectively (p=0.682). The mean serum level of IP10 in healthy control was obtained at 15.98±28.03 pg/mL (Figure 3c).

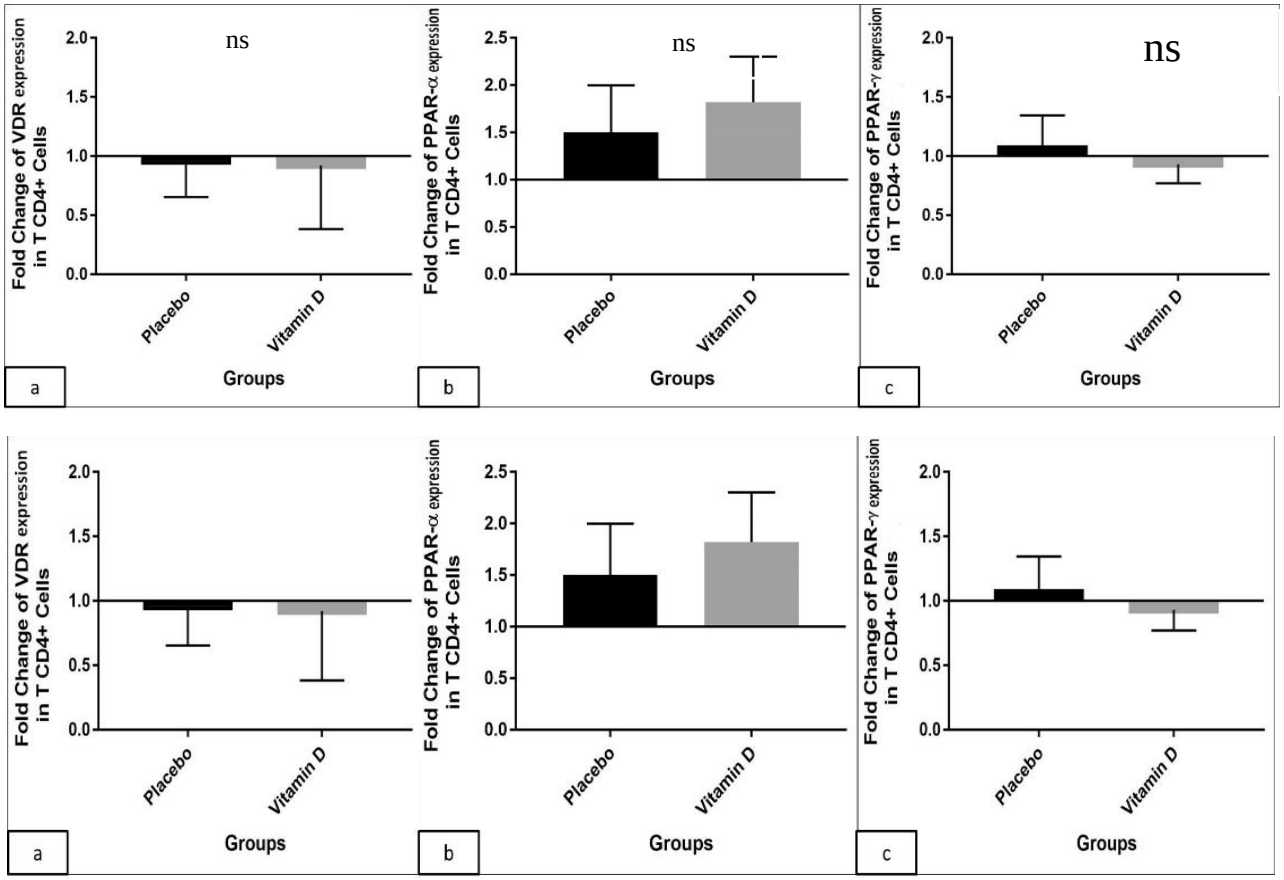

Figure 2. The results of vitamin D receptors (VDR) (a), peroxisome proliferator-activated receptor (PPAR) -α (b), and PPAR-γ (c) gene expression changes in $CD4^+$ T cells in the study groups. After supplementation, the expression of the VDR gene was non-significantly decreased in the vitamin D and placebo groups (a). After supplementation, the PPAR-α gene expression was non-significantly increased in vitamin D and placebo groups (b). The expression level of the PPAR-γ gene non-significantly decreased in the vitamin D group; also, in the placebo group, it was increased non-significantly after supplementation (c). Data are presented as means ± SD. ns: non-significant.

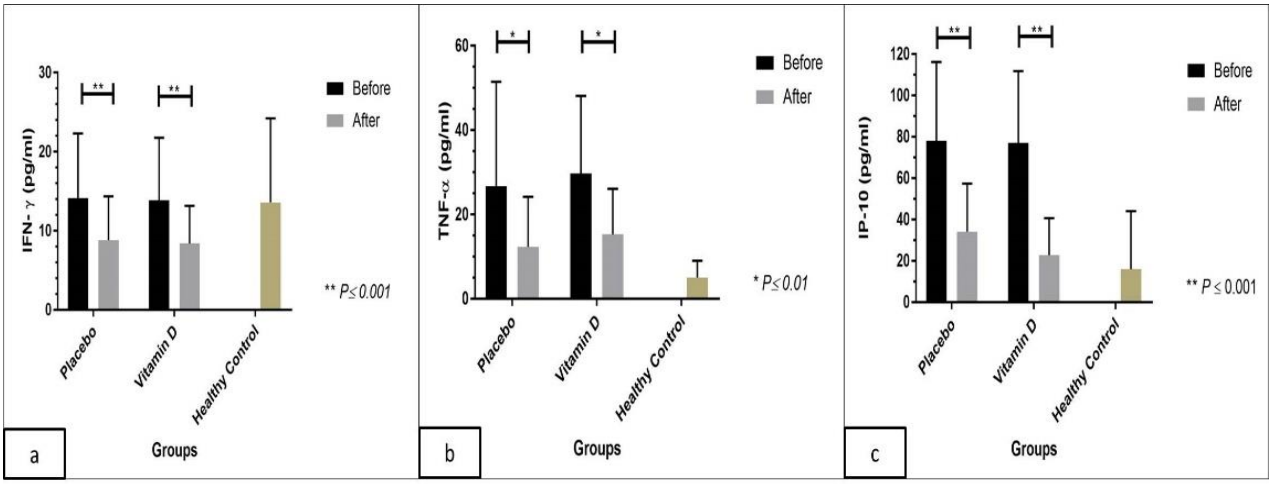

Figure 3. Evaluation of the difference in interferon-gamma (IFNγ) (a), *tumor necrosis factor-alpha* (TNF-α) (b), and induced protein 10 (IP10) (c) levels before and after three months of vitamin D supplementation in the study groups. Moreover, the mean serum level of these cytokines in healthy control has been shown. After the intervention, the serum concentration of IFNγ (a), TNF-α (b), and IP10 (c) were significantly decreased in the vitamin D and placebo groups. Data are presented as means ± SD. *: $p<0.05$, **: $p<0.001$. Significant compared with the control group

Correlation Test

The results of the Pearson's correlation test showed a moderate negative correlation between the dose of LT-4 and the serum level of thyroid-stimulating hormone (TSH) (r=-0.528, p=0.002), indicating a decrease in the serum level of TSH with an increase in the dose of LT-4 (Figure 4a). On the other hand, a correlation was found between the levels of TSH and IFNγ in HT patients (r=0.378, p=0.036), indicating a linear low positive correlation between these two variables in the same direction, i.e., the level of IFNγ decreased with a decrease in the serum TSH (Figure 4b). In addition, there was a linear low positive correlation between the serum levels of TSH and IP10 (r=0.383, p=0.018), indicating a decrease in the serum IP10 with a reduction in serum TSH (Figure 4c).

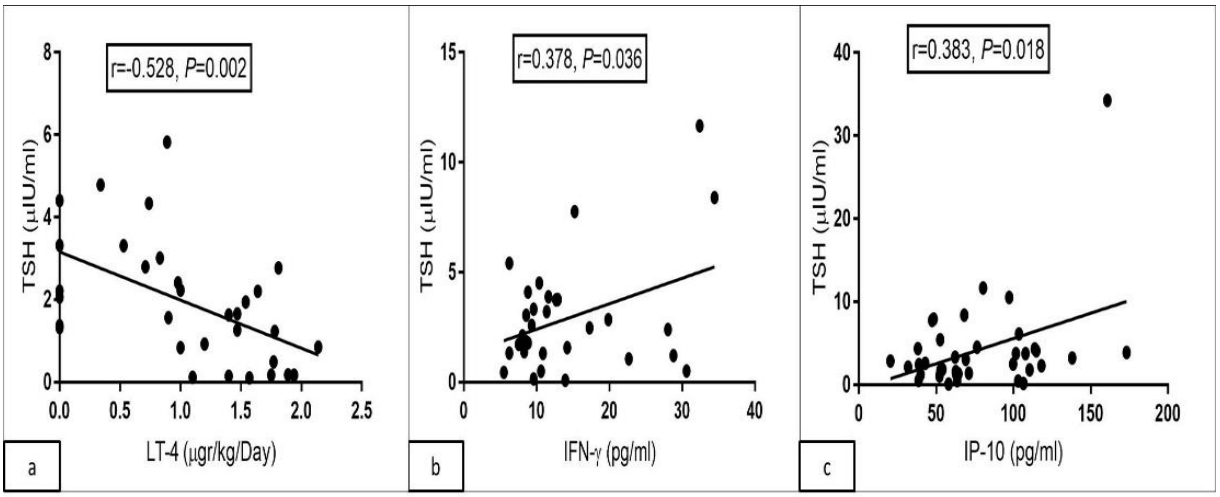

Figure 4. The linear regression between the dose of levothyroxine (LT-4) and thyroid-stimulating hormone (TSH) (a), IFN-γ and TSH (b), and IP10 and TSH (c) in serum of Hashimoto's thyroiditis (HT) patients. The results of the Pearson's correlation test showed a moderate negative correlation between the dose of a LT-4 and serum level of TSH (r=-0.528, p=0.002) (a) and a linear low positive correlation between the levels of TSH and IFNγ in HT patients (r=0.378, p=0.036) (b). Moreover, there was a linear correlation between the serum levels of TSH and IP10 (r=0.383, p=0.018) (c).

DISCUSSION

Vitamin D supplements have the fewest side effects compared to drugs currently used for HT management. Therefore, if the positive effects of vitamin D are confirmed in terms of IFNγ-IP10 axis modulation through decreasing their secretion in the serum, increasing the expression of their inhibitors, and preventing disease progression, vitamin D supplementation can be included in the treatment protocol for these patients. Moreover, it can be recommended as a supplement to improve other autoimmune disorders involving IP10 overproduction. According to the results, vitamin D supplementation was effective and significantly increased in the intervention group. The serum level of vitamin D was improved from insufficiency (before the intervention) to sufficiency status (after the intervention). At the same time, the subjects in the placebo group remained at the insufficiency level. In 2011, Kivity et al, evaluated the serum level of vitamin D, the relationship between vitamin D deficiency and AITD, thyroid function, level of anti-thyroid autoantibodies, and demographic characteristics in a population of patients with autoimmune thyroid disease. They found that the thyroid function was more disturbed in patients with a vitamin D level below 10 ng/ml (deficient) than those with a vitamin D level above 10 ng/mL.[30] As vitamin D deficiency and AITD are more common in women; their relationship is also very probable. The higher levels of autoantibodies against TG and TPO and the lower serum levels of vitamin D are more prevalent in women versus men.[31] Some researchers have reported a correlation between vitamin D and estrogen in developing AITD. However, the relationship between lower levels of vitamin D and higher prevalence of AITD is much more common before menopause compared to the postmenopausal period.[32] In addition, Zhang et al, found that the serum level of TSH had an inverse correlation with the level of vitamin D independent of thyroid hormones.[33] The above evidence indicates a correlation between vitamin D deficiency and hypothyroidism.[34] IP10 is one type of CXC chemokines that is inducible by IFNγ and has an important role in the chemotaxis of lymphocytes during inflammation.[35] IP10 is a ligand of the CXCR3 receptor. There is a close relationship between CXCR3 expression and the differentiation of CTL and Th1 cells.[36-38] CXCR3 expression by Th1 cells plays an important role in the immune responses and induces autoimmune responses in specific organs. IP10 stimulates chemotaxis and directs the movement of CXCR3 expressing Th1 cells to the target tissues, which causes more inflammation there. On the other hand, producing IFN-γ by the called Th1 cells would strengthen the immune response at the site of inflammation. The production of IFN-γ is one of the most important inflammatory factors in target tissues.[39] In patients with HT, Th1 cells are the primary and most effective cell populations. These cells can activate CTL cells and macrophages and destroy the follicular thyroid cells. The severity of HT disease is directly related to the increase in the Th1 cell population relative to Th2.[40]

Using immunohistochemistry techniques, Garcia Lopez et al, showed that the protein expression of IP10 in HT thyroid glands was increased.[2] Moreover, the results of the Kemp et al, study showed that the IP10 chemokine expression in HT patients was higher than in patients without AITD.[12] It was also demonstrated that serum IP10 levels in HT patients were higher than those with AITD-like multi-nodular goiter and were correlated to TSH levels.[41] High serum IP10 levels in patients correlate to a hypoechoic pattern on ultrasound. The hypoechogenicity indicates a robust association between lymphocyte infiltration and poor thyroid function. Thus, IP10 is probably considered an important and strong factor in the inflammatory process, which leads to the destruction of the thyroid gland and ultimately hypothyroidism.[41-43] The results of previous studies on the impacts of thyroid autoimmunity or hypothyroidism on serum levels of IP10 showed that the impaired thyroid function correlated to higher IP10 levels that were obtained more than euthyroidism.[44] These findings are consistent with previous experimental data and show that the microenvironment of Th1 cells leads to apoptosis of thyroid cells and severe forms of hypothyroidism.[45] Since serum levels of IP10 in HT patients treated with LT4 showed no changes; it can be concluded that the autoimmune disease process is responsible for increasing serum IP10 concentrations in these patients.[44] In this study, we observed that the increased vitamin D levels in the patients' group reduced serum levels of IFNγ and IP10. Our findings showed that vitamin D reduces the serum level of IFNγ and affects the production of IP10. Presumably, decreased serum levels of IFNγ reduce the differentiation of Th1 cells,

which is one of the main pathogen agents in the HT and plays an important role in the destruction of the thyroid gland by causing inflammation and the production of inflammatory cytokines. In addition, decreased serum level of IP10 reduces the chemotaxis of inflammatory lymphocytes into the thyroid gland. According to these data, vitamin D is important in modulating the inflammatory axis of IFNγ-IP10 and can be a useful supplement in treating HT patients. TNF-α is another solid pro-inflammatory cytokine that is very impressive in the immune system during inflammation. Monocytes and macrophages produce TNF-α.[46,47] Compared with ordinary people, TNF-α is differentially expressed in autoimmunity disorders. As shown recently, in HT patients, TNF-α is overexpressed.[48] A study by Botelho et al, showed that vitamin D might play an important role in preventing HT by reducing the Th1 and Th17 cells cytokines such as IFNγ and TNF-α.[49] A meta-analysis study showed that lower vitamin D levels increased the risk of HT.[50] Studies have shown that an imbalance between Th1/Th2 cells, which ultimately leads to increased Th1 cell activity, leads to an increase in HT prevalence.[51] Vitamin D may suppress the immune system of HT patients by several mechanisms.[52] Binding of vitamin D to its receptor prevents the activation of T cells by DC, ultimately reducing the production of proinflammatory cytokines such as TNF-α and IFNγ, reducing the immune response.[53] By reducing the differentiation of naïve T cells to Th17, vitamin D causes an increase in the Treg cells population and reduces thyroid inflammation.[54,55] Another study showed that decreases in serum vitamin D levels were directly related to the pathogenesis of HT disease.[56] Another meta-analysis study showed that vitamin D plays an important role in the regulation of HT disease by reducing the expression of primary histocompatibility class II, stimulatory molecules (CD40, CD80, CD86) as well as the reduction of inflammatory cytokines TNF-α and IFNγ.[57] In the present study, we also observed that the serum levels of TNF-α were significantly decreased in the vitamin D group after supplementation. Probably vitamin D could inhibit the inflammatory cytokines by reducing serum levels of TNF-α and can be used to control the HT disease.

One of the critical limitations of this study is the psychobiological placebo effect. In the present study, some results were obtained similarly to the vitamin D supplementation group after supplementation. Unfortunately, we did not find a convincing reason for this response in the placebo recipients. We suggest that this exception be eliminated in future studies by increasing the sample size.

The results of the present study in the vitamin D group suggested that the inflammatory axis of IFNγ-IP10 and TNF-α are highly expressed in the serum of HT patients, and it can be reduced by vitamin D supplementation. However, because of the presence of some common complications in clinical trials, including the limitations of the sample size, the dose, and duration of supplementation, the other medications that patients receive during the intervention, and the existence of similar responses in the placebo group, more studies are needed to clarify the exact effect of the vitamin D on HT patients.

CONFLICT OF INTEREST

The authors declare no conflicts of interest for this work.

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