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THE ASSOCIATION BETWEEN SEVERITY OF VITAMIN D DEFICIENCY AND HASHIMOTO'S THYROIDITIS

Running title: Vitamin D and thyroiditis

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ABSTRACT

Objective: The relation between vitamin-D and autoimmune disorders has long been investigated regarding to important roles of this hormone in immune regulation. We evaluated 25-hydroxy-vitamin-D (25OHD) status of subjects with Hashimoto's thyroiditis (HT) and healthy controls.

Methods: Group-1 consisted of 180 euthyroid patients (123 females/57 males) with HT who were on stable dose of L-thyroxine (LT). Sex, age and BMI matched 180 euthyroid subjects with newly diagnosed HT were considered as Group-2 and healthy volunteers (n=180) were enrolled as controls (Group-3). All subjects (n=540) underwent thyroid ultrasound and were evaluated for serum 25OHD, anti-thyroid peroxidase(anti-TPO) and anti-thyroglobulin(anti-TG) levels.

Results: Group-1 had the lowest 25OHD levels (11.4±5.2 ng/mL) compared to newly diagnosed HT subjects (Group-2) (13.1±5.9 ng/mL, p=0.002) and to control subjects (15.4±6.8 ng/mL, p<0.001). Serum 25OHD levels directly correlated to thyroid volume (r=0.145, p<0.001) and inversely correlated to anti-TPO (r=-0.361,p<0.001) and anti-TG levels (r=-0.335,p<0.001). The 48.3% of group-1, 35% of group-2, and 20.5% of controls had severe vitamin-D deficiency (25OHD <10ng/ml). Female chronic HT patients had the lowest serum 25OHD levels (10.3±4.58ng/mL), while male control subjects had the highest (19.3±5.9ng/mL) (p<0.001).

Conclusions: We showed that serum 25OHD levels of patients with HT were significantly lower than controls and severity of vitamin-D deficiency correlated with duration of HT, thyroid volume and antibody levels. These findings may suggest a potential role of 25OHD in development of Hashimoto's thyroiditis and/or its progression to hypothyroidism.

Key words: vitamin D, autoimmunity, Hashimoto's thyroiditis

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Abbreviations:

25OHD = 25-hydroxy-vitamin-D_3 ; **Anti-TG** = anti-thyroglobulin; **Anti-TPO** = anti-thyroid peroxidase; **fT_3** = free tri-iodothyronine; **fT4** = free thyroxine; **HT** = Hashimoto's thyroiditis, **LT** = L-thyroxine; **TSH** = thyrotrophic hormone; **VDR** = vitamin D receptor

INTRODUCTION

Vitamin D is an important nutrient which has widespread effects on cellular proliferation, differentiation, apoptosis and angiogenesis. Exploration of vitamin D receptors (VDR) on immune system cells such as monocytes, macrophages, antigen presenting cells, dendritic cells and lymphoyctes, has recently opened wide possibilities for studies on the immune modulator role of vitamin D (1). Results of epidemiological studies indicated a significant association between decreased levels of serum 25-hydroxy vitamin D (25OHD) and increased incidence of several autoimmune diseases such as type 1 diabetes mellitus, multiple sclerosis, systemic lupus erythematosis (2). On the other hand, experimental data showed that administration of vitamin D prevented or suppressed some kinds of autoimmune diseases such as encephalomyelitis, arthritis, and inflammatory bowel disease (2-4). In several studies VDR polymorphism has long been suspected to associate with thyroiditis, based on the evidence that auto reactive T cells developed in the absence of active or functional VDR; however the results were conflicting or lack of the relation between vitamin D levels (5).

Hashimoto's thyroiditis (HT) is the most common autoimmune thyroid disease with a strong genetic predisposition involved in the etiology. The anti-thyroid immune response begins with activation of thyroid antigen specific helper T cells (6). Thyroid autoimmunity is also suspected to confer with human leukocyte antigen (HLA) class-II genes, as in animal models active vitamin D was shown to prevent autoimmune thyroiditis by down regulating the expression of HLA class II molecules on thyrocytes, inhibiting lymphocyte proliferation

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and secretion of inflammatory cytokines (7). Regarding to the potential casual relation between vitamin D deficiency and development of autoimmune diseases, we investigated those two prevalent issues in a group of Turkish people. In this study, we evaluated the relation of serum 25OHD levels with anti-thyroid antibody levels and thyroid volume of patients with Hashimoto's thyroiditis, and compared to healthy control subjects.

METHODS

Study subjects

Total number of 540 individuals was included in this cross-sectional research. Study population was evaluated for thyroid function, thyroid autoimmunity and ultrasonographic features before enrollment. Subjects; [1] who were not euthyroid [2] who had diabetes, malignancy, chronic renal and/or hepatic failure or autoimmune diseases [3] who were on oral contraceptive, antiepileptic or anti-osteoporotic medication had been excluded. None of the subjects were taking vitamin and/or calcium supplements. Subjects enrolled consecutively into three groups. Group-1 composed of 180 euthyroid follow-up subjects who were previously diagnosed with primary hypothyroidism due to Hashimoto's thyroiditis and on stable dose of L-thyroxine at least for six months. Group-2 included age, sex and BMI matched 180 euthyroid subjects who admitted to our clinic for routine check-up and who have been found to have Hashimoto's thyroiditis according to presence of thyroid auto-immune antibodies and ultrasonographic characteristics of HT. Group-3 (control group) consisted of 180 healthy volunteers with negative anti-thyroid antibodies and normal thyroid ultrasound. The study was performed between June and August, 2011. All subjects gave informed consent prior to study. Study was started after being approved by Diskapi YB Training & Research Hospital's local research ethics committee.

Measurements

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Serum samples were taken after an overnight fasting for routine biochemical investigation including renal and hepatic functions, serum electrolytes (sodium, potassium, calcium, phosphorus) and lipid profile. Thyroid tests including free tri-iodothyronine (fT3), free thyroxine (fT4), thyrotrophic hormone (TSH), anti thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibody were assessed by ECLIA (enzyme chemiluminescence immunoassay) method with a commercially available kit (Immulite 2000, Bio DPC, Los Angeles, CA, USA) with normal ranges for TSH: 0.55-4.78 mIU/mL, fT4:0.74-1.52 ng/dL, fT3:2.3-4.2 pg/mL, Anti-TPO: 0-35 IU/mL, and for Anti-TG: 5-40 U/mL. Any subjects who had not normal TSH, fT3 and fT4 levels (in other terms who were not euthyroid) and/or who had abnormal hepatic or renal functions were not included. Subjects with Anti-TPO <35 IU/mL and anti-TG<40 U/mL were considered as "negative" for thyroid autoimmunity. The diagnosis of Hashimoto's thyroiditis was based on [1] anti-TPO and anti-TG positivity [2] reduced parenchymal echo and healthy control group was negative for these antibodies. Thyroid ultrasound was performed by an expert and thyroid volume was calculated according to formula; thyroid volume: [right lobe (a x b x c) ml x 0.502] + [left lobe (a x b x c) ml x 0.502] /2. Measurements were done twice and the average value of each dimension was put in the formula.

The serum 25OHD concentration was determined by using a commercial ELISA kit (Immuno-biological laboratories, Minneapolis, USA) with normal ranges of 11.1-42.9 ng/mL. Vitamin D deficiency was described as "severe" for <10 ng/mL, "moderate" for 11-20 ng/mL, "mild" for 21-30 ng/mL serum levels of 25OHD for the summer period.

Statistical analyses

Data analysis was performed by using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normally or not was determined by Shapiro Wilk test. Levene test was used for the evaluation of

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homogeneity of variances. The mean differences among groups were compared by using One-Way ANOVA and Kruskal Wallis test was applied for comparisons of the median values. When the p value from Kruskal Wallis test statistics are statistically significant Conover's non-parametric multiple comparison tests was used to know which group differ from which others. The difference between gender groups was evaluated by Mann Whitney U test. Nominal data were analyzed by Pearson's Chi-square test. Degree of association between continuous variables was analyzed by Spearman's correlation test. A p value less than 0.05 was considered statistically significant.

RESULTS

Each of the study groups were consisted of 180 subjects (123 females, 57 males) who were not different in terms of mean age and body mass index (Table 1). The group-1 patients, who have chronic (Hashimoto's) thyroiditis and are taking L-thyroxine replacement, presented significantly lower 25OHD levels than the group-2 subjects, who have not been hypothyroid yet (11.4±5.2 ng/mL versus 13.1±5.9, $p<0.001$) and than the control subjects (15.4±6.8 ng/mL, $p<0.001$). All subjects were euthyroid as mentioned in the selection criteria. When compared to control population, all subjects with HT (Group-1 and Group-2) had significantly lower 25OHD levels (12.2±5.6 ng/mL versus 15.4±6.8 ng/ml, $p<0.001$). There was a significant negative correlation between serum 25OHD levels and Anti-TPO ($r= -0.361$, $p<0.001$) and Anti-TG levels ($r= -0.335$, $p<0.001$). On the other hand, significant positive correlation was found between serum 25OHD levels and thyroid volume ($r=0.145$, $p<0.001$).

When compared between genders, the mean serum 25OHD level of all female subjects (n=369) was significantly lower than males (n=171) (11.71±5.5 ng/mL versus 16.6±6.3 ng/mL, $p<0.001$) (Table 2). Male control subjects had the highest 25OHD levels (19.3±5.9 ng/mL) while female patients with HT and on L-thyroxine replacement (Group-1) had the lowest (10.3±4.5 ng/mL) ($p<0.001$). The female group-2 subjects presented the second

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lowest 25OHD levels (11.4±6.4 ng/mL) and followed by control female subjects (13.5±4.9 ng/mL).

The comparison of characteristics of all study subjects according to severity of vitamin D deficiency is shown on Table-3. Majority of all study subjects (49.6%) presented moderate vitamin D deficiency (10≤ 25OHD <20ng/ml). While the majority (48.3%) of HT patients on L-thyroxine therapy (group-1) presented severe vitamin D deficiency (25-OH-D_3 <10ng/ml), 35% of Group-2 and 20.5% of controls had severe vitamin D deficiency (p<0.001). Only 10 subjects had 25OHD ≥ 30 ng/ml and 60% of them were in the control group.

DISCUSSION

Vitamin D deficiency is a prevalent health problem in Turkey. Erkal et al had showed that more than 78% of Turkish people had below 25 ng/ml serum 25OHD levels independent of where they resided (9). In another study, Ergür and colleagues revealed the frequency of severe vitamin D deficiency as 27% among women in reproductive age (10). However there is not enough data about vitamin D levels of patients with Hashimoto's thyroiditis. The higher prevalence of vitamin D deficiency in our study population can be attributable to presence of Hashimoto's thyroiditis. In our study population 94.4% of subjects had 25OHD < 25 ng/mL, while 42.8% of females presented severe deficiency. Tamer and colleagues reported lower serum vitamin D levels in HT patients (16.3±10 ng/ml) compared to healthy control subjects (29.6±25.5) in a younger population with mean age 34.8 (11). Similarly, Kivity et al found below 10 ng/ml 25OHD levels in 79% of patients with HT (12). Distinct from these two studies, we excluded the HT patients who were not in euthyroid status because of the potential effects of hypo-or-hyperthyroidism on vitamin D metabolism directly and/or through effecting lipid metabolism and renal functions (13). On the other hand, LT4 replacement therapy may influence the metabolic clearance of vitamin D, as a matter of fact which should be evaluated by in further analyses. Seasonal variation and body mass index are other two

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important confounding factors which must be excluded before evaluating the vitamin D levels, as we did in our study.

We found statistically significant negative correlation between serum 25OHD and anti-TPO, anti-TPO levels in HT patients. The results of previous studies about the correlation between anti-thyroid antibody levels and vitamin D deficiency were pertained to the immune modulator role vitamin D (12,14). The activation of thyroid antigen specific helper T cells induces B cells to secrete thyroid antibodies which are associated with thyroid damage and lymphocytic infiltration (6). T-lymphocytes and macrophages are known to present higher concentrations of vitamin D receptors (VDRs) than B cells (15). Active form of vitamin D regulates T cell response through its receptors as [1] decreasing the proliferation of T helper cells and production of cytokines such as IL-2, IL-5, IFN-γ and TNF-α which are essential for cell-mediated immune response and antibody mediated immunity (15,16), [2] increasing the production of IL-4 and transforming growth factor in T helper 2 cells which may suppress inflammatory activity (4), [3] inhibiting dendritic cell differentiation and maturation and promoting their apoptosis, down regulating the expression of MHC-II (17). In experimental models of autoimmune thyroiditis, thymically derived T cells, which express the highest amounts of VDRs, mediated protection against autoimmunity (4,18). Most of the studies concerning with the relation between VDR polymorphism and autoimmune diseases revealed that the absence of functional VDR associated with increased prevalence of autoimmune thyroiditis, however some did not (5, 19, 20). These results suggested that vitamin D deficiency might affect induction of thyroid autoimmunity via up regulating the immune response of helper T cells and expression of MHC-II antigens on thyrocyte surface. These data may explain why the subjects with the lowest vitamin D levels present the highest anti thyroid antibody titers. On the other hand, the lowest vitamin D levels may be related to LT4 replacement as a result of an increment in vitamin D metabolism. Thyroid volumes of the

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group 1 subjects who are on LT4 replacement were smaller, as expected. However, anti body positive euthyroid subjects (group 2) would have greater thyroid volumes than control group. Although statistically non significant, this discordance may be due to sample size or to iodine deficiency of the subjects in the control group. Further placebo controlled prospective studies and laboratory analyses are needed to evaluate how vitamin D replacement therapy would influence serum anti body titers or how LT4 replacement may influence vitamin D metabolism. The urinary excretion of iodine must be kept in mind as a factor to evaluate, especially in the epidemic areas for iodine deficiency such as Turkey, while designing studies.

In conclusion, we showed that patients with Hashimoto's thyroiditis present lower vitamin D levels inversely correlated to antibody levels and directly correlated to thyroid volume. Finally, our results suggested that there might be a casual relation between vitamin D deficiency and development of Hashimoto's thyroiditis. On the other hand, there might be a possible relation between severity of vitamin D deficiency and progression of thyroid damage. However further studies are needed especially about the effects of vitamin D supplementation on prevention and/or progression of autoimmune thyroid disease.

DISCLOSURE

The authors of this manuscript have no relevant interests to disclose

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Table 1: Characteristics of study population

	Group-1	Group-2	Controls	Total	*P* value
Number *(n)*	180	180	180	540	
Female	123	123	123	369	
Male	57	57	57	171	
Age *(mean ±SD)*	42.8±12.4	42.3±10.2	42.5±10.4	42.5±11	0.919
BMI *(kg/m^2)*	28.1±4.3	28.4±4.5	28.9±4.1	28.4±4.3	0.176
TSH *(mIU/mL)*	2.7±1.5	2.6±1.4	2.1±0.1	2.4±1.3	0.87
Anti-TPO*(IU/mL)*					
Mean	630.4	411	20.4		
Median	433.5	75.5	9		***<0.001***
Maximum	1755	1647	51		
Anti-TG *(U/mL)*					
Mean	168	137	18		***<0.001***
Median	56	91	13		
Maximum	1300	1200	65		
Thyroid volume					
Mean	11867.6	14766.5	13701.9		
Median	10786.9	13039	13053.5		***<0.001***
Maximum	32541.6	52161.4	39651.7		
25OHD *(ng/mL)*	11.4±5.2	13.1±5.9	15.4±6.8	13.3±6.2	***<0.001***

For definition of study groups see "Subjects and Methods"
TSH: 0.55-4.78 mIU/mL, Anti-TPO: 0-35 IU/mL, Anti-TG: 5-40 U/mL, 25OHD: 11.1-42.9 ng/mL
Thyroid volume: [right lobe (a x b x c) x 0.502] + [left lobe (a x b x c) x 0.502] /2
P value <0.05 is statistically significant

Table 2: Comparison of men and women study subjects.

	WOMEN			MEN			
	Group-1	Group-2	Control	Group-1	Group-2	Control	***P***
Number *(n)*	123	123	123	57	57	57	
Age *(mean ±SD)*	42.3±12.6	42.1±10.3	42.2±11.6	43,7±11.8	42,7±9.9	43,2±7.2	
BMI *(kg/m^2)*	27.9±4.6	28.2±4.8	28.8±4.3	28,3±3.6	28,6±3.9	29,1±3.8	
TSH *(mIU/mL)*	2.74±1.47	2.68±1.44	2.14±1.02	2.5±1.5	2.35±1.3	1.8±0.75	
free-T$_4$ *(ng/dL)*	1.13±0.2	1.13±0.2	1.12±0.3	1.17±0.1	1.2±0.2	1.26±0.1	
Anti-TPO*(IU/mL)*							
Mean	694±568	499±543.9	27.8±18.4	493.1±581	221±405	4.54±1.4	***<0.001**
Median	500	177	28	64	52	4	
Anti-TG *(U/mL)*							
Mean	190.4±227	164.3±242	23.4±13.1	119.8±154	80.8±150	9.0±0.0	***<0.001**
Median	107	78	22	50	27	9	
Thyroid volume							
Mean	11230	13574	11585	13241	17337	18271	
St deviation	5269	6698	5388	6314	9275	5977	***<0.001**
Median	10385	12267	10822	12920	14563	17547	
25OHD ***(ng/mL)***	**10.3±4.5**	**11.4±4.9**	**13.5±6.46**	**13.9±5.6**	**16.5±6.4**	**19,4±5.9**	***<0.001**

For definition of study groups see "Subjects and Methods"
TSH: 0.55-4.78 mIU/mL, Anti-TPO: 0-35 IU/mL, Anti-TG: 5-40 U/mL, 25OHD: 11.1-42.9 ng/mL
Thyroid volume: [right lobe (a x b x c) x 0.502] + [left lobe (a x b x c) x 0.502] /2
P value <0.05 is statistically significant. P values indicate the difference between "men" and "women"

Table 3: Comparison of subjects' characteristics according to severity of vitamin D deficiency

	Severe 25OHD <10ng/mL	**Moderate** 10≤ 25OHD <20ng/mL	**Mild** 20≤25OHD <30 ng/mL	**Normal** 25OHD ≥30 ng/mL	**p**
Total Cases*(n,%)*	187 (34.6%)	268 (49.6%)	75 (13.9%)	10 (1.9%)	
Women *(n,%)*	158 (%42.8)	181 (%49.1)	25 (%6.8)	5 (%1.3)	<0.001
Men *(n,%)*	29 (%16.9)	87 (%50.9)	50 (%29.3)	5 (%2.9)	
Age *(mean ±SD)*	42.6±11.5	41.7±10.9	45.3±10.1	44.4±10.4	0.01
Group-1*(n,%)*	87 (48.3%)	81 (45%)	10 (5.6%)	2 (1.1%)	<0.001
Group-2*(n,%)*	63 (35%)	90 (50%)	25 (13.9%)	2 (1.1%)	
Control *(n,%)*	37 (20.5%)	97 (53.9%)	40 (22.2%)	6 (3.4%)	
Anti-TPO *(IU/mL)*	526.6±561.8	297.1±486.3	135.8±331.6	284.2±536.6	0.03
Anti-TG *(U/mL)*	139.8±189.6	98.5±186.9	67.9±178.1	82.1±107.9	<0.001
Thyroid volume	12512.0	13640.7	14704.4	16227.2	<0.001

For definition of study groups see "Subjects and Methods"
TSH: 0.55-4.78 mIU/mL, Anti-TPO: 0-35 IU/mL, Anti-TG: 5-40 U/mL, 25OHD: 11.1-42.9 ng/mL
Thyroid volume: [right lobe (a x b x c) x 0.502] + [left lobe (a x b x c) x 0.502] /2
P value <0.05 is statistically significant. P values indicate the difference between "men" and "women"
P values demonstrate the statistical difference between severe, moderate and "mild+normal" groups