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Serum vitamin D level in healthy individuals versus patients with symptomatic and asymptomatic oral lichen planus

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ABSTRACT

The aetiology of oral lichen planus (OLP) is multifactorial, having variable triggers. A role for vitamin D related to the immune system has been established. Vitamin D modulating effect is on the adaptive and innate immune responses. Our study aimed to compare serum levels of vitamin D in patients having different clinical symptoms of OLP (symptomatic or asymptomatic) with healthy individuals. Also, in this study, for further evaluation, the expression level of interleukin-17A and interleukin-6 (IL-17A and IL-6) was evaluated because the presence of active vitamin D reduces the expression of these pro-inflammatory factors. This study was included three groups with 30 volunteers in each. The first group included asymptomatic oral lichen planus patients (reticular or plaque-like lesions). The second group consisted of symptomatic oral lichen planus patients (atrophic or bullous-erosive lesions). In contrast, the third group consisted of healthy control subjects. The serum 25-hydroxyvitamin D was measured between the three groups and then correlated with clinical manifestation of oral lichen planus, either symptomatic or non-symptomatic. The Real-Time PCR technique was used to evaluate the expression of IL-17A and IL-6. Patients with symptomatic OLP (second group) had statistically significantly lower Vitamin D levels than asymptomatic OLP patients (first group). Healthy Controls (third group) exhibited statistically significantly higher vitamin D levels than OLP groups. The results of IL-17A and IL-6 genes expression showed that the presence of vitamin D had a statistically significant effect on reducing the expression of these two pro-inflammatory cytokines among symptomatic and asymptomatic OLP patients. Also, the results showed that there was a statistically significant difference between OLP patients (group I and II) and the control group (group III). In general, the current study results showed that lack of vitamin D had an important role in initiating or increasing the OLP's severity.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory oral disease having a prevalence from 0.5% to 2.0% of the general population (1). The percentage of lichen planus patients having both skin and oral lesions is between 50% to 70%, while the percentage of patients having OLP alone is 25% of total lichen planus patients (2). OLP is an immune-related disorder that dominantly occurs among women between 30 and 60 years of age. Commonly appears clinically as bilateral symmetrical lesions with multifocal involvement in the oral mucosal sites affecting mainly buccal mucosa, tongue, lips, gingivae and the alveolar ridge (3). Asymptomatic types of OLP include reticular, papular and plaque-like lesions while symptomatic types include atrophic or bullous-erosive lesions (4). The erythematous and erosive types are associated with pain and an increased risk of malignant transformation (5).

The actual aetiology of OLP is not obviously known but is mostly considered as immune-mediated disorder, so any factor that influences the immune system may act as a trigger for initiating the disease (6). These factors include mechanical, psychological,

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electrochemical, immunological, infectious, malnutrition, endocrine disorders as well as genetic susceptibility and increased oxidative stress (7). The histopathological picture of OLP shows apoptosis of basal keratinocytes in association with liquefactive degeneration (8). A dense band-like from T-lymphocytic infiltrate exists subepithelial to the disrupted basement membrane which suggests a role for T lymphocytes in the initiation and pathogenesis of OLP (9).

Vitamins and micronutrient deficiencies may be effective in exacerbating or initiating OLP disease. Vitamin D present naturally in nutrients and in the form of dietary supplements (10). It is considered a biomarker of overall health and 1,25-dihydroxyvitamin D (1,25(OH)2D3 is the active form of it. A role of vitamin D related to the function of the immune system has been established (11). Receptors of Vitamin D (VDRs) are expressed on multiple immune cells such as antigen-presenting cells, T cells or B cells. Vitamin D can mediate both the down and upregulation of immune cell differentiation and affects T cells regulation and immunoglobulin secretion (12).

An immunomodulatory and therapeutic effect was found for vitamin D in autoimmune disorders (13). Experimental animal studies showed that vitamin D can suppress or prevent the clinical manifestation of diseases such as autoimmune encephalomyelitis, diabetes mellitus and systemic lupus erythematosus by reducing the level of T helper 2 (Th2) and enhancing the production of interleukin-4 and Transforming growth factor-beta which in turn suppresses T-cell inflammatory activities (14). Other studies report a probable role for vitamin D deficiency through pathogenesis or in exacerbation of autoimmune bullous mucocutaneous and pemphigus vulgaris with various immune-related mechanisms (15, 16).

Meanwhile, studies have shown that vitamin D leads to a reduction in the production of IL-1β and interferon-gamma (IFN-γ) in the epithelium suggesting that vitamin D deficiency may be involved in OLP pathogenesis (17). The role of serum vitamin D levels in the OLP aetiology should be adequately evaluated as it may have implications for the treatment of OLP, given that erosive and atrophic cases of OLP have an increased risk for malignant transformation (18). Our study aimed to compare serum levels of vitamin D in patients having different clinical symptoms of OLP (symptomatic or asymptomatic) with healthy individuals. Also, in this study, for further evaluation, the expression level of interleukin-17A and interleukin-6 (IL-17A and IL-6) was evaluated because the presence of active vitamin D reduces the expression of these pro-inflammatory factors.

Materials and methods

The current study was operated at the outpatient clinic of the Faculty of Dentistry, Umm Al-Qura University.

Inclusion Criteria

Patients from 35 to 60 years of age (both genders), with OLP patients who have not undergone any prior treatment for the disease and diagnosed of OLP were carried out according to clinical presentation and histopathological examinations (19) were included in this study. Three groups were included in this study each composed of 30 volunteers.

Group 1: Asymptomatic oral lichen planus (reticular, popular and plaque-like lesions)

Group 2: Symptomatic oral lichen planus (atrophic or bullous-erosive lesions)

Group 3: Healthy control subjects

Exclusion Criteria

Patients with age under 18 years old or had a history of taking any type of corticosteroid or other immunosuppressive therapy through the past 4 weeks or any drug that might produce a lichenoid reaction. Patient with history of taking any medications that alter vitamin D serum level such as supplementation of vitamin D, multivitamin, calcium and sunscreen or sun blockers cream. Patients with a history of chronic diseases such as diabetes mellitus, renal or hepatic diseases, malignancies and thyroid or parathyroid disease.

Blood Sampling and testing

A 5-mL blood sample was taken from all participants at the outpatient clinic. Collected blood was centrifuged for 15 min at 4 °C for serum separation and then stored at −20 °C till the analysis of vitamin D levels was completed. Diagnosis of oral lichen planus was based on medical, dental history,

review of symptoms, and presence of lesions in the mouth. Lab tests were done to look for indications of oral lichen planus.

Serum Vitamin D Level

Measurements were done for the total 25-hydroxyvitamin D (25(OH)D) levels. It is considered the most accurate marker for vitamin D (20). This was done by the commercially available enzyme-linked immunosorbent assay (ELISA) kits (25 (OH) Vitamin D ELISA kit (ab213966), Abcam, UK).

Serum vitamin D levels were considered as "severe deficient" if the levels were lower than 10 ng /ml and levels from 10 to 20 ng /ml were considered "deficient", while between 20-30 ng /ml were considered "vitamin D insufficient" level. Serum vitamin D levels between 30-100 ng/ml were considered "normal" level and subjects with levels higher than 100 ng/ml were considered "hyper-vitamin D" (21). A comparison of vitamin D serum levels were done between healthy individuals and patients having either symptomatic or asymptomatic OLP. Meanwhile, the distribution of vitamin D serum levels was done in each group according to the classification of vitamin D serum levels (21).

RNA extraction, cDNA synthesis, and Real Time-PCR

5 ml of blood samples were collected in tubes containing EDTA anticoagulants. According to the manufacturer's instructions, total RNA was extracted using a MACHEREY-NAGEL extraction kit (Germany). The extracted RNAs' integrity, concentration, and purity were confirmed using spectrometry, nanodrop, and electrophoresis. 5µg of total extracted RNA was used for cDNA synthesis with hexamer random primer via HyperScriptTM Reverse Transcriptase (GeneAll, South Korea) in a total volume of 20µl. Real-time PCR was performed using Roche Light cycler 96 (version: 1.1.0.1320, Germany), primers, and specific probes for IL-17A, IL-6, and beta-actin as housekeeping genes. The used primer and probe sequences for Real-Time PCR are shown in Table 1.

The total volume of the reaction was about 25µl, of which 4µl was related to the synthesized cDNA solution, 12.5µl of RealQ Plus 2x Master Mix for probe (Ampliqon, Denmark), 500nM of each forwarding and Reverse primers and 250nM TaqMan probe. The instructions were as follows: A. pre-warming step (10 min at 94°C); B. denaturation step (15s at 94°C); C. annealing/extension step (60°C for 60s).

Table 1. The primer and probe sequences for Real-Time PCR

Target	Primer	sequence (5'-3')
IL-17A	Forward	AATCTCCACCGCAATGAGGA
	Reverse	ACGTTCCCATCAGCGTTGA
	Probe	FAM-CGGCACTTTGCCTCCCAGATCACA
IL-6	Forward	GGTACATCCTCGACGGCATCT
	Reverse	GTGCCTCTTTGCTTTCAC
	Probe	FAM-TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT
Beta-actin	Forward	TCACCCACACTGTGCCCATCTACGA
	Reverse	CAGCGGAACCGCTCATTGCCAATGG
	Probe	FAM-ATGCCCTCCCCCATGCCATC

Statistical analysis

Analysis of the collected Data was done using "version 22" SPSS - Windows (IBM, Corp., Chicago, IL, USA)., Comparing the frequency regarding gender was done by using Chi square test. Comparing between the three groups regarding age and Vitamin D levels was done by One-way ANOVA test. Post hoc Tukey test made the pairwise comparisons. The p-value < 0.05 was deliberated as statistically significant. The expression of mRNA was calculated based on ΔΔCT method, and fold change was evaluated by $2^{-\Delta\Delta CT}$ for each gene.

Results and discussion

In this study, each group was composed of 30 participants. Group I was composed of 6 males (20%) and 24 females (80%) with mean age + SD (47.47 ± 6.39) having asymptomatic oral lichen planus either popular, reticular, or plaque-like lesions. The second group was composed of 5 males (16.7%) and 25 females (83.3%) their mean age + SD is (48.70 ± 7.20) having symptomatic oral lichen planus either atrophic or bullous-erosive lesions. The third group was composed of 6 males (20%) and 24 females (80%) with mean age + SD (47.10 ± 7.71). No statistically significant difference existed among the three groups regarding gender distribution or age (p-value=0.661, 0.254 respectively) (Table 2).

Table 2. Comparison among the study groups regarding gender, age and vitamin D concentration

Variables / Groups	Gender No (%)		Age Mean ± SD	Vit. D Mean ± SD
	Male	Female		
Group I	6 (20.0)	24 (80.0)	47.47 ± 6.39	23.14 ± 5.28^{AB}
Group II	5 (16.7)	25 (83.3)	48.70 ± 7.20	15.51 ± 5.77^{AC}
Group III	6 (20.0)	24 (80.0)	47.10 ± 7.71	29.87± 4.81^{BC}
P value	0.254		0.661	0.000

Vitamin D level

The result of this study showed statistically significant differences among the three groups regarding vitamin D serum levels (p=0.000). The first group had mean ± SD equal to (23.14 ± 5.28) while group 2 showed mean ± SD equal to (15.51 ± 5.77) and the third group had mean ± SD equal to (29.87± 4.81). Both groups 1 and 2 showed lower vitamin D serum levels when compared to the third group. At the same time, serum vitamin D levels of group 2 were lower than group 1 levels (p=0.000 for all comparisons) (Table 2 and Figure 1).

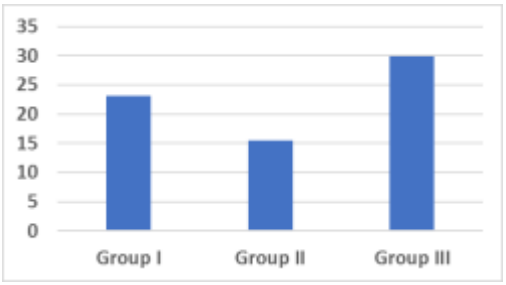

Figure 1. Vitamin D levels (ng/ml) among the three groups; Group I: Asymptomatic oral lichen planus (reticular, papular and plaque-like lesions); Group II: Symptomatic oral lichen planus (atrophic or bullous-erosive lesions); Group III: Healthy control subjects

Regarding the classification of vitamin D serum levels, group III had the highest percentage of participants with normal vitamin D level (43.3%), followed by the group I (13.3%) and group II (3.3%). At the same time group II had the highest percentage of participants with deficient (50%) and severely deficient (16.7%) cases in vitamin D levels, followed by group I showed (20%) of participants with a deficient level of vitamin D and (3.3%) severely deficient cases, while group III showed the lowest level of deficient cases (10%) and (0%) for severely deficient cases (Table 3).

Table 3. The number (percentage) of participants in each group according to the classification of vitamin D level

Variables / Groups	Vitamin D level				Total No (%)
	Normal No (%)	Insufficient No (%)	Deficient No (%)	Severely deficient No (%)	
Group I	4 (13.3)	19 (63.3)	6 (20)	1 (3.3)	30 (100)
Group II	1 (3.3)	9 (30.0)	15 (50.0)	5 (16.7)	30 (100)
Group III	13(43.3)	14 (46.7)	3 (10.0)	0 (0.0)	30 (100)
Total	18(20.0)	42 (46.7)	24 (26.7)	6 (6.7)	90 (100)

Expression of IL-17A and IL-6 genes

As shown in Figure 2, the expression results of IL-17A and IL-6 genes showed that for both genes there were statistically significant differences between group I and group II (P<0.05), also there was statistically significant differences between group I and II with control group (group III)(P<0.001).

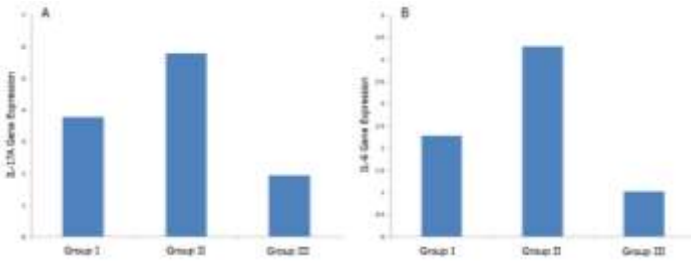

Figure 2. The expression results of Pro-inflammatory cytokines; "A" is level of IL-17A gene expression and "B" is level of IL-6 gene expression; Group I: Asymptomatic oral lichen planus (reticular, papular and plaque-like lesions); Group II: Symptomatic oral lichen planus (atrophic or bullous-erosive lesions); Group III: Healthy control subjects

OLP is an inflammatory disease associated with excessive infiltration of sub-epithelial lymphocytes and destruction of basal keratinocytes (5). Clinical features of OLP include symptomatic (atrophic or bullous-erosive lesions) and asymptomatic (reticular and plaque-like lesions) types (22). Symptomatic types involve pain ranging from mild to severe discomfort and are considered as a potentially precancerous lesions and life-threatening conditions.[33] Vitamin D is classically related to the regulation of calcium and phosphate metabolism; however, it is also considered as an immune-modulatory hormone (23-25). It may have a role regarding the initiation or the severity of OLP by regulating the body immune system.

Deficiency of vitamin D may result in a decrease in the numbers of Th2 cells when compared to the other

T cells as Th1 and Th17 which are involved in the inflammatory pathway, and this will aggravate the inflammatory conditions as in LP (23). Vitamin D prevents the keratinocytes apoptosis by regulating cytokine production and preventing the antigen presentation for T lymphocytes. Also, it was suggested that vitamin D has anti-cancerous effects and its levels were reported to be severely deficient in precancerous lesions and oral squamous cell carcinoma (18).

Different studies determined the deficiency of vitamin D serum level in multiple autoimmune diseases (26). El-Komy *et al*. (15) and Joshi *et al*. (27) in their studies on patients suffering from pemphigus vularis (PV) found out that these patients showed decreased serum vitamin D levels by comparing them with healthy individuals. These results showed that deficiency of vitamin D may act as a predisposing factor in PV by different immune-mediated effects which regulate the function of T- lymphocyte.

In the current study, we aimed to compare the levels of serum vitamin D in healthy individuals with the levels in patients having either symptomatic or non-symptomatic OLP. Our results showed a significant difference in vitamin D levels between either symptomatic (15.51 ± 5.77) or non-symptomatic (23.14 ± 5.28) lichen planus patients when compared with the healthy subjects (29.87± 4.81). OLP patients showed lower levels of serum vitamin D than in healthy controls, with much lower levels noted in symptomatic OLP patients compared to those with asymptomatic disease.

These results were consistent with the results obtained by Gupta *et al*. (28) who reported levels of vitamin D in OLP patients equal to 20.40 ng/ml while the level of the control subjects was 32.67 ng/ml. Similar to our study, this was a statistically significant difference. Seif *et al*. (29) compared vitamin D serum levels in 30 patients having OLP with 66 healthy subjects, their results showed a high percentage decrease in vitamin D serum levels for OLP patients. On the contrary, Bahramiyan *et al*. (30) found an insignificant difference between both groups regarding vitamin D serum levels. The mean vitamin D serum levels in OLP patients were 30.38 + 20.38 ng/ml and 36.45 + 15.33 ng/ml in healthy subjects (P = 0.34).

In the current study, statistically significant differences exist in vitamin D serum levels between symptomatic (15.51 ± 5.77) and asymptomatic OLP patients (23.14 ± 5.28). This was consistent with a study by Ahmed (31) who corroborated our results showing further decreases in vitamin D serum levels in those with the symptomatic disease compared to asymptomatic disease. The mean serum vitamin D levels were equal to (13.11 ng/ml) in patients with atrophic OLP cases and (23.53 ng/ml) in patients without atrophic lesions. Also, the cases with erosive lesions had mean serum vitamin D levels equal to (14.42 ng/ml) which was significantly lesser than the mean in patients without erosive lesions (24.82ng/ml). Tak *et al*. (17) also found out that vitamin D serum levels in patients diagnosed with OLP were less than the levels in healthy subjects, significantly lower levels of vitamin D were shown in cases that suffer from erosive lichen planus. Taken all these results together will support the role of vitamin D in initiating or increasing the severity of OLP disease (32).

Many studies showed that Vitamin D has an important role in regulating the immune system (33-35). Actually, vitamin D inhibits the expression of inflammatory cytokines such as IL-6 in monocytes (35). It also directly alters the cytokine profile of T cells by inhibiting the production of inflammatory cytokines such as IL-17A (34, 36). Therefore, increasing the amount of vitamin D should lead to a decrease in these inflammatory cytokines (37). The results of IL-17A and IL-6 genes expression showed that the presence of vitamin D could have a significant effect to reduce the expression of these two pro-inflammatory cytokines among patients with asymptomatic OLP than symptomatic OLP. Also, the results showed that there was statistical significant between OLP patients (group I and II) and control group (group III).

Deficiency or insufficiency of vitamin D serum levels is a common situation in many populations potentially due to increased indoor activity, excess use of sunscreen, skin coverage related to various cultural beliefs or as protection from skin cancer following direct sun exposure (38). It is necessary to examine and monitor vitamin D levels while OLP disease.

Extra studies are required to investigate the effects of using vit D supplementation after OLP diagnosis for mitigating severity in the course of disease. This

may be useful for instructing future therapeutic protocols for patients diagnosed with OLP.

Acknowledgments

None.

Conflict interest

None.

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