ORIGINAL ARTICLE

In vitro Effect of Vitamin D on the Levels of Cytokines and Apoptosis Related Markers in Systemic Lupus Erythematosus

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ABSTRACT

Background: *Both genetic and environmental factors are responsible for systemic lupus erythematosus (SLE) pathogenesis. Knowledge about the roles of cytokines and apoptosis in SLE has led to new clinical points of view in its pathogenesis.* ***Objective:*** *This work aimed to study the in vitro effects of vitamin D3 on the level of IL-6, TNF-α, INF-γ, and IL-10 cytokines, as well as the expression of Bax and Bcl-2 in peripheral blood mononuclear cells (PBMCs) cultures of SLE patients.* ***Methodology:*** *Isolated PBMCs from 50 SLE patients were cultured with 1,25(OH)2D3. Vitamin D treated cultured cells were assessed for detection of the levels of cytokines by ELISA and gene expression of apoptotic markers by real time-quantitative reverse transcription PCR (RT-qPCR).* ***Results:*** *It was found that vitamin D significantly decreased the levels of IL-6, TNF -α, IFN-γ and IL-10 in vitamin D treated cells of SLE patients relative to the untreated cells (P value<0.001 for each). Vitamin D3 treated cells showed that expressions of Bax (0.67±0.12) and Bcl-2 (19.83±6.6) underwent fold change compared to that in untreated cells. It was found down regulation of Bax expression (3.5%) and up regulation of Bcl-2 expression (1.7 fold increase) in vitamin D treated cells.* ***Conclusion:*** *Vitamin D is an immuno-modulator agent and its administration in SLE patients may exert a good therapeutic effect. It had both inhibitory effect on cytokine levels and anti-apoptotic effect in PBMCs of SLE patients.*

INTRODUCTION

Systemic lupus erythematosus is an autoimmune disease, causing chronic inflammation with damage of several organs. Both genetic and environmental factors are responsible for SLE pathogenesis[1]. Vitamin D insufficiency is one of such factors[2]. Without vitamin D supplementation, the skin represents the primary source of cholecalciferol (vitamin D3) upon ultraviolet-B radiation exposure. Diet represents the source of ergocalciferol (vitamin D2) which is characteristically a minor provider to the overall vitamin D[3]. SLE patients should stay away from direct sunlight, which is not only a frequent trigger of the disease flare but also the primary source of vitamin D3. So, the risk of vitamin D deficiency in SLE patients is even more than in general population[4]. It was reported that more than 90% of SLE patients exposed to UV radiation showed abnormal reactions. Importantly, solar radiation, principally 280–315 nm UVB, is considered a risk factor of SLE or SLE-associated mortality[5].

The levels of vitamin D3 in 2/3 of SLE patients with high activity were significantly lower (<10 µmol) in comparison to those with minimal activity or control group[6]. This may be due to lack of sunlight exposure, sun block use, or the disease itself. Excessive metabolism or defective 25-hydroxylation of 1,25(OH)2D3, due to drugs or even by the disease itself, are other explanations[7]. So, SLE could result in low vitamin D level and also lack of vitamin D could have a role in etiology and aggravation of SLE[8].

Vitamin D decreases Th1 cell response and Th1 cytokines release (such as INF-γ, TNF-α) with subsequent reduction of B-lymphocytes proliferation and differentiation[9]. Additionally, vitamin D modulates dendritic cells differentiation and up-regulates T-reg (T-regulatory) cells with preservation of innate immune response[10,11].

Lymphocyte apoptosis deregulation in SLE has been focused since 1990s, when two SLE murine models were tested. One had defective *Fas* and *FasL* genes and another had excessive expression of an anti-apoptotic

gene *Bcl-2*. In both mice, decreased lymphocyte apoptosis resulted in auto-reactive lymphocytes accumulation with subsequent autoimmunity[12,13]. However, such mutations were not detected in SLE patients[14]. Dissimilarly, Emlen, et al detected evidence of increased peripheral blood lymphocytes apoptosis in SLE patients [15], that was confirmed by later studies[16]. Besides, defect in apoptotic debris clearance has been detected in SLE patients[17]. These consequences initiated a hypothesis which states that SLE is a disease of excess cellular wastes, which could be caused by both enhanced apoptosis and decreased clearance[18]. Definitely, it was found that lymphocyte apoptosis increases in vasculitis and other rheumatic disorders[19]. Furthermore, the presence of apoptosis together with SLE-associated lymphopenia and anti-nucleosomal antibodies could confirm the causality. Regarding to lymphopenia in SLE, it has been suggested to be resulted from increased apoptosis[20]. Anti-nucleosomal antibodies are considered a good marker of lupus and the disease activity and considered to be pathogenic. These act against nucleosomes formed by apoptosis and therefore supposed to be related to apoptosis too[21].

The aim of this study is to investigate the in vitro effect of vitamin D3 on the level of pro-inflammatory cytokines IL-6, TNF-α and INF-γ, and anti-inflammatory cytokine IL-10, as well as the expression of apoptotic marker *Bax* and anti-apoptotic marker *Bcl-2* in P genes PBMC cultures of SLE patients.

METHODOLOGY

Subjects

This cross sectional study was done over a period of six months, and included 50 SLE patients (47 women and 3 men, with a mean age of 35.7 ± 7.5 years), admitted to the rheumatology unit or attending the rheumatology out- patient clinic of Mansoura University Hospitals, Egypt. All SLE patients fulfilled no less than 4 of revised SLE characters of American College of Rheumatology for SLE classification according to revised criteria in 1997[22]. Each SLE patient was assessed by a physician, with these inclusion and exclusion criteria:

Inclusion criteria

- Newly diagnosed patients, before starting treatment.
- SLE patients during remission taking only prednisolone not more than 10 mg/day.

Exclusion criteria

- SLE patients during remission taking hydroxylchloroquine.
- SLE patients during remission taking a dose of prednisolone higher than 10 mg/day.
- SLE patients taking cytotoxic drugs.
- SLE patients had overlap syndromes.
- SLE patients had malignancy.
- SLE patients had infections.
- SLE pregnant female patients.

Ethical approval

The study was approved by Institutional Review Board of the Faculty of Medicine, Mansoura University, Egypt, code number: R. 24.10.2833.

Preparation of peripheral blood mononuclear cell culture

PBMCs were isolated from heparinized blood within one to two hours following collection by density gradient centrifugation using Ficoll-Hipaque (Sigma-Aldrich, USA), and 2×10^6 cells were put into every well of the plate with 1 mL RPMI 1640 medium, FCS 10%, 100 mg/mL streptomycin, and 100 IU penicillin. Lymphocytes (5×10^5 cells) were incubated with 50 nM 1,25(OH)2D3 (vitamin D3) for 24 hours (Sigma-Aldrich, USA). Vitamin D3 treated cultured cells were assessed for detection of cytokines and apoptotic markers. Cultured cells without vitamin D3 were used as controls.

Measurement of cytokines by ELISA

The levels of pro-inflammatory cytokines IL-6, TNF-α and INF-γ, and anti-inflammatory cytokine IL-10 were measured by ELISA kits (Mabtech, Sweden) according to the manufacturer's recommendations.

Detection of gene expression of apoptotic markers by RT-qPCR

TriPure kit (Sigma-Aldrich, USA) and RNase Free DNase Kit (Thermo Fisher, USA) were used for RNA extraction and removal of genomic DNA contamination. The cDNA was synthesized and the expression level of *Bax* and *Bcl-2* genes were assessed by SYBR Green real-time PCR master mix (Thermo Fisher Scientific, USA). A set of primers was used to amplify the apoptotic *Bax* (BCL2 associated X-protein) gene: 5'- TCTGACGGCAACTTCAACTG-3', reverse 5'-TTGAGGAGTCTCACCCAACC-3', the antiapoptotic *Bcl-2* (B-cell lymphoma2) gene: forward 5'-GGATGCCTTTGTGGAACTGT-3', reverse 5'-AGCCTGCAGCTTTGTTTCAT -3' and the internal reference *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene: 5'-AGCCGGGCATGTTCTTCAAC-3', 5'-AGGGAGCTTCACGTTCTTGTAT-3' (Promega, USA). Real time PCR were performed using Light Cycler platform (Roche, Switzerland) and *bax*, *bcl-2* and *GAPDH* genes were achieved by melting curve analysis (85.7°, 83.9°C, 87.2°C respectively)[23].

Statistical analysis

All data are reported as mean±SD, median, minimum and maximum. Data of cytokines analysis and genes expression were analyzed using Wilcoxon signed rank test using SPSS software version 27. *P*-value ≤0.05were considered as significant.

RESULTS

Effect of vitamin D3 on TNF, IFN-γ, IL-6 and IL-10 levels

It was found that vitamin D3 significantly decreased the levels of IL-6, TNF -α, IFN-γ and IL-10 (*P* value<0.001 for each) in vitamin D treated cells of SLE patients relative to the untreated cells (table.1& Figure 1).

Effect of vitamine D3 on gene expressions of *Bax* and *Bcl-2*

RT-qPCR analysis of *Bax* and *Bcl-2* expression in vitamin D3 treated PBMC cultures of SLE patients showed that expressions of *Bax* (0.67±0.12) and *Bcl-2* (19.83±6.6) underwent fold changes compared with that in untreated cultures. It was found down regulation of *Bax* expression (3.5%) and up regulation of *Bcl-2* expression (1.7 fold increase) in vitamin D3 treated cells (table.2& Figure 2). The data were expressed as mean transcripts expression fold changes over untreated cultures, standardized to *GAPDH*.

Table 1: Comparison of the levels of cytokines in vitamin D3 treated and untreated PMNC cultures of SLE patients

	Vitamin D3 non-treated PMNCs		Vitamin D3 treated PMNCs		*P*
	No=50		No=50		
	mean±SD	Median (range)	mean±SD	Median (range)	
TNF-α	1.99±1.1	1.89(0.01-4.99)	1.31±0.57	1.42(0.31-2.45)	<0.001
INF-γ	3.36±0.99	3.29(1.15-5.31)	1.52±0.90	1.51(0.24-3.41)	<0.001
IL-6	2.88±1.89	2.61(0.38-9)	1.01±0.41	1.1(0.11-1.84)	<0.001
IL-10	1.34±0.62	1.34(0.13-2.78)	0.83±0.63	0.58(0.01-2.57)	<0.001

SD; standard deviation.

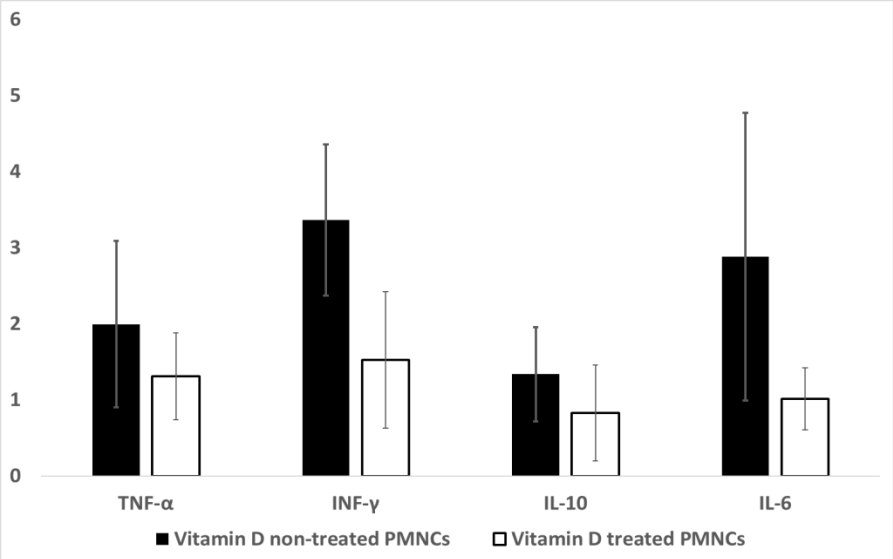

Fig. 1: Treatment of PMNC cultures of SLE patients by 50 nM vitamin D3 (1,25(OH)2D3) for 24 hours significantly decreased the levels of TNF -α, IFN-γ, IL-6 and IL-10 compared to control untreated cells as detected by ELISA

Table 2: Comparison of the levels of gene expression of apoptotic markers in vitamin D3 treated and untreated PMNC cultures of SLE patients

	Vitamin D3 non-treated PMNCs		Vitamin D3 treated PMNCs		*P*
	No=50		No=50		
	mean±SD	Median (range)	mean±SD	Median (range)	
Bax	0.67±0.12	0.66(0.5-1.3)	0.19±0.06	0.19(0.08-0.34)	<0.001
Bcl-2	19.83±6.6	21.26(0.23-28.32)	36.11±14.59	35.69(1.13-58.29)	<0.001

SD; standard deviation.

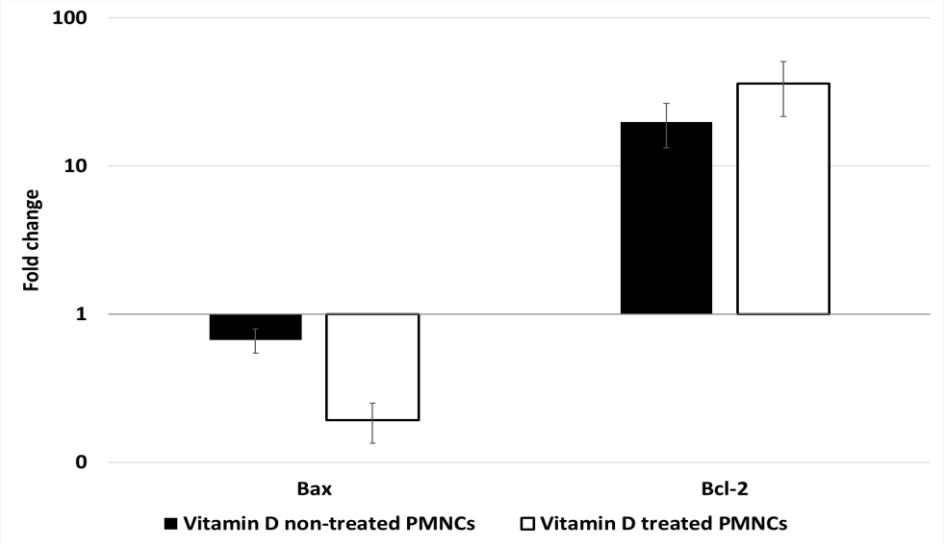

Fig 2. Treatment of PMNC cultures of SLE patients by 50 nM vitamin D3 (1,25(OH)2D3) for 24 hours significantly decreased *Bax* expression and increased *Bcl-2* expression compared to control untreated cells as detected by RT-qPCR

DISCUSSION

SLE is chronic autoimmune disorder of (immune complex mediated) type III hypersensitivity of unidentified etiology[24]. The role of vitamin D as an immuno-modulatory agent has been an interesting topic in recent studies[25]. However, the exact mechanism of this modulation necessitates more research. So, this research was conducted to investigate the in vitro effects of vitamin D3 on the levels of pro-inflammatory cytokines TNF-α, INF-γ and IL-6 and anti-inflammatory cytokine IL-10, and also the expressions of apoptotic marker *Bcl-2* and antiapoptotic marker *Bax* in PMCs cultures of SLE patients.

Pro-inflammatory cytokines such as IL-6 is responsible for B-lymphocytes stimulation and autoantibodies production in SLE. In SLE, high serum levels of IL-6 were detected, possibly because of immune imbalance, based on the inflammatory mechanisms[26]. IL-6 is produced by many cells, both lymphoid (Th-2 and B-cells) and non-lymphoid (endothelial cells, macrophages, etc.), and it is a multifunctional cytokine that acts on many target cells associated with inflammatory response (such as acute phase proteins synthesis) and hematopoiesis. IL-6 plays a considerable role in the antibodies production in SLE, through triggering polyclonal hyperactivity of B-cells. Together with TNF-α and IL-1; IL-6 has complementary and synergic actions, representing a key factor in the amplification of both anti-infectious and inflammatory responses. Nevertheless, IL-6 can function as a remote messenger, unlike TNF-α and IL-1 that control local reaction[27].

Another key inflammatory mediator is TNF-α which has the same effects as IL-6. TNF-α triggers the differentiation of macrophages and enhances their cytocidal ability, through activation of neutrophils chemotaxis. Consequently expression and activation of adhesion molecules occur to initiate the transdothelial migration toward the inflammatory site. TNF-α also activates the release of IL-1, IL-6, IL-8 and TNF-α itself. It stimulates hepatocytes to synthesize acute-phase proteins; stimulates arachidonic metabolism and forms both pro-inflammatory, pro-coagulant and vasodilating prostaglandins. It stimulates IL-2, IL-2R and IFN-γ production in activated T-cells and stimulates proliferation and differentiation of activated B-cells[28].

IFN-*γ* is mainly produced by Th-1 cells and NK cells. Earlier bioinformatics analysis reported that interferon signaling pathway against viral infection was powerfully associated with SLE pathogenesis and development. Interestingly, IFN-*γ* enhances MHC-I expression, so stimulates peptides recognition by cytotoxic-T lymphocytes and encourages initiation of cell- mediated reaction of SLE. Furthermore, IFN-*γ* activates both MHC-I and MHC-II molecules transcription, which afterward participate in both development and severity of SLE[29].

In our research, vitamin D3 significantly decreased the levels of inflammatory cytokines; IL-6, TNF–α and IFN-γ in vitamin D treated cells of SLE patients relative to the untreated cells (*P* value<0.001, for each). Similar results were detected by Chegni et al[30] who reported that vitamin D3 significantly reduced expressions of IFN-γ gene by 73% ($P \leq 0.05$). In another study done on vitamin D3 as an immuno-modulatory agent, vitamin D3 was found to reduce the inflammatory cytokines TNF-α, and IL-6 significantly[31]. Several researches confirmed that IL-6 could reduce both activity and numbers of T-reg cells which was associated with active SLE[32]. Additionally, Stockinge reported that vitamin D3 decreased IL-6, which has a crucial role in the switch of

CD4+ Tcell into TH-17 cell, which is essential in pathogenesis of autoimmune disorders[33]. A similar study done by by Abo-Shanab et al[34] in which they found an inverse relationship between serum IFN-γ levels and vitamin D3 in newly diagnosed juvenile SLE compared with healthy individuals.

Regarding IL-10 in our research, it was found that vitamin D3 significantly decreased the IL-10 in vitamin D treated PMCs cells of SLE patients relative to the untreated cells (*P* value<0.001). Similar result was reported in a previous study in which vitamin D3 reduced expressions of IL-10 gene by 29% ($P \leq 0.05$)[30]. IL-10 is a strong anti-inflammatory cytokine produced by many cells such as CD4+ cells, cytotoxic T cells, B-cells, NK cells, monocytes, mast cells, neutrophils and eosinophils. Contrary to expectations, severity of tissue damage in SLE patients is linked to higher levels of serum IL-10[27]. Increased production of IL-10 is coupled with an increase in IFN-γ expression that may result in Th1/Th2 cell imbalance in SLE. Within CD40 and CD40L signaling pathway, T and B lymphocytes interaction results in release of both IL-6 and IL-10 causing B-cells polyclonal activation with production of autoantibodies. Consequently, immune complexes are formed and precipitate in tissues in SLE leading to immune cells stimulation and complement activation[30,35]. It was found that injection of IL-10 in mouse models of SLE could increase incidence of renal illness, although treatment of the same mouse model with monoclonal antibody directed against IL-10 stopped the illness, confirming effect of IL-10 on SLE pathogenicity[36]. One of the points to be taken into consideration is the phases of SLE. Vitamin D3 administration in different SLE phases may have variable effect on the levels of cytokine expressions. As confirmed in one study, vitamin D significantly reduced IL-10 in newly onset SLE[31].

As previously reported, vitamin D has inhibitory effects upon cell proliferation, but these effects were not well characterized whether exerted through inhibition of apoptosis, cell cycle or both[37]. In our research, we studied the in vitro effect of vitamin D3 treatment on apoptosis in SLE patients, through expression of *Bax* and *Bcl-2* genes. We found that vitamin D decreased the expression of *Bax* and increased the expression *Bcl-2*, and so it could inhibit the apoptosis in SLE patients. Similar results were obtained by Tabasi et al[38] who found that vitamin D3 treatment of PBMCs of SLE participants increased expressions of apoptotic *Bax* and *FasL* and decreased expression of anti-apoptotic *Bcl-2* genes. Another study suggested that T-cell apoptosis was more in SLE patients compared with control group and was associated with lower T-lymphocyte levels in lupus patients. They also found that patients with active lupus had higher T-cell apoptosis compared with patients with inactive disease. Therefore, T- lymphocyte apoptosis could correlate directly with SLE activity and inversely with T- lymphocyte levels[39].

Apoptosis can play a controversial role in SLE. Impairment of apoptosis and reduced removal of autoreactive lymphocytes, during development of immune system, can result in of self tolerance loss and autoimmunity. However, many studies stated that T-reg cells are very liable to apoptosis, and so increased apoptosis can reduce their number resulting in active lupus. This is because reduced number or impaired function of T-reg cells is correlated with autoantibodies production, inflammatory mediators release and immune tolerance disruption[32,40].

Finally, it can be concluded that vitamin D is an immuno-modulator agent and its administration in SLE patients may exert a good therapeutic effect. It had both inhibitory effect on cytokine levels and anti-apoptotic effect in PBMCs of SLE patients. Further researches are important to determine the effects of vitamin D on apoptosis and cytokines expressions in each phase of SLE discretely and to understand the alterations according to each phase.

Conflicts of interest

- The authors declare that they have no financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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