

# Research Article:

## Vitamin D3 Reduction in Individuals Exposed to Sulfur Mustard



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## ABSTRACT

**Background:** Inflammatory responses in individuals exposed to sulfur mustard occur in some organs such as lung, skin and eyes. These organs manifest overreaction in cellular and humoral immune responses. Over a long period, the immune responses often continues toward chronic inflammatory processes with some interventions. Obviously some elements have been deregulated. Therefore, this study evaluated Vitamin D3 levels as a modulatory agent that could first regulate inflammatory interactions, then switch to suppress inflammatory mechanisms. Several studies showed that Vitamin D3 plays an undeniable role in suppressing inflammatory responses. In addition, some studies showed that calcium and phosphorus could play some role in inflammatory responses, too. Also, levels of serum calcium and phosphorus are associated with serum Vitamin D3 level, because it promotes absorption and reabsorption of calcium and phosphorus in intestine and kidney.

**Materials and Methods:** Levels of vitamin D3, calcium and phosphorus were measured by ELISA and spectrophotometric method, respectively. 114 exposed individuals as case group and 79 unexposed as control group were evaluated.

**Results:** The results showed that the level of serum Vitamin D3 is low in individuals exposed to sulfur mustard ( $P < 0.001$ ). Also, there is a significant difference between exposed and control group in calcium level ( $P < 0.001$ ). However, serum phosphorus assay result showed no significant difference between two groups.

**Conclusion:** According to our findings, chronic inflammation has correlation with status of serum Vitamin D3 deficiency in individuals exposed to sulfur mustard.

## Introduction

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ulfur mustard as a war weapon was used during the past two decades [1]. It has been used more than other chemical gases in wars, thereby its effects has better

known than other poisons [2]. Sulfur mustard gas was a mass killer in the final years of the First world war [3]. Also, this chemical agent was used by Iraqi army against Iranian soldiers during 1984-1988 imposed war [4]. Sulfur mustard is a strong alkylating agent with cytotoxic, carcinogenic and mutagenic properties. The

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toxicity of this vesicatory agent has been identified since the past century [5, 6].

Sulfur mustard effects could occur as early complications (acute) and late complications (delayed) in a variety of organs such as lung, eyes, skin, nervous and digestive system, bone marrow, and immune system [7]. Delayed problems in lung, eyes and skin in exposed individuals indicate an interaction between the immune system and chronic inflammation [8-10]. Several reports show that functional disorder in humoral and cellular immunity would happen by contact with sulfur mustard [11-13]. In addition, inflammatory reactions could appear followed by reduction in level of serum Vitamin D3. Because of inflammation, people with Chronic Obstructive Pulmonary Disease (COPD) encounter difficulties in transportation of Vitamin D receptor to cytoplasm. Furthermore, Histone Deacetylase 2 (HDAC) with vitamin D3 regulate NFkB, the transcription factor in expression of inflammatory cytokines, but expression of HDAC reduces followed by stress oxidative. Therefore, Vitamin D3 could not contribute in interaction of inflammatory mechanisms in order to modulate inflammatory responses and switching to equilibrium situation [14].

Vitamin D3 is an effective tool in suppressing inflammatory responses and also plays an essential role in keeping extracellular calcium and phosphorus concentration [15]. Moreover, its non-classical role in modulation of inflammatory state acts through inflammatory responses of immunity [16]. Vitamin D3 (active form of vitamin D) creates heterodimer with retinoic acid then binds to vitamin D receptor and stays on its promoter that is so-called Vitamin D binding element [17]. Consequently, inflammatory transcriptional factors expression such as NFkB, NFAT and so on were inhibited that decreases inflammatory cytokines and increases anti-inflammatory cytokines such as Interleukin (IL)12, IL23 and IL10 [18].

Generally, studies show that vitamin D deficiency exists in individuals with Chronic Obstructive Pulmonary Disease (COPD) [19-21]. Furthermore, calcium and phosphorus are other necessary elements in inflammation. Several studies showed that levels of serum and dietary calcium could reduce inflammation and also treatment with 1,25(OH)2D3 suppresses TNF- $\alpha$  (tumor necrosis factor-alpha) expression. Some studies report that phosphorus and hypophosphatemia is depleted in people with COPD and has correlation with respiratory failure [22, 23]. In the present study, the level of serum calcium and phosphorus were measured.

On the other hand, individuals exposed to sulfur mustard are similar to people with COPD with respect to lung complications. Because of the importance of Vitamin D in reducing inflammatory responses and few reports on investigating Vitamin D level in individuals exposed to sulfur mustard, we decided to evaluate the levels of serum Vitamin D3 in individuals exposed to sulfur mustard.

## Materials and Methods

### Study population

This was a case-control study with 193 participants, including 114 people exposed to sulfur mustard and 79 unexposed people as the control subjects. All exposed and control subjects were male.

### Inclusion and exclusion criteria

Inclusion criteria were having 30 to 60 years old and voluntarily participated in the study. The subjects had no observed diseases which intervened in our experiments. Furthermore, Exclusion criteria were those with using immunosuppressive drugs or Vitamin D supplement, having acute infectious diseases, missed laboratory samples, or developed Vitamin D disorder metabolism.

### Serum preparation

Because of seasonal changes in serum Vitamin D, the blood was taken from all individuals in December which the procedure will be described briefly in the following sections. Initially, a sample of 5 mL of blood was taken and frozen to measure Vitamin D3 levels.

### Vitamin D3, Phosphorus and Calcium measurement

Serum level of Vitamin D3 was measured with competitive ELISA method (bioactive diagnostic, GmbH). At first, anti-vitamin D coated in each well and incubated with standard, control, and sample. Conjugated vitamin D-biotin was conjugated at room temperature for 90 min. After washing, TMB solution was dispensed and incubated at room temperature for 30 min. Consequently, H2SO4 as stop solution was used and the result was read at 450 nm wavelength.

Amount of 10  $\mu$ L of 25-OH VD standards, controls and samples were dispensed into each well, as required. Then 100  $\mu$ L working solution of biotinylated 25 (OH) D reagent was added into each well. Next, the plates were incubated at room temperature for 90 minutes. Afterwards, the contents of the wells were shaken out and 300  $\mu$ L of

wash buffer was added into each well for 5 times. Then, 100 µL of conjugated enzyme (streptavidin-HRP) was added into each well. After that, they were incubated at room temperature for 30 minutes and then 300 µL of wash buffer was added into each well for 5 times. In this step, the multi-channel pipette was used and then 100 µL of TMB substrate was added into each well and incubated for 33 minutes at room temperature (in a dark place). Subsequently, 100 µL of stop solution was added into each well to stop the enzymatic reaction and mixed plate contents for 20-30 s. The result was read at 450 nm wavelength. Level of serum phosphorus was measured by spectrophotometer based on routine methods.

**Cytokine assay**

In this study, IL-4 and IFN-γ (Interferon gamma) cytokines were measured by Sandwich ELISA method (R&D kit, Germany) which will be described briefly. Initially, 100 µL of anti-mouse IL-4 and IFN-γ were dispensed into each well. After incubating for 12 h (overnight) and washing 5 times, various concentrations of standard and sample solutions were added to each well. After incubating for 1 h at 37°C, the plates were washed five times and then conjugated antibody was added to each well. The plates were incubated at 37°C for 2 h again, then TMB substrate solution was added to each well. After

washing, the reaction was stopped by H2SO4 and optical density was read in 450 wavelength.

**Results**

Vitamin D3, calcium and phosphorus levels from 114 exposed and 79 unexposed individuals were analyzed in this study. The results demonstrated significant differences between two groups (Table 1).

**Vitamin D3**

The result showed a significant decrease in Vitamin D3 serum level in exposed group compared with that in the control group (P<0.001) (Figure 1).

**Calcium and phosphorus**

The results showed that level of serum calcium has increased remarkably in the exposed group in comparison to the control group (P<0.001). However, the results of serum phosphorus assay showed no significant differences between two groups (P=0.636) (Figure 2 & 3).

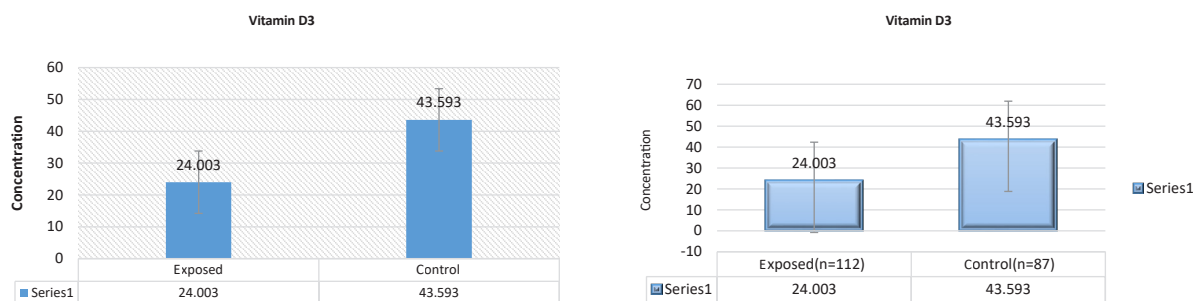
**Vitamin D3, Calcium and Phosphorus correlation**

Results demonstrated no correlation between levels of serum vitamin D3 and calcium in the control and exposed groups (P=0.097). Also, there is no correlation

**Table 1.** Characteristics of study individuals exposed to sulfur mustard and control group

Characteristics	Exposed	Control
Number	125	108
Age, y	30-60	30-60
Sex	Male	Male
1, 25(OH)2D3 (ng/mL)	24.003±18.324	34.111±24.783
Serum calcium (mg/dL)	9.63±0.38	9.27±0.40
Serum phosphorus (mg/dL)	3.59±0.62	9.27±0.40

\*All data were shown as No. or Mean±SD



**Figure 1.** Vitamin D3 level measurement by ELISA method

There is a significant decrease in Vitamin D3 in exposed group as compared to the control group (P<0.001).

**Table 2.** Results of vitamin D3, calcium and phosphorus correlation

Factors		VITD	
		Exposed (n=112)	Control (n=87)
Serum calcium	r*	-0.039	0.180
	P	0.700	0.097
Serum phosphorous	r*	-0.094	-0.377
	P	0.351	<0.001

\* The Spearman rank correlation coefficient

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**Table 3.** Results of vitamin D3, calcium and phosphorus correlation obtained by regression analysis

Model B	Coefficients <sup>a</sup>				t	Sig.
	Unstandardized Coefficients		Standardized Coefficients			
	Standard Error	Beta				
(Constant)	7.736	45.622			0.170	0.866
Group	19.949	4.210	0.430		4.739	0.000
Serum calcium	2.676	4.121	0.049		0.649	0.517
Serum phosphorous	-9.622	2.580	-0.247		-3.729	0.000
Age	0.217	0.230	0.074		0.943	0.347

a: Dependent Variable: VITD CON

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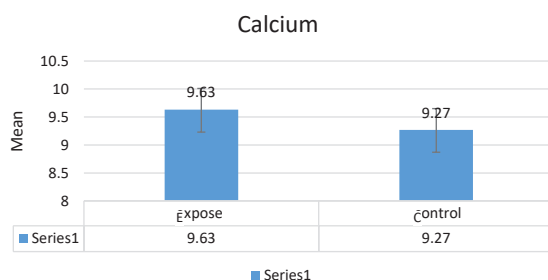
between phosphorus and vitamin D3 in exposed group (P=0.300). However, there is a significant and negative correlation between the control group and serum phosphorus level (P<0.001, r=-0.377) (Table 2). The results of regression assay showed that vitamin D status has a significant correlation with group and level of serum phosphorus (P<0.0001) (Table 3).

**Statistical analysis**

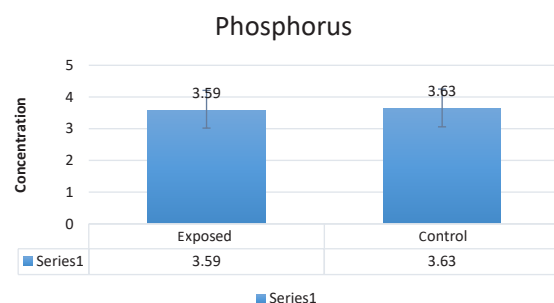
The obtained data were expressed as Mean±SD and analyzed in SPSS Vrsion 22. The Independent t-test, regression and Mann-Whitney test was used for comparison of the means of two groups. P values less than 0.05 were considered statistically significant.

**Discussion**

Vitamin D3 as an immunomodulatory agent enhances immune responses against infections and suppresses excessive inflammatory reactions. In addition, studies show that Vitamin D3 could suppress inflammatory cytokines and utilized as supplementary drug to treat inflammatory disorders such as autoimmune diseases. On the other hand, individuals who are exposed to sulfur mustard suffer from chronic pulmonary infections and their bronchus were obstructed and inflammatory cytokines were up-regulated. Furthermore, some studies indicate that people with COPD suffer from Vitamin D deficiency. Therefore, vitamin D3 has a critical role in suppressing inflammatory cytokines and establish homeostasis in



**Figure 2.** Serum level of calcium in exposed and control groups



**Figure 3.** Serum level of phosphorus exposed and control groups

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body with prevention of inflammatory reactions. However, this equilibrium was perturbed when Vitamin D3 level is not sufficient. Also, calcium and phosphorus as functional components act within the network of Vitamin D3 performance [24-26].

In the present study, we showed a significant correlation between vitamin D3 status and lung chronic inflammation caused by sulfur mustard in exposed individuals to sulfur mustard as compared with the control group. Moreover, the level of serum calcium reveals a significant difference between the exposed group and control group. However, there are no significant differences between the exposed group and control group with regard to phosphorus level.

As we know, vitamin D has a classical role to absorb calcium and phosphorus in intestine [15, 27, 28]. Also, vitamin D3 operates as a regulator of immune system. Thus, vitamin D illustrates a new aspect of natural component of immunomodulation in this respect. As mentioned above, several studies revealed that individuals with COPD are deficient in vitamin D [20]. Also, Person et al. studied on the levels of vitamin D and lung function Forced Vital Capacity (FVC) and reported that vitamin D deficiency in individuals with COPD increases inflammatory cytokine, decreases FVC, and makes them susceptible to infection pulmonary [29]. In another study, Janssens et al. measured levels of serum Vitamin D, and reported a correlation between Vitamin D deficiency and COPD. Furthermore, they demonstrated a clear connection between levels of serum Vitamin D and lung function in individuals with COPD [21].

Barker et al. addressed the relationship between Vitamin D and pro-inflammatory cytokines. They concluded that Vitamin D deficiency coincide with increase in secretion of pro-inflammatory cytokines such as IFN- $\gamma$  and IL1 $\beta$  [30]. As we known, serum vitamin D is deficient in individuals exposed to sulfur mustard. Zhu et al. reported that serum and dietary calcium could reduce inflammation and also treatment with 1,25(OH) $_2$ D $_3$  suppress TNF $\alpha$  (tumor necrosis factor-alpha) expression synergically. Hyder et al. stated that phosphorus and hypophosphatemia were depleted in people with COPD and had correlation with respiratory failure [22, 23].

In the present study, calcium and Vitamin D level were low in exposed group as compared to control group. Therefore, inflammatory responses increases following vitamin D and calcium deficiency. On the other hand, phosphorus level has an important role in respiratory failure in individuals with COPD. Vitamin D deficiency

could produce inflammation, meanwhile its molecular mechanism is not fully understood. Generally, there is an interaction between Vitamin D receptor with FoxO (Forkhead box) protein and its regulator, SIRT1, which inhibits inflammatory status in the lung [31, 32]. This mechanism in individuals with inflammatory reactions in their lungs leads to lower activity of regulation of HDAC2 via production of stress oxidative [14].

As a result, lower SIRT1 in inflamed lung followed by enhancement of NF $\kappa$ B activity could lead to more production of inflammatory cytokines. Also, Vitamin D function reduces followed by lack of Vitamin D receptor translocation into cytoplasm. Thereby, pro-inflammatory cytokines are expressed more. Taken together, in the present study individuals exposed to sulfur mustard showed insufficient levels of vitamin D3. Additionally, measurement of expression and activity of Vitamin D receptor are necessary to understand its mechanism of action.

As levels of serum Vitamin D3 reduces in individuals exposed to sulfur mustard, higher inflammatory responses in the lung could be seen in these people. Thus, Vitamin D3 deficiency is correlated to deregulation in Vitamin D3 immunomodulatory function to prevent excessive inflammatory responses in individuals exposed to sulfur mustard.

## Ethical Considerations

### Compliance with ethical guidelines

The study was approved by the Ministry of Health of Iran and the Board of Research Ethics of Veterans Medical and Engineering Research Center (Shahed. REC. 1392. 49). A written informed consent was obtained from all subjects in this study.

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### Conflict of interest

The authors declare they have no conflict of interest.

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