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# Effect of Vitamin D on the Expression Level of the IL-10 and IL-4 in Asthmatic Mice

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**Abstract:** Asthma is a chronic disease that affects the airways in the lungs and is common in many countries. Studies have shown that vitamin D levels are associated with the onset and exacerbation of asthma symptoms. Given the role of vitamin D in regulating immune responses and the importance of the two major cytokines IL-4 (an inflammatory cytokine) and IL-10 (an anti-inflammatory cytokine) in immunological processes, in this study, the relationship between vitamin D intake and the level of expression of these ILs in asthmatic mice was investigated. In the case-control study performed in three groups of mice, 10 experimental asthma mice, 10 asthmatic vitamin D-treated mice, and 10 healthy mice as the control group were studied. For analyses, after RNA extraction and cDNA synthesis, the expression level of two *IL-4* and *IL-10* genes was evaluated by real-time PCR. Finally, the results were analyzed by SPSS software. The results showed that in the control group, the expression level of *IL-4* and *IL-10* genes was 0.01 and 0.02, respectively. Accordingly, the expression level of the *IL-4* gene in asthmatic mice and asthmatic mice treated with vitamin D was 0.6 and 0.2, respectively, while the expression level of the *IL-10* in these groups was 1.2 and 2.8, respectively, which showed significant changes ( $p$ -value < 0.05) in treated mice compared to the asthmatic mice without treatment. Based on results, in asthmatic mice treated with vitamin D, a significant increase in IL-10 expression was observed (1.2 to 2.8), while IL-4 expression was decreased from 0.6 to 0.2. It appears that vitamin D supplementation in asthmatic patients by affecting T-cell maturation and increasing anti-inflammatory IL-10 and suppressing IL-4 may play a role in reducing the symptoms of asthma.

**Keywords:** Asthma; IL-4; IL-10; Vitamin D.

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## 1. Introduction

Asthma is a chronic disease characterized by variable symptoms such as airway obstruction and bronchitis. The airways become swollen, inflamed, and tender in subjects with asthma, and they react vigorously to certain inhalers [1-4]. World Health Organization (WHO) reported that the number of asthma patients is 300 million among all over the world, and it has been predicted to reach 400 million by 2025 [5]. Some believe that asthma is caused by a combination of genetic and environmental factors [6-8]. Moreover, it has been shown that alterations in epigenetics and lifestyle changes increase the incidence of asthma [9-11]. In asthma, as a heterogeneous disorder, cells like Th17, Th2, Th1, Treg, macrophages, neutrophils, dendritic, airway smooth muscle cells, and epithelial cells produce cytokines

associated with this disease. One of the essential cytokines is interleukin-4 (IL-4) [12]. IL-4 cytokine, through its function in regulating Th2 cell proliferation and survival, inhibiting Th1 differentiation, and synthesizing IgE, is crucial at the beginning of allergic airway and humoral responses at multiple levels [13]. In humans, it can be considered as the most important cytokine in the pathogenesis of allergic diseases and isotype changes from IgG to IgE [14].

Other important cytokines include interleukin 10 (IL-10) that produced from CD4<sup>+</sup> Th2 cells known as a cytokine synthesis inhibitory factor. IL-10 inhibits the production of IL-5 and IL-4 by Th2 cells [15, 16], as well as IL-2 and *IFN-γ* produced by Th1 lymphocytes [17]. Therefore, pro-inflammatory responses of both adaptive and innate immune cells are inhibited by IL-10 as a fundamental anti-inflammatory cytokine. Moreover, IL-10 represses the proliferative T cell responses to different antigens in peripheral blood mononuclear cells [18].

Recent evidence suggests that vitamin D status may play a role in regulating inflammatory processes contributing to the development of chronic diseases [19-21]. Recently, the *non-calcitropic* role of 1, 25-(OH)<sub>2</sub>-D, especially its anti-inflammatory and immunomodulatory action, has been drastically regarded. Animal studies have shown the beneficial effects of vitamin D and their compounds on various immune disorders (Mathieu C). Moreover, the recent investigations have explained some of the underlying mechanisms, proposing a potential therapeutic function of vitamin D, its analogs, and its metabolites for immune disorders in humans [22-26].

According to the results of previous studies, the prevalence of asthma is increasing worldwide [27]. Besides, according to studies on vitamin D deficiency and its effects on allergic reactions, it is necessary to investigate how this vitamin affects immunological factors and the control of allergic reactions. It seems that vitamin D treatment has anti-inflammatory effects in asthma by increasing the IL-10 expression and ultimately leading to the reduction of the IL-4 expression as an inflammatory cytokine. Therefore, the present study aimed to investigate the effect of vitamin D on IL-4 and IL-10 expression in a mouse model of asthma.

## 2. Materials and Methods

### 2.1. Mice and Grouping.

In the present study, 30 male C57BL/6 mice with a weight of 200-250g were used at about 3 months of age. The experiments were approved by the Institutional Animal Care and Use Committee at Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1398.197). All mice were caged and maintained according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Department of Health and Human Services). Mice were randomly divided into three different groups of 10 mice in each: A- healthy mice (normal control), B- mice model with asthma (positive control), and C- mice model with asthma treated by vitamin D. Mice in group C were injected intraperitoneally with 5 µg/kg vitamin D<sub>3</sub>, dissolved in 150 µl of sesame oil, three times a week.

### 2.2. Development of mice model with asthma.

On the first day of the experiments, ovalbumin solution was injected intraperitoneally into all mice except the control group (1 mg ovalbumin + 50 mg 10% Al (OH)<sub>3</sub> + 0 ml normal saline). Furthermore, on the 8th day, the ovalbumin solution (0.02 mg ovalbumin + 0.5 mg 10% Al (OH)<sub>3</sub> + 0.5 ml normal saline) was injected intraperitoneally. For inhalation, each

mouse received 100 mg of ovalbumin powder dissolved in 2.5 ml of normal saline, which was sprayed by a nebulizer and aerosol device into a closed chamber for 18 days.

### 2.3. Sampling.

On day 29, mice were anesthetized, and blood samples were collected in EDTA tubes by cardiac puncture.

### 2.4. RNA extraction.

Total RNA was extracted using the YTA RNA Extraction kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. Finally, the extracted RNA was eluted with 50 µl RNAase- free water and stored at -70°C. The RNA quality and quantity were assayed using spectrophotometric optical density measurement (wavelength, 260, and 280 nm).

### 2.5. cDNA synthesis.

For cDNA synthesis, the mixture containing 1 µl random hexamer primer (SinaClon, Iran), 5 µl extracted RNA, and 6 µl DEPC water incubated at 65°C for 5 minutes. Then, 4 µl 5X M-MLV RTase reaction buffer (Yekta Tajhiz, Iran), 2 µl dNTP mix (SinaClon), 1µl DTT (100 mM), 1µl M-MLV RTase (Yekta Tajhiz, Iran) were added to each tube, resulting in a final volume of 20 µl; followed by incubation at 50°C for 60 min and 95°C for 5 min. The synthesized cDNA was stored at -20°C until use.

### 2.6. Real-time PCR.

SYBR Green real-time polymerase chain reaction technique (RT-PCR) was employed to quantify IL-4, IL-10 mRNA expression, and GAPDH as the internal control. Amplification was performed on a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) using SYBR Green PCR Master Mix (Fermentas). The 25 µl of PCR mixture included 12.5 µl 2x SYBR Green Master Mix, 2 µl cDNA, 1 µl forward-primer, 1 µl reverse-primer, and 8.5 µl DEPC water. The PCR condition was 95°C for 15 minutes, followed by 35 cycles of 95°C and 57°C (63°C for GAPDH) for 20 and 63 seconds, respectively. For Non-template Negative Control (NTC) samples, the 2 µl of DEPC water was added instead of a 2 µl cDNA template. All the samples were tested in duplicate. The information of specific primers is shown in Table 1. The accuracy and specificity of primers activity in the real-time PCR were confirmed by examining the size of the PCR products on 2% agarose gel electrophoresis.

**Table 1.** Primers used for real-time PCR in this study.

Genes	Sequence (5' to 3')	Size of PCR Products (bp)	Annealing Temperature (°C)
<b>IL-4</b>	Forward:5'-TCCGACCACCACTACAGCAA-3' Reverse:5'-ATCTTTCAACACGCAGGACA -3'	416	57
<b>IL-10</b>	Forward:5'-ACACATGGTATAGATGCAGC-3' Reverse:5'-TTCCAAGACCTCAGGCAAGA-3'	102	57
<b>GAPDH</b>	Forward: 5'-TCGACAGTCAGCCGCATCTTCTTT-3' Reverse: 5'-ACCAAATCCGTTGACTCCGACCTT-3'	98	63

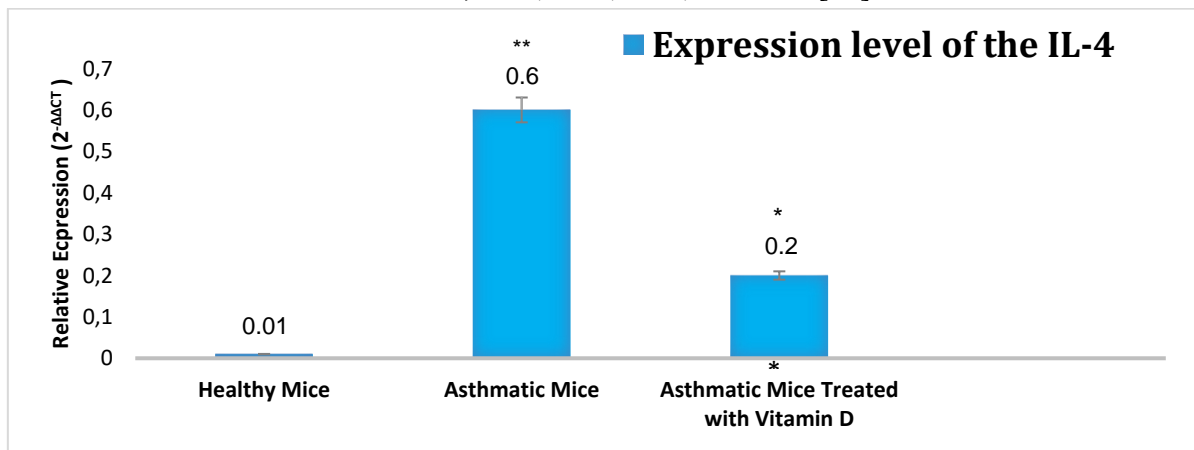
### 2.7. Calculations for relative quantification and data analysis.

The levels of IL-4 and IL-10 mRNA were calculated and analyzed using the  $2^{-\Delta\Delta CT}$  method. Data are expressed as mean  $\pm$  SD. Statistical t-test was conducted for pairwise comparison, and the P-values resulted in less than 0.05 were considered significant.

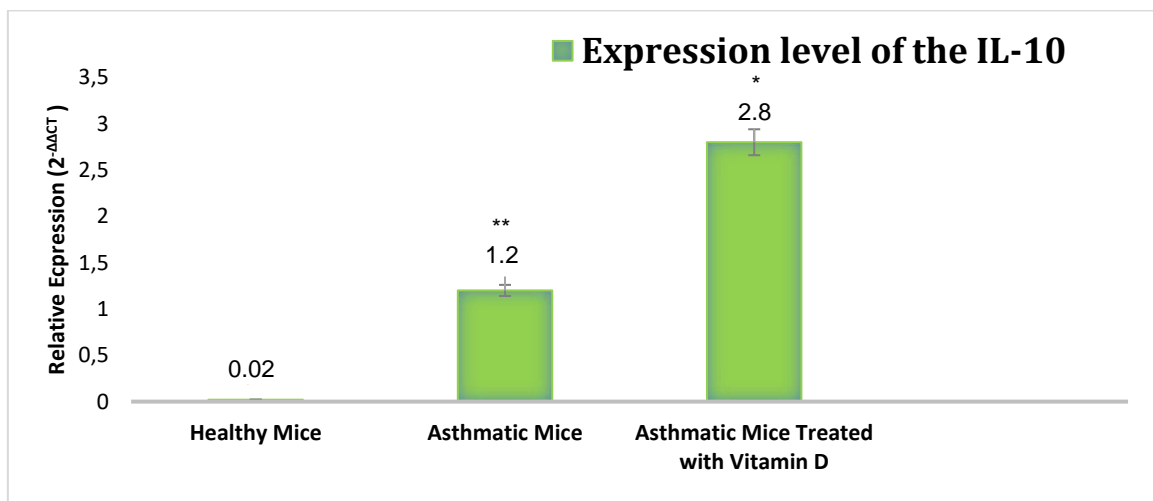
### 3. Results and Discussion

The healthy group samples were used as reference samples to compare changes between other groups. The expression of target genes in healthy mice was measured relative to GAPDH resulted in 0.01 and 0.02 for *IL-4* and *IL-10* genes, respectively. *IL-4* and *IL-10* expression in both asthma group without vitamin D3 administration and asthma group with vitamin D3 administration were significantly increased in comparison to the healthy control group ( $p < 0.05$ ) (Fig. 1 & 2). Furthermore, the relative expression of *IL-4* in the group with asthma that treated with vitamin D3 was significantly decreased (0.6 to 0.2) in comparison to the group with asthma without vitamin D3 treatment ( $p < 0.05$ ) (Fig. 1) while *IL-10* expression was significantly increased (1.2 to 2.8) the group with asthma treated with vitamin D3 than those in the asthma group ( $p < 0.05$ ) (Fig. 2). In addition, the real-time PCR products of GAPDH, *IL-4*, and *IL-10* showed a single band and expected size (Fig. 3).

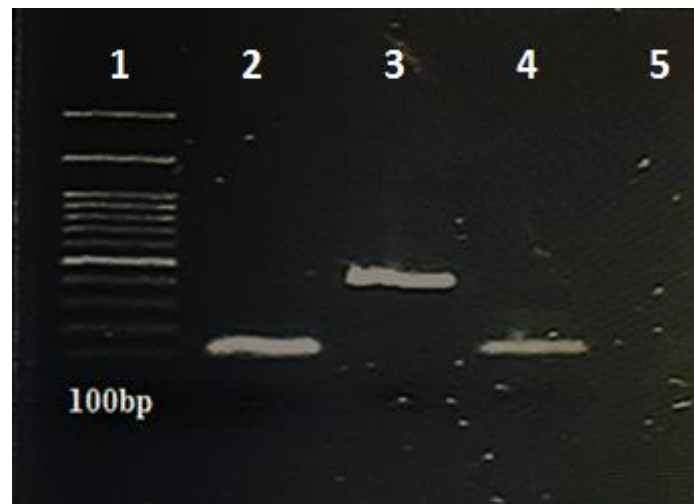
In recent decades, asthma has become one of the most common non-communicable diseases in the world. It is increasing due to the volume of air pollutants [28]. The pathogenesis of asthma includes the role of chemical mediators, mast cells, basophils, and platelets. Mast cells and basophils are stimulated due to the secretion of IgE from plasma cells, leading to the secretion of mediators such as  $IFN\alpha$ , *IL-1*, *IL-2*, *IL-3*, and *IL-4* [29].



**Figure 1.** Comparison of the relative expression of the *IL-4* gene between three different studied groups. \* $p < 0.05$ : Compared with the healthy group; \*\* $p < 0.05$ : Compared with the asthma group.



**Figure 2.** Comparison of the relative expression of the *IL-10* gene between three different studied groups. \* $p < 0.05$ : Compared with the healthy group; \*\* $p < 0.05$ : Compared with the asthma group.



**Figure 3.** Real-time PCR products on 2% agarose gel. 1: 100 bp Ladder, 2: Internal Control GAPDH, 3: IL-4, 4: IL-10, 5: NTC.

However, several asthma-related concerns remain poorly recognized. Consequently, investigation of asthma pathogenesis is crucial to find innovative preventive and therapeutic targets for asthma. In the present study, we studied the expression level of IL-4 as one of the crucial and key cytokines in the development of asthma together with the IL-10 as a regulator of the immune system by dividing the experimental samples into three groups of mice. According to the results of this study, the expression of IL-4 was significantly increased in mice model of asthma compared to healthy mice, which was following the results of the investigation conducted by Tachdjian *et al.* in 2019. They indicated an increased level of IL-4 in mice model with asthma, which may have exacerbated asthma symptoms and remodeled airways [30]. Furthermore, Steinke reported that the expression level of IL-4 elevated in most patients with asthma [29]. Moreover, in this study, a considerable increase of IL-10 expression was observed among mice model of asthma compared to healthy mice.

Various studies have published that vitamin D plays a crucial role in many aspects of allergic and autoimmune disorders and acts as an influential factor in regulating the immune system [31, 32]. Hatami *et al.* studied the relationship between vitamin D levels and asthma among children. They reported that most children with asthma had vitamin D deficiency. Only 5% of the subjects had adequate levels of vitamin D. Their results also show that vitamin D deficiency was associated with the severity of asthma [33]. In 2013, Rachel *et al.* examined the role of vitamin D in asthma. They demonstrated that vitamin D could reduce or control asthma symptoms, and vitamin D treatment reduced IL-4 concentrations and the inflammatory responses [5]. Whereas, the influence of vitamin D on IL-10 synthesis is controversial among scholars. Several previous reports state a decrease, while others declare elevated production [34, 35].

In this research, the impact of vitamin D on IL-4 and IL-10 expression was studied, and the results showed the relative expression of IL-4 was significantly decreased in the asthma group treated with vitamin D in comparison with the asthma group without any treatment. Also, the relative expression of IL-10 was substantially higher in the vitamin D treated group than the mice model with asthma. Based on the results of several studies, it can be expected that vitamin D treatment can help balance the levels of Th<sub>2</sub> and T regulatory lymphocytes. As a result, high levels of IL-10 secreted by Treg lead to decreased levels of IL-4. Regarding the alarming prevalence of vitamin D deficiency and its effects on the incidence of allergic complications and asthma, it is necessary to examine the relationship between these two

factors. Since asthma is a kind of Th2-type immune responses, it can be expected that this vitamin has a remarkable impact on atopic allergic responses through the induction of regulatory T-cells and the secretion of inflammatory cytokines [36]. As a result, vitamin D treatment can reduce responses and improve symptoms. However, evidence of the anti-inflammatory effect of vitamin D is rare in human clinical studies. Accordingly, it is suggested that further studies must be conducted to investigate the effect of vitamin D in reducing the complications of asthma in human samples.

#### 4. Conclusions

According to studies, increasing the level of vitamin D in mice by affecting the function of interleukins can be reduced the symptoms of the asthmatic disease. Since mice are mammalian models similar to humans, it can be concluded that Vitamin D can be also effective in strengthening the immune system in the human body. However, more studies are needed.

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#### Conflicts of Interest

The authors declare no conflict of interest

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