ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2023; 22(3):254-264.
DOI:10.18502/ijaai.v22i3.13053

Effect of Vitamin D on the HMGB1/RAGE Pathway and Adipokines Levels in Obese Asthmatic Mice

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Received: 11 January 2022; Received in revised form: 14 March 2023; Accepted: 19 March 2023

ABSTRACT

Compared to common asthma, obese asthma is difficult to control. Previous studies have shown that vitamin D (Vit D) has a therapeutic effect on asthma. Nevertheless, the action mechanism of Vit D for obese asthma are not well known.

In this study, we, therefore, induced obesity and established an obese asthma mouse model using ovalbumin (OVA) stimulation and applied treatment with Vit D (100 ng/kg). Accordingly, thirty mice were randomly divided into 5 equal groups of normal control, asthma, obese asthma, asthma+Vit D, and obese asthma+ Vit D. The levels of inflammatory factors and adipokines were measured by the ELISA assay; then the quantitative reverse transcription PCR (qRT-PCR) method was used to evaluate the expression of high mobility group box 1(HMGB1) and receptor for advanced glycation end products [RAGE] genes.

The results showed that OVA sensitization significantly increased airway resistance, the levels of inflammatory cytokines, and HMGB and RAGE expression in asthmatic and obese asthmatic mice, as compared to the control group. Also, these changes in the obese asthmatic group were notably higher than those in the asthmatic one. In addition, the treatment of asthmatic and obese asthmatic mice with Vit D significantly reduced the raw, serum and BALF levels of inflammatory cytokines, as well as the expression of HMGB1 and RAGE mRNA.

To conclude, the present study showed that vitamin D might attenuate lung injury by up-regulating HMGB1 and RAGE expression. Our findings, thus, may offer new concepts and approaches for the treatment and prevention of obese asthma.

Keywords: Asthma; group box1; High mobility; Inflammation; Obesity; Vitamin D3

INTRODUCTION

Asthma is recognized as an inflammatory disease affecting the airways of the lungs; it is recognized by reversible airflow obstruction, variable and recurring symptoms, and easily triggered bronchospasms.[1] Despite the widespread pharmacologic treatment including inhaled corticosteroids and bronchodilators in the treatment of patients with asthma, its management is still unacceptably poor.[2,3]

Over the last period, substantial hard work has been done to discover the molecular and cellular mechanisms of asthma. Animal models and clinical studies have established a role for high-mobility group box 1 (HMGB1) and its receptors in asthma. HMGB1 is an

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inflammatory mediator abnormally expressed in asthmatic patients.[4] As a result, the HMGB1/RAGE/NF-κB signaling pathway is an important part of immunoregulatory processes.[5] However, the precise role of HMGB1 in obese asthmatic mica is unclear. So, regulation and intervention of any part of the signaling pathway may affect the development and occurrence of asthma.

Recently, research has tended to focus on obesity as a risk factor for both prevalent and incident asthma [6]. Obesity can deteriorate the condition of asthma, and it may alter the effectiveness of standard asthma medications, leading to the poor response of obese asthma to the treatment of glucocorticoids.[6,7] Obesity-induced disorders involve endocrine function alterations of the adipose tissue, including adipokines, leptin, leptin receptor and adiponectin, which, in turn, worsen the condition of asthma.[8] An additional question is related to the effect of vitamin D on obese asthma.

Nowadays, there is a large body of data indicating that Vit D can serve as a supplementary medicine via exhibiting a remarkable immunoregulatory effect that may improve asthma symptoms.[9,10] As previously reported, Vit D3 can downregulate the expression of MHC class II molecules and co-stimulatory molecules on the antigen-presenting cell surface, inhibit antigen presentation and T cell immune response, and impede the expression of IL-4 and interferon-γ, thus alleviating airway inflammation.[11] The present study, therefore, examined the role of Vit D in a mouse model of asthma and the underlying action mechanism of HMGB1/RAGE in obese asthma; this may then serve as a beginning hypothetical point for the clinical application of targeted drug therapy and development of new drugs against obese-asthma phenotypes.

Although previous studies have suggested the preventive effect of Vita D in the management of asthma, there are still inconclusive results. This raises many questions regarding the effect of Vit D on obese asthmatic mice. To better understand the direct effects of Vit D on asthma and pursue further clinical studies, we first established the mouse model of obesity and asthma, and examined the asthma severity of the obese mice. Furthermore, we inspected the therapeutic effect of Vit D and the main action mechanism for obese asthma. Further, we will discuss knowledge gaps and provide suggestions for future studies.

MATERIALS AND METHODS

Drugs and Kits

The ovalbumin, vitamin D, Trizol reagent, and pentobarbital sodium were purchased from Sigma Chemical Co. (Sigma, Germany). The Mouse adiponectin (ab108785), leptin (ab199082), IL-4 (ab100710), IL-6 (ab222503), IL-10 (ab108870), IL-17 (ab100702), IL-1β (ab197742), TNF-α (ab208348), and IgE (ab157718) ELISA kits were obtained from Abcam company (Cambridge, UK). The DNA Synthesis Kit was purchased from Jienuo Inc, Shanghai, China.

A total of 30 male Balb/c mice, weighing 22 to 25 g, were purchased from the Qinglongshan Animal Breeding Base, Nanjing, China. The animals were fed either a standard chow diet or a high-fat one that induced obesity and tap water *ad libitum.* Mice were acclimatized in a well-ventilated room with a 12-hour light/12-hour dark cycle, and a normal temperature of 25 ± 3°C. The animal handling procedures were conducted in conformity with the international guidelines for the care and use of experimental animals and approved by the local Research Ethical Committee at Jiaxing University.

Experimental Procedures

Mice were randomly allocated into five equal groups (n=6): normal control group (group I), asthma group (group II), obese asthma group (group III), asthma + Vit D group (group IV), and obese asthma + Vit D group (group V). Groups I, II and IV were fed the standard mice chow and groups III and V were fed by applying the high-fat diet (16 kJ% from protein, 29 kJ% from carbohydrates, and 55 kJ% energy from fat) to achieve a diet-induced obesity model. After 8 weeks of feeding, the asthma model was established using a previously described method. Briefly, in groups II-V, 0.2 ml of a freshly prepared antigen solution (50 μg ovalbumin (OVA) + 0.15 ml 10% Aluminum hydroxide + 0.05 ml normal saline) was intraperitoneally (i.p) injected on days 1, 8 and 15. Then, the mice were placed in a closed container and challenged with aerosol OVA (5% in normal saline) for 45 min daily, from day 21 to day 28. The control group was stimulated and challenged with normal saline. In groups IV and V, the mice were given l ml of Vit D (100 ng/ml, dissolved in corn oil) orally 45 min before the OVA challenge, while the other groups were treated with 1 ml corn oil at the same time.

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Sample Collection

Twenty-four h after the last stimulation, the mice were anesthetized with xylazine/ ketamine (i.p) and the blood samples were collected by cardiac puncture and centrifuged at 3000 rpm for 10 min at 4 °C; the separated sera were stored at - 80°C for next biochemical analysis. After sacrificing the animals, their lungs tissues were removed and rinsed with 0.5 ml of PBS three times through tracheal intubation; bronchoalveolar lavage fluid (BALF) was isolated and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant fluids were stored at - 80°C for inflammatory factors and the precipitated cells were resuspended, fixed, stained with Giemsa staining, and examined under light microscopy. The lung tissues were then kept in liquid nitrogen for the next gene expression analysis.

Measurement of Airway Resistance (Raw)

To evaluate the airway resistance, the increase in pulmonary resistance was evaluated in response to enhancing the concentrations of acetylcholine in anesthetized mice. Briefly, the animals were anesthetized with xylazine/ ketamine (190–4.25 mg/kg). Trachea was exposed and cannulated with an 18-gauge tracheal tube. Then a stitch was placed around the trachea to prevent air leakage. The mice were mechanically ventilated using a ventilator for small animals (VentElite, Harvard Apparatus, Holliston, MA, USA) with a respiratory rate of 150 breaths/minute, inhalation and exhalation ratio of 2:3, tidal volume of 10 ml/kg, and positive end-expiratory pressure of 2-3 cm H2O. Then the ascending concentrations of acetylcholine (1-5 mg/ml) were given to the mice and a reading was carried out every 3 minutes for 10 seconds in each concentration. Raw was calculated using the flexiVent software.

Real-time Polymerase Chain Reaction Analysis

Total RNA from lung tissues was extracted by using Trizol reagent and total extracted mRNA was reverse-transcribed using the DNA Synthesis Kit according to their manufacturer's protocols. Quantitative reverse transcription PCR (qRT-PCR) reactions were performed with SYBR green and LightCycler 96 System (Roche Life Science, Italy). The following specific primer sequences were used: HMGB1: F: 5′-TAT CTA AAT ACG GAT TGC TCA GGA A-3′ and R: 5′-AGG GAC AAA CCA CAA TAT AGG AAA A-3', RAGE: F: 5′-TGA ACT CAC AGC CAA TGT CCC TAA-3′ and R: 5′-CGA AGC GTG AAG AGA CCC GT-3’, and GAPDH: F: 5′-AAC ATC GAT CTC GAG GTC-3′; R: 5′-TTC AAC TGC CGC AGG GTT-3′. The $2^{-\Delta\Delta Ct}$ method was used for the analysis of raw data and the housekeeping gene GAPDH was used as an internal control [12].

Measurement of Inflammatory and Obesity-related Factors

Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of IL-4, IL-6, IL-10, IL-17, IL-1β, TNF-α, and IgE in serum, and IL-4 and IgE in BALF, according to the manufacturer’s instructions. Also, the serum concentrations of adiponectin and leptin were determined by the ELISA assay. Briefly, 100 µL of standards or samples were added in duplicate into the reaction well and the plate was covered, shaken slightly, and incubated at room temperature for 60 min. After aspiration, 100 µL of biotin-conjugated secondary antibody was added to each well and incubated at room temperature for 30 min. Next, each well was aspirated, washed three times, supplemented with substrates A and B (each 50 µL), gently mixed, and incubated for 30 min at room temperature in the dark. Finally, 50 µL of the stop solution was added to each well and the optical density[13] was read using an ELISA microplate reader at 450 nm

Statistical Analysis

The differences between the obtained values (Mean±S.E.M) were tested by one-way analyses of variance (ANOVA) and this was followed by the Tukey post hoc test using the SPSS 16.0 software. *p* values of 0.05 or less were considered to show significant differences for all comparisons made.

RESULTS

The Body Weight of Mice

As shown in Figure 1, 12-week feeding of mice with a high-fat diet meaningfully increased their body weight, so that the mean body weights in the obese asthmatic group and obese asthmatic treated with Vit D were significantly higher than those of the control group ($p<0.001$).

Effect of Vit D on Airway Resistance

The effect of Vit D on airway resistance (Raw) is shown in Figure 1. As can be seen, the Raw in asthmatic

mice and obese asthmatic mice groups was significantly higher than that in the control group (p<0.001). Also, the results showed that airway resistance was notably higher in asthmatic obese mice than in the asthmatic group. In addition, the treatment of asthmatic mice with Vit D significantly reduced airway resistance (p<0.001), as shown in Figure 2.

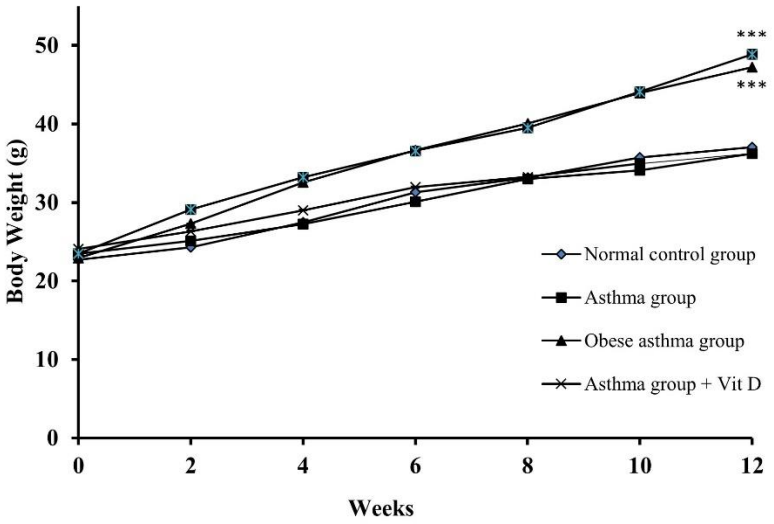

Figure 1. Body weight of mice in each group during the study. To investigate the effect of high fat diet (HFD) and Vit D on animals' body weight changes, we measured the weight of the mice of all groups every week. Values are presented as Means±SD (n=6), ***p<0.001, as compared to the normal control group.

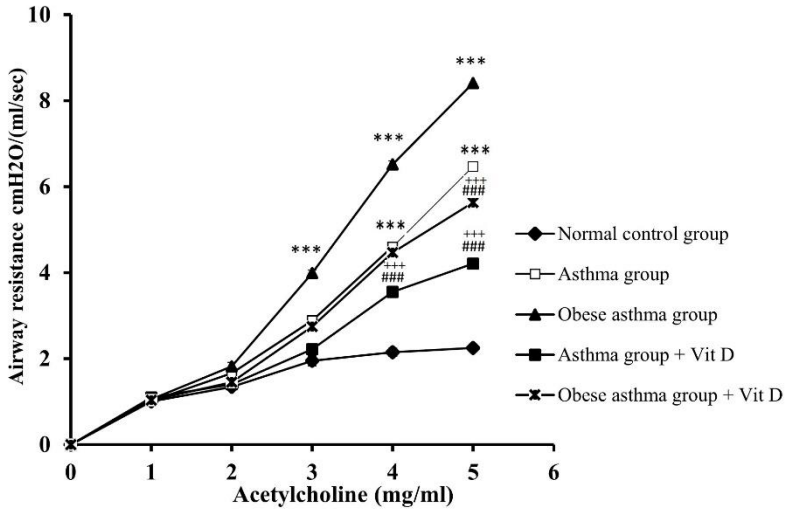

Figure 2. Effect of Vit D on airway resistance. To evaluate the effect of Vit D on airway resistance, the increase in pulmonary resistance was evaluated in response to enhancing the concentrations of acetylcholine in anesthetized mice. All values are expressed as Mean±SD. ***p<0.001, as compared to the control; $^{###}p$<0.05, as compared to the obese asthma group; $^{+++}$ p<0.001, as compared to the asthma group.

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Effect of Vitamin D on BALF Composition

To investigate the effect of Vit D on asthma in obese mice, we examined the number of leukocytes and the percentage of eosinophils in BALF. As shown in Figure 3a, OVA-induced a significant influx of white blood cells into BALF in the asthmatic and obese asthmatic groups, as compared to the control group (p<0.001). Also, the level of eosinophils was significantly increased in the BALF of asthmatic and obese asthmatic mice groups (Figure 3b). Our results, thus, showed that treating asthmatic or obese asthmatic mice with vitamin D significantly decreased leukocytosis and absolute eosinophilia, as compared to the obese asthma group (p<0.001, Figure 3 a-b).

Effect of Vitamin D on IL-4 and IgE in the BALF

ELISA results showed that the levels of IL-4 and IgE in BALF were significantly increased in the asthma group and obese asthmatic mice than in the normal control one (p<0.05, Figure 4 a-b). The level of IgE in the obese asthma group was significantly higher than that in asthma one (p<0.001). After Vit D treatment, BALF levels of IL-4 and IgE were significantly decreased in the asthma group and obese asthma groups (p<0.05). These results, thus, show that Vit D can effectively decrease the inflammatory response in obese asthmatic mice.

Effect of Vitamin D on the Serum Levels of Inflammatory Factors

ELISA results displayed that the serum levels of IL-4, IL-6, IL-17, IL-1β, IgE and TNF-α were significantly increased (Figure 4 c, d, f - i), while and IL-10 was significantly decreased in asthma and obese asthma groups, as compared to the normal control group (p<0.05) (Figure 4d). After Vit D treatment, the serum levels of IL-4, IL-6, IL-17, IL-1β, IgE and TNF-α were significantly decreased, while IL-10 was significantly increased in the Vit D treated group, as compared to the obese asthma one (p<0.05).

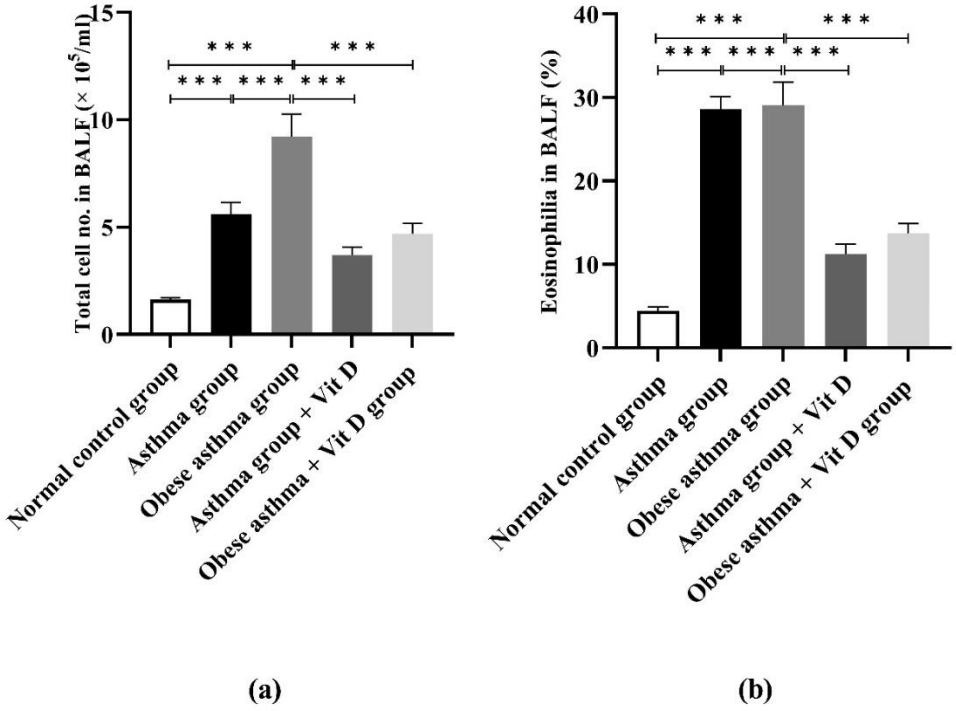

Figure 3. The total number of leukocytes and the percentage of eosinophils in the bronchoalveolar lavage fluid (BALF). To investigate the effect of Vit D on asthma in obese mice, we examined the number of leukocytes and the percentage of eosinophils in BALF. a) Total cells number in BALF and b) The percentage of eosinophil. ***p<0.001

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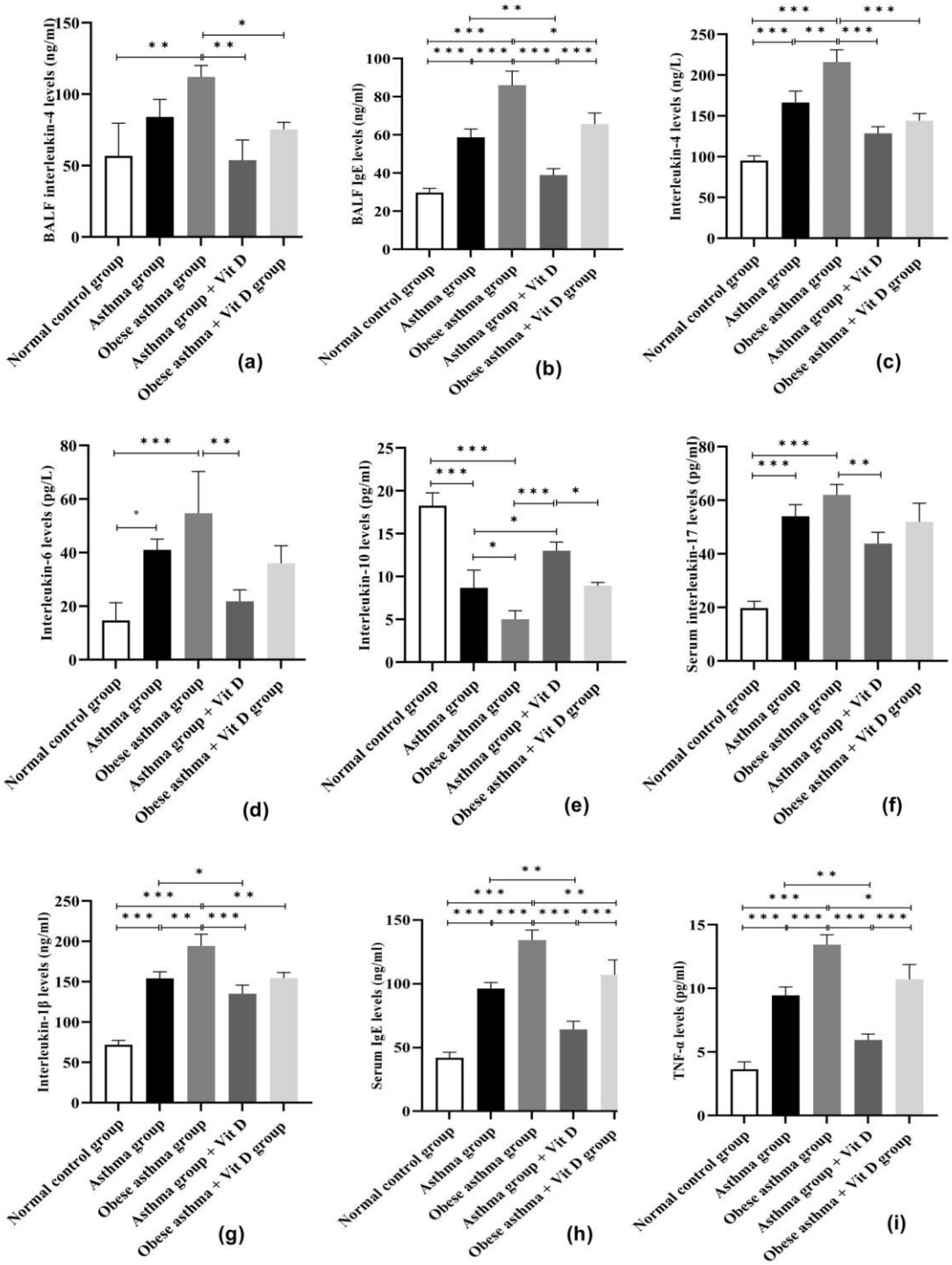

Figure 4. ELISA assay was used to evaluate the effects of vitamin D (Vit D) on the inflammatory and obesity-related factors in bronchoalveolar lavage fluid (BALF) and serum. (a) Interleukin-4 in BALF, (b) IgE in BALF, (c) Interleukin-4 in serum, (d) Interleukin-6 in serum, (e) Interleukin-10 in serum, (f) Interleukin-17 in serum, (g) Interleukin-1β in serum, (h) IgE in serum, (i) tumor necrosis factor (TNF)-α in serum. *p<0.05, **p< 0.01, ***p<0.001

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Comparison of Adiponectin and Leptin in the Serum

The levels of adiponectin in the obese asthma group were significantly lower than those of the normal control and asthma groups (p<0.001). However, the serum levels of adiponectin were increased in the Vit D treated groups (p<0.001, Figure 5a). The Leptin levels in the obese asthma group were significantly higher, as compared to the normal control and asthma groups (p<0.001). Treatment of obese asthmatic mice with Vit D noticeably decreased the serum levels of leptin (p<0.001, Figure 5b).

Effect of Vitamin D on the Expression of HMGB1 and RAGE

The results of qRT-PCR showed that the mRNA expression of HMGB1 and RAGE was significantly increased in the lung tissues of asthmatic and obese asthmatic groups, as compared to the normal control group (p<0.05); the levels were further increased in the obese asthmatic group, as compared to the asthma one (p<0.05). Treatment of asthmatic and obese asthmatic mice with Vit D also noticeably decreased the expression levels of HMGB1 and RAGE (p<0.001, Figure 6 a, b).

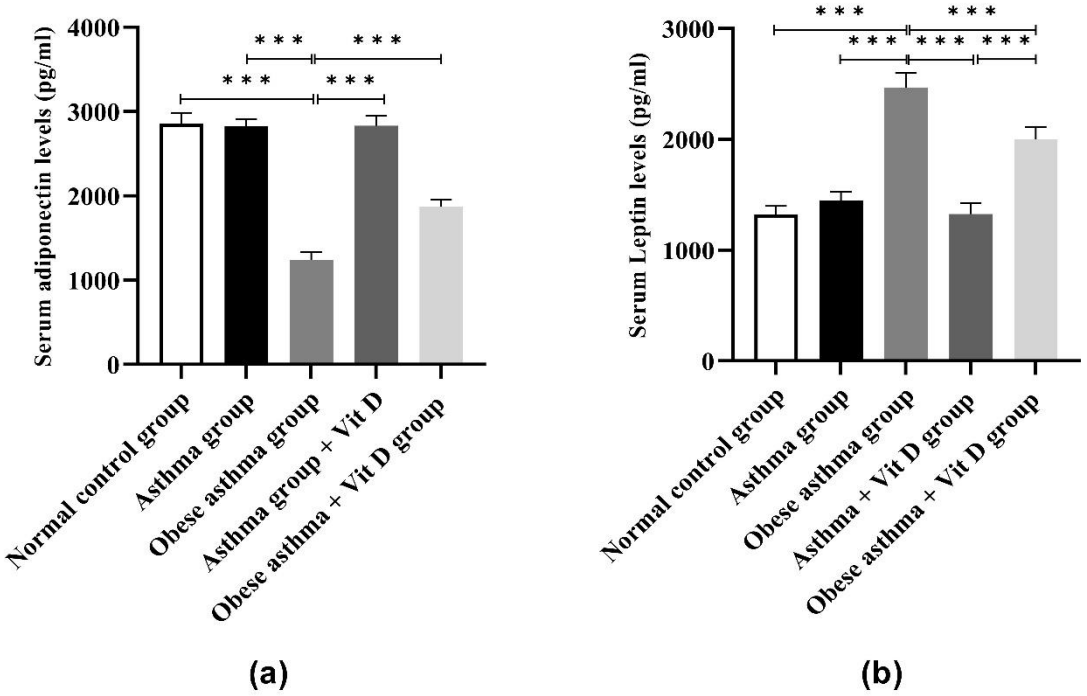

Figure 5. ELISA assay was used to evaluate the effects of vitamin D (Vit D) on the serum levels of adiponectin and leptin study groups (a) adiponectin (b) leptin. ***p<0.001

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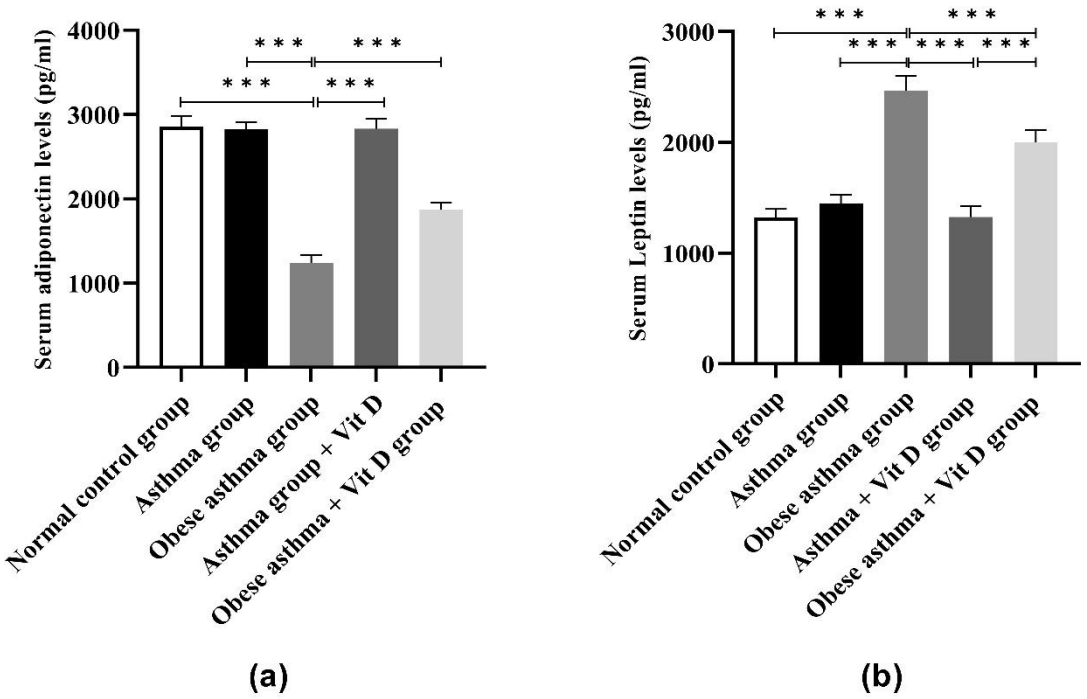

Figure 6. Quantitative reverse transcription PCR (qRT-PCR) assay was used to examine the effects of vitamin D (Vit D) on the expression levels of High mobility group box 1 (HMGB1) and Receptor for Advanced Glycation Endproducts [RAGE] mRNA. (a) HMGB1, (b) RAGE, ***p<0.001

DISCUSSION

The main results of this study could be summarized here: (1) compared to the asthma group, the obese asthmatic mice had more severe asthma symptoms and increased expression of HMGB1 and RAGE; (2) *in vivo* Vit D supplementation noticeably decreased the airway resistance, leukocyte infiltration, HMGB1 and RAGE expression, as well as the levels of inflammatory factors in obese asthmatic mice.

Asthma in obese individuals is usually more severe than in the normal weight subjects, and the data obtained from translational and clinical studies proposed that obese patients could respond to therapies, predominantly corticosteroids.[14] Therefore, finding another option for asthma in obesity is a matter of great importance. In the present study, we found that Vit D could modulate adipokines in obese asthmatic mice; it can also ameliorate asthma symptoms and airway inflammation, reducing inflammatory markers. To the best of our knowledge, this is the first report comparing HMGB1/RAGE signaling in obese asthma. In this study, an obese asthma mouse model was established; the obtained data showed that Vit D greatly attenuated the inflammatory response. This effect of Vit D was attained through the HMGB1/RAGE signaling pathway, but the control group was not significantly improved. Vitamin D3 treatment also suppressed leptin expression in the obese asthma mice model. Overall, these results revealed that Vit D could protect airway injury from obesity asthma via the suppression of HMGB1/RAGE signaling and inhabitation of the adipokines pathway; therefore, Vit D may be considered effective in the control of obese asthma.

Various studies have shown Vit D effects on many aspects of asthma; this vitamin, thus, acts as a powerful factor in regulating the immune system.[9-11] Vit D has been reported to exhibit several pharmaceutical properties, including anti-inflammatory[15] and immunoregulatory activities.[16] Recently, Rigo et al,[17] reported that vitamin D3 deficiency exaggerated the features of airway disease in asthmatic mice, concluding that vitamin D3 supplementation led to the modulation of the innate immune defense of airway epithelium in asthmatic mice.[18] A previous study also demonstrated that vitamin D could downregulate the expression of

HMGB1/TLR4 by alleviating airway inflammation.[11] HMGB1 chiefly acts as a pro-inflammatory factor, contributing to the process of oxidative stress and inflammatory response; it also directly contributes to the pathogenesis of asthma [5]. The expression of HMGB1 can also increase the endothelial cell gap and destroy the cytoskeleton. So, decreasing the expression of HMGB1 can effectively slow the pulmonary inflammatory response.[19] The qRT-PCR results also showed that the relative expression of HMGB1 and RAGE mRNA in the obese asthma group was significantly higher, as compared to the control and asthma groups. Our results, thus, proved that RAGE and HMGB1 were involved in the pathogenesis of obese asthma. In this research, for the first time, the impact of Vit D on HMGB1/RAGE signaling pathway expression was studied in obese asthmatic mice; the results revealed the relative expression of HMGB1/RAGE was significantly decreased in the Vit D-treated obese asthma group, as compared to the control group. The promising mechanism includes HMGB1 activation of the downstream signal transduction pathway through RAGE signaling, promotion of the release of inflammatory mediators and cytokines, aggravation of lung inflammation and airway remodeling. Our findings, thus, established that HMGB1 and RAGE were involved in the pathogenesis of obese asthma, serving as a hypothetical basis for the clinical use of targeted drug therapy and the development of new drugs against the obese asthmatic phenotype.

Previous studies have also shown that adipocyte abnormality is one of the important mechanisms of obese asthma.[6,8] The most common immune abnormality in asthma is functional imbalance and the proportion of leptin/adiponectin.[20]

Previously, Vit D has been identified as having typical anti-inflammatory properties against pro-inflammatory cytokines by the adipose tissue. Therefore, we speculated that Vit D could elicit protection against inflammation in obese asthma by the inhibition of serum leptin. Our results also indicated that Vit D could decrease and increase the levels of leptin and adiponectin, respectively. Also, the relative expression of leptin was substantially higher in the obese asthma group than in the mice model with asthma. We, thus, hypothesized that leptin might further promote the immune response and further aggravate the severity of obese asthma. Based on our results, Vit D treatment can help to balance the levels of leptin/adiponectin. The obese asthmatic phenotype of asthma is associated with additional symptoms, more severe exacerbation, and decreased response to standard medication.[7,8,20,21] Airway remodeling is the most common pathophysiological feature of obese asthma, which is characterized by the obstruction, airway hyper-responsiveness and mucus hyper-production.[22] Obesity-associated hormone, leptin, plays a crucial role in stimulating the inflammation of allergic airways in asthma by activating lung immune cells and modulating the influx of circulating immune cells. Since obese asthma is a kind of imbalance of adipokines, Vit D has a remarkable impact on obese asthma through leptin and adiponectin regulation.[13] As a result, Vit D treatment can reduce responses and improve symptoms. However, there is not still much evidence regarding the role of Vit D in the regulation of adipokines in human clinical studies.[23] Therefore, further studies should be conducted to investigate the effect of Vit D on reducing the complications of obese asthma in human samples. Therefore, Vit D3 may attenuate obesity airway remodeling via the inhibition of leptin levels. The results also confirmed that HMGB1 and RAGE were involved in inducing asthma by obesity. Nevertheless, the current study used only qRT-PCR to identify HMGB1 and RAGE; further experimental methods should be investigated in future works. Our study faced some limitations; these included not assessing the Western blot analyze; as well, and the duration of the study was relatively short. Finally, the relationship between the HMGB1/RAGE/cascade and adipokines was not addressed, which should be further investigated in our forthcoming work.

To conclude, the present study showed that vitamin D might attenuate lung injury by decreasing adipokines levels and also, up-regulating HMGB1 and RAGE expression; these, in turn, lead to decreasing the inhibition of downstream inflammatory cascades.

STATEMENT OF ETHICS

A total of 30 male Balb/c mice, weighing 22 to 25 g, were purchased from the Qinglongshan Animal Breeding Base, Nanjing, China. All animal experiments were conducted according to the Chines guidelines for experimental animal welfare and approved by the local Research Ethical Committee at Jiaxing University (Approval No.: SYXK-2020-0007). The study was carried out in accordance with the ARRIVE guidelines.

FUNDING

This work was supported by grants from the Jiaxing City Science and Technology Plan (Project+2022AD30023)

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank Jiaxing University for their providing advice to this study.

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