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Role of 1,25-dihydroxyvitamin D_3 in the treatment of asthma

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Abstract. – OBJECTIVE: This study aims to observe the influence of 1,25-$(OH)_2D_3$ on airway inflammation and chemokine expression in asthmatic rats and to explore its significance in the treatment of asthma.

MATERIALS AND METHODS: Wistar rats were randomly divided into a normal control group (N), an asthma group (A), a 1,25-$(OH)_2D_3$ group (VD), a budesonide group (P) and a 1,25-$(OH)_2D_3$ + budesonide treatment group (L). The acute asthma models were established through ovalbumin sensitisation and challenge. Lung tissues were stained with haematoxylin and eosin to observe pathologic changes, whereas an enzyme-linked immunosorbent assay was used to examine serum IgE, as well as the eosinophil chemoattractant protein (eotaxin) and interleukin-8 (IL-8) expression levels in bronchoalveolar lavage fluid and the serum.

RESULTS: VD treatment partially reversed the characteristic pathological changes of airway inflammation. The IgE, eotaxin, and IL-8 expression levels in the VD group were significantly lower than those in the A group ($p < 0.05$) but remained higher than those in the control group ($p < 0.05$).

CONCLUSIONS: 1,25-$(OH)_2D_3$ reduces airway inflammation, airway hyperresponsiveness and airway remodeling by partially inhibiting chemokine production during airway inflammation, and 1,25-$(OH)_2D_3$ synergises with hormone therapy.

Key Words:
Airway inflammation, Asthma, EOS, Eotaxin, IgE, IL-8, 1,25-$(OH)_2D_3$.

Introduction

Bronchial asthma is a chronic airway inflammatory disease[1] that involves eosinophils (EOS), lymphocytes, neutrophils, mast cells and other cells and components. It is characterised by chronic inflammatory disorder of the airway, airway hyperresponsiveness (AHR) and irreversible airflow obstruction. Asthma and airway inflammation are closely related and different types of airway inflammations occur in asthma. When tissues are damaged or inflamed, the local increase in inflammatory chemokine concentration directs inflammatory cells to achieve and maintain a high concentration of partial local concentration of inflammatory cells that promote inflammation and aggravate asthma. EOS infiltration is a main feature of airway pathology and the EOS chemoattractant protein (eotaxin) promotes EOS migration to the airway and EOS activation[2]. Interleukin-8 (IL-8) is an effective activating factor and chemokine for neutrophils and EOS that may indicate severe asthma. Inhaled corticosteroids effectively control asthma; thus, inhaled corticosteroid therapy is recommended as the primary treatment for maintenance and mitigating asthma exacerbation. Nonetheless, glucocorticoid treatment is ineffective in some children. Taher et al[3,4] determined that 1,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$] is the most important active metabolite of vitamin D (VD); it has a complex role in regulating chronic airway inflammation, autoimmune diseases and chemokine expression. However, its effect on the regulation of chemokine eotaxin and IL-8 expression in asthma has not been reported. Therefore, we established an experimental rat model of acute asthma by administering 1,25-$(OH)_2D_3$, glucocorticoids and their combination to observe the relationship between airway inflammation and chemokine expression. Determining the effect of 1,25-$(OH)_2D_3$ on airway inflammation will help develop new therapeutic approaches for treating asthma.

Materials and Methods

Model and Grouping

Fifty healthy, female, SPF-grade Wistar rats weighing 150 g to 160 g were purchased from

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the Experimental Animal Center of China Medical University (Shenyang, China). This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University. The rats were randomly divided into normal control group (N), asthma group (A), 1,25-$(OH)_2D_3$ group (VD), budesonide (Pulmicort group, P) and 1,25-$(OH)_2D_3$ and budesonide combination therapy group (L), n = 10. The Wistar rat asthma models were established based on our previous study[5] and that of Vanacker et al[6]: the asthmatic rats were intraperitoneally injected with antigen sensitising liquid (1 mg of ovalbumin, OVA; Grade V; Sigma, St. Louis, MO, USA), and 200 mg/ml aluminium hydroxide (Sigma), was dissolved in 1 ml of phosphate buffer (phosphate-buffered saline, PBS). Meanwhile, 1 ml (6×10^9) of inactivated pertussis vaccine (Beijing Institute of Biological Products, Beijing, China) was injected intraperitoneally to the rats for sensitisation. For the PBS-treated control group, the rats were placed daily for 14 days in a non-completely transparent sealed glass container (20 cm × 20 cm × 20 cm) connected to an ultrasonic nebuliser (Shanghai Medical Instrument Factory, Shanghai, China). Once a day for 7 d, 4 rats were sensitised by spraying 20 ml of 1% OVA for 20 min. For the control group, PBS was used instead of atomised OVA. For the treatment group, the rats were treated 1 h before OVA sensitisation. The VD group received 1,25-$(OH)_2D_3$ (Roche, Shanghai, China) 2.5 μg/d gavage, whereas the B group was given 0.5 mg of budesonide (Pulmicort) suspension (AstraZeneca Pty Ltd, North Ryde, Australia) for inhalation. Budesonide and 1,25-$(OH)_2D_3$ were used in the combined treatment group at previously described doses and modes of administration.

Lung Histopathology

The rats in each group were collected within 24 h after the challenge. The rats where sacrificed with an overdose of phenobarbital and the middle lobe of the right lung was collected and fixed with 4% paraformaldehyde for 18 h to 24 h. The lung tissues were embedded in paraffin and cut in 5 μm sections. The lung tissues were stained with haematoxylin and eosin (HE) to detect pathologic changes.

Plasma-Specific IgE, Eotaxin, and IL-8 Levels

The rats in each group were anaesthetized with 10% chloral hydrate (40 mg), their chest cavities were opened, and blood was collected from the heart. Serum was collected by centrifugation and frozen at –80°C, and subsequently used to quantify the OVA-specific IgE (OVA-specific IgE, OVA-sIgE), eotaxin and IL-8 levels using an enzyme-linked immunosorbent assay (ELISA) detection kit (R & D Systems, Minneapolis, MN, USA).

Bronchoalveolar Lavage and Cell Counts, and the Eotaxin and IL-8 Levels

Each rat was bled and executed with an excess of phenobarbital. The trachea was separated and the right main bronchus was ligated. The left bronchus was intubated and injected with saline. The intubation was maintained for 30 s and slowly withdrawn. This procedure was repeated three times and the bronchoalveolar lavage fluid (BALF) was collected (75% to 80% recovery rate). After measurement, 0.1 ml of the BALF was subjected to a WBC count on a counting board and the remaining BALF was centrifuged at 4°C and1000 r/min for 10 min. The supernatant was frozen at –80°C for subsequent determination of the eotaxin and IL-8 levels. The cell pellet was collected for smear and the cells were stained with Wright-Giemsa and observed under a microscope using an oil immersion objective for counting and classification, At least 100 cells per field for at least four horizons were counted. The EOS percentage was calculated and multiplied by the percentage of total cells to determine the absolute number of EOS.

Statistical Analysis

SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All measurement data are presented as ± s, and a one-way ANOVA was used to determine homogeneity, whereas an SNK-q test was used for pairwise comparisons. Moreover, a Kruskal-Wallis test was used to test for heterogeneity. Differences with $p < 0.05$ were considered statistically significant.

Results

Asthma Attack After Excitation

Asthma mostly occurred within 30 min after OVA excitation. The rats in the asthma group exhibited cyanotic lips, shortness of breath, abdom-

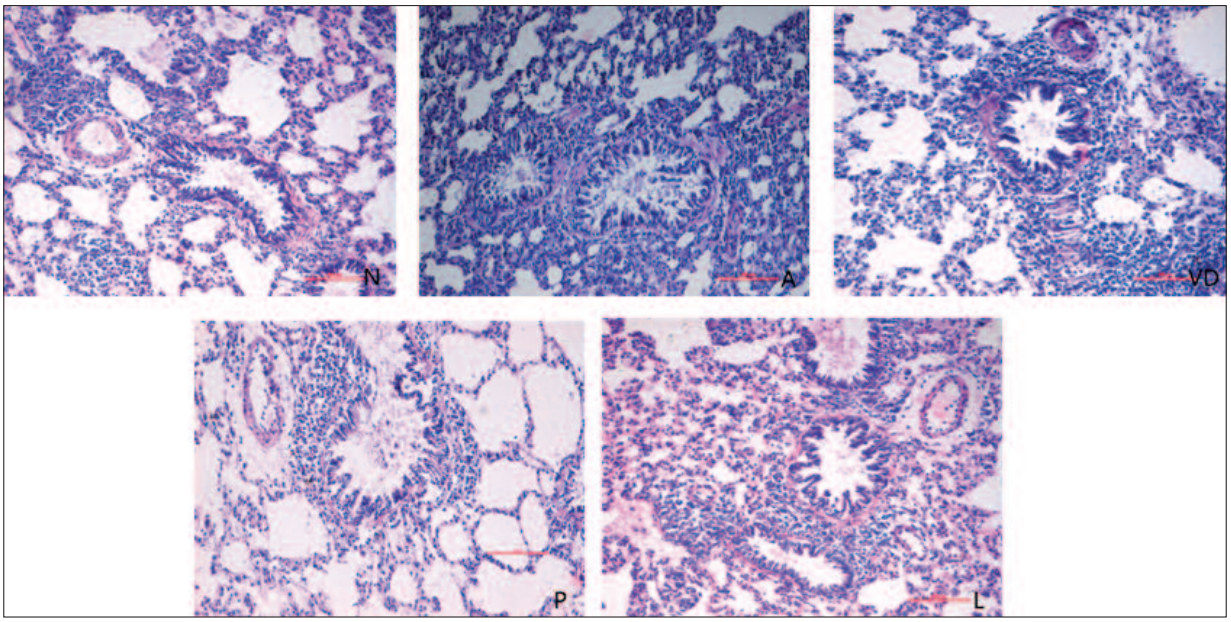

Figure 1. Pathological changes of lung tissue rats in each group (HE staining × 200). N: normal control group; A: asthma group; VD: 1,25-$(OH)_2D_3$ group; P: budesonide (Pulmicort group); L: combination group.

inal flaring, irritability, and lied motionless. The rats in the VD group, the budesonide group and the combination group exhibited slightly less intense exacerbation, with no abnormal symptoms. The rats in the control group were agile and showed no apparent abnormalities.

Histopathologic Changes in the Lung

Under light microscopy, the tracheal epithelial cells in the control group were intact, with scattered goblet cells in the trachea and all levels of the bronchi exhibited no gland hyperplasia and significant inflammatory cell infiltration (Figure 1N). A large number of inflammatory cells and increased bronchial goblet cells were observed in the asthma group under the submucosa and tubes (Figure 1A). The VD group, budesonide group and combined group exhibited partial reversal these lesions and the combination group was more effective than the single treatments (Figure 1 VD, P and L).

Comparison of OVA-Specific Serum IgE Levels of Rats in Different Groups

OVA-sIgE was not detected in the serum of rats in group N. The OVA-sIgE content in group A was significantly higher than group N ($p < 0.01$). The VD group was significantly lower than in group A, but was still significantly higher than those in groups N, P and L ($p < 0.01$). Group L was significantly lower than groups A and VD ($p < 0.01$), but still higher than group N. Moreover, the difference between groups L and P was not statistically significant ($p > 0.05$; Table I).

Inflammatory Cell Counts in BALF

The total number of cells in the BALF and the EOS counts in group A were significantly higher than group N and treatment group ($p < 0.01$). The number of cells in group VD was significantly lower than in group A, but significantly higher than in groups N, P, and L ($p < 0.01$). The total

Table I. Serum levels of OVA-specific IgE in different group rats (n = 8, $\bar{x} \pm s$).

Group	N	A	VD	P	L
OVA-sIgE	0	333.22 ± 10.91^{a}	$270.45 \pm 10.94^{a,b}$	$200.38 \pm 36.46^{a,b,c}$	$169. \pm 19.47^{a,b,c,d}$

Note: a: each group compared with group N, $p < 0.01$; b: each group compared with group A, $p < 0.01$; c: each group compared with group VD, $p < 0.05$; d: each group compared with group P, $p > 0.05$.

number of cells in group L was still significantly higher than group N ($p < 0.01$). The difference in EOS between group L and group N was not statistically significant ($p > 0.05$; Table II).

Eotaxin and IL-8 Levels in the Serum and BALF of Different Groups

The serum eotaxin expression in group N was significantly different compared with other groups ($p < 0.01$). The eotaxin expression in group A was significantly higher than those in group N and the treatment group ($p < 0.01$). The eotaxin expression in group VD was significantly lower than in group A, but significantly higher than groups N, P, and L ($p < 0.01$). The eotaxin expression in group L was significantly lower than those in groups A, P and VD ($p < 0.01$), but not significantly different from that in group N ($p > 0.05$; Table III, Figure 2). The eotaxin expression in the BALF in group N was statistically significant compared with the other groups ($p < 0.05$). The eotaxin expression in group A was significantly higher than that in group N and the treatment group ($p < 0.01$). The eotaxin expression in group VD was significantly lower than that in group A ($p < 0.05$) and significantly higher than those groups N, P and L ($p < 0.01$). The eotaxin expression in group L was significantly lower than those in groups A, VD and P ($p < 0.05$) but not significantly different from that in group N ($p > 0.05$; Table III, Figure 3).

Serum IL-8 expression was not detected in group N and significantly different from the other group ($p <0.01$). The serum IL-8 expression in group A was significantly higher than those in group N and the treatment group ($p < 0.01$). The serum IL-8 expression in group VD was significantly lower than in group A and significantly higher than those in groups N and L treatment group ($p < 0.01$) and significantly higher than group P ($p < 0.05$). The serum IL-8 expression in group L was significantly lower than those in groups A, VD and P ($p < 0.01$; Table III, Figure 4). BALF IL-8 expression was not detected in group N and significantly different compared with the other group ($p < 0.01$). The BALF IL-8 expression in group A was significantly higher than those in group N and the treatment group ($p < 0.05$). The VD group was significantly lower than group A ($p < 0.05$), but significantly higher than those in groups N, P, and L treatment group ($p < 0.01$). The BALF IL-8 expression in the L treatment group was significantly lower than those in groups A, VD, and P ($p < 0.01$; Table III, Figure 5).

Table II. Inflammation cells in different group rat's BALF (n = 8, $\bar{x} \pm s$) $\times 10^3$/ml.

Group	Total cells	Eosinophils
N	169.1 ± 11.9	31.1 ± 5.1
A	471.0 ± 26.2[a]	136.5 ± 11.5[a]
VD	367.4 ± 23.1[a,b]	95.3 ± 11.8[a,b]
P	278.1 ± 22.3[a,b,c]	56.1 ± 10.6[a,b,c]
L	199.8 ± 15.9[a,b,c,d]	39.3 ± 9.9[b,c,d,e]

Note: a: each group compared with group N, $p < 0.01$; b: each group compared with group A, $p < 0.01$; c: each group compared with group VD, $p < 0.01$; d: each group compared with group P, $p < 0.01$; e: each group compared with group N, $p > 0.05$.

Discussion

Asthma is a major allergic disease during childhood and its incidence is increasing rapidly. However, the mechanism of asthma inflammation is still unclear. In asthma patients, stimulation with endogenous or exogenous factors, aller-

Table III. Levels of Eotaxin (Eu/l) and IL-8 (ng/l) in different group rats (n = 8, $\bar{x} \pm s$).

Group (n = 8)	Eotaxin (Eu/l)		IL-8 (ng/l)	
	Serum	BALF	Seru	BALF
N	26.5 ± 2.1	14.15 ± 1.2	0	0
A	82.9 ± 5.1[a]	117.0 ± 19.5[a]	330.9 ± 21.3[a]	307 ± 36.3[a]
VD	69.9 ± 6.4[a,b]	66.1 ± 12.4[a,b]	243.9 ± 15.8[a,b]	250.8 ± 15.3[a,b]
P	57.9 ± 13.0[a,b,c]	33.8 ± 11.4[a,b,c]	184.7 ± 39.8[a,b,c]	182.0 ± 27.8[a,b,c]
L	33.36 ± 4.53[b,c,d,e]	17.3 ± 1.4[a,b,c,d]	127.4 ± 26.9[a,b,c,d]	128.5 ± 31.4[a,b,c,d]

Note: a: each group compared with group N, $p < 0.01$; b: each group compared with group A, $p < 0.01$; c: each group compared with group VD, $p < 0.01$; d: each group compared with group P, $p < 0.01$; e: each group compared with group N, $p > 0.05$.

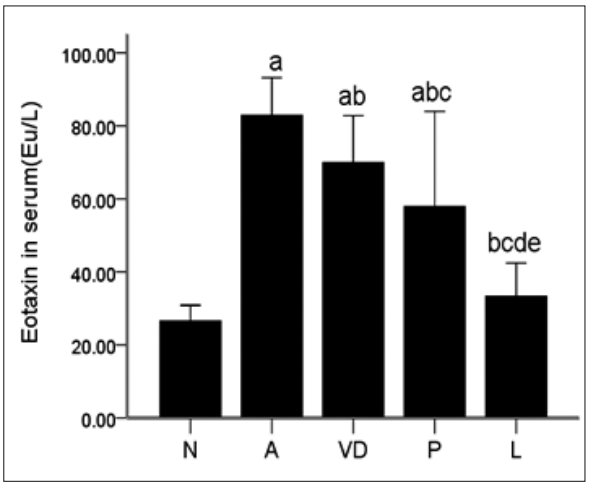

Figure 2. Serum eotaxin content value.

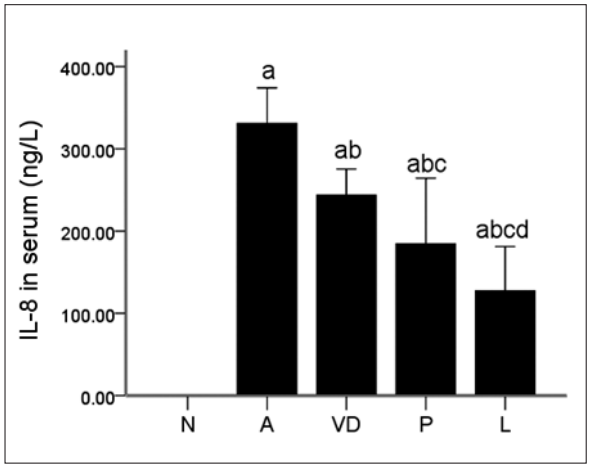

Figure 4. Serum level of IL-8.

gens or mental factors, bronchial smooth muscle spasm, inflammatory cell infiltration, airway mucosal oedema and increased secretions increases airway resistance and the occurrence of asthma[7]. Airway inflammation is the common pathologic feature for asthma. IgE is an immunoglobulin synthesised by B cells that mediates type I hypersensitivity. Synthesised IgE activates mast cells and basophils to degranulate and release inflammatory mediators, thereby, causing airway inflammation and resulting in speed hairstyle asthmatic reaction. Increased total serum IgE levels and specific IgE levels are the specific and primary identifying features of bronchial asthma[8]. Our study found that normal rats have no OVA-specific IgE in the serum. The asthma group had significantly higher serum OVA-specific IgE than the normal group and the treatment group, and significantly decreased the levels in the three groups after treatment. Thus, IgE is involved in the pathogenesis of asthma. The AHR and airway inflammation of asthma patients are closely related percentage of specific IgE-positive cells; thus, the IgE levels in the peripheral blood is an important indicator for diagnosing asthma, similar to EOS.

The recruitment and activation of inflammatory cells in the airway among asthma patients is a key step in the development of disease. The airways of asthmatics are usually exhibit CD4 + Th2 lymphocytes and EOS aggregation[9]. EOS are recruited into the lungs and released by the damaged tissue, which is the central link in airway inflammation, airway remodelling and AHR[10]. Convention-

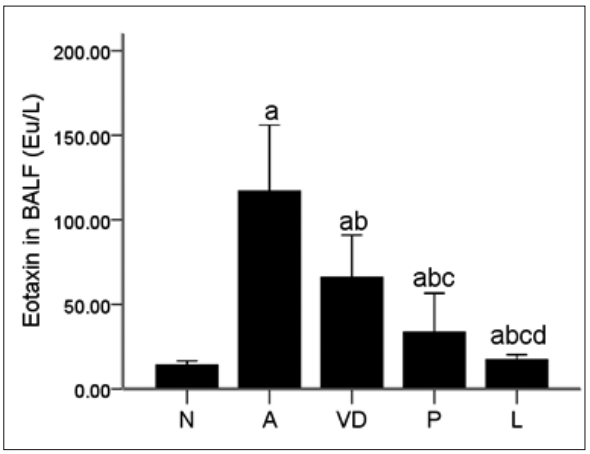

Figure 3. BALF content value of eotaxin.

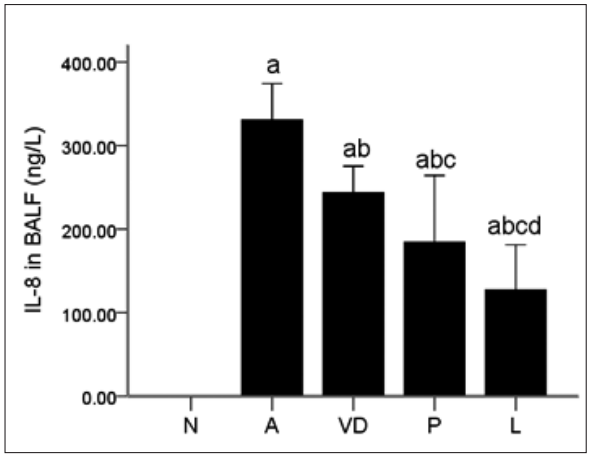

Figure 5. BALF level of IL-8.

ally, EOS inflammation is considered as an important feature of asthma. However, the development of the application of induced sputum and other non-invasive techniques for airway inflammation research show that many other cells are involved in airway inflammation, airway inflammation heterogeneity and asthma disease aside from EOS. Airway inflammation can be classified into eosinophilic asthma and non-eosinophilic asthma. Studies have shown that mild and moderate asthma mostly show EOS infiltration, whereas heavy airway inflammation exhibit significantly increased neutrophils. Therefore, neutrophils participate in acute asthma onset and exacerbation process[11] and are related to insensitivity to hormone therapy.

Chemokines are chemotactic cytokines produced by neutrophils, monocytes and other cells. Chemokines are classified according to their molecular structure, and different types of chemokines direct chemotaxis for certain inflammatory cells. Shannon et al[12] have shown that inflammatory cell infiltration of the airway in asthma is closely related to the functions of eotaxin and IL-8. A large number of chemokines promote aggregation and maintain the local concentration of inflammatory cells, which aggravate inflammation and asthma. Halwani et al[13] showed that eotaxin, RANTES, and IL-8 increase the rate of ASMC proliferation, and inhibit their apoptosis, and they contribute to epithelial cell migration towards ASMC. These effects promote airway remodelling. Eotaxin is a chemokine and its receptor CCR3 is mainly involved with EOS. A variety of inflammatory stimuli significantly increase eotaxin expression. Eotaxin involves increasing the EOS infiltration and damage to the tissue organisation, inducing AHR and airway remodelling[10,14].

IL-8 is a multi-derived cytokine, belonging to CXC chemokine subfamily that is mainly produced by monocytes and macrophages. IL-8 is the major chemoattractant and neutrophil activator, which are considered to exhibit the most potent chemotactic activity for neutrophils in CXC subfamily. IL-8 activates neutrophils and triggers degranulation. The medium contained dissolved oxygen radicals, arachidonic acid and other inflammatory mediators are released to induce airway secretory cells to secrete mucus and to promote the inflammatory process. Oxygen radicals can inactivate α1- antitrypsin and promote lung matrix degradation mediated by protease, which causes the development of lung injury and emphysema and decreases lung function. IL-8 also has weak chemotactic activity for basophils and EOS has weak chemotactic activity. In the presence of IL-3, IL-8 causes basophil degranulation, thereby releasing histamine and leukotrienes, and mediates IgE-dependent allergic reactions, thereby causing delayed asthma. IL-8 also exerts chemotaxis and activates EOS for cationic protein synthesis and release, which increases the microvascular permeability of the airway submucosa, as well as causes glandular hypersecretion, smooth muscle contraction and detachment of airway epithelial cells, thereby causing airway damage and injury, and inflammation. IL-8 positively regulates the airway inflammation of asthma. Moreover, neutrophils and EOS also secrete IL-8 after aggregation in the airway to form a positive feedback network, which amplifies the effect of inflammation and aggravates the asthma[15]. The analysis of different disease markers of airway inflammation by Shannon et al[12] showed that airway inflammation exhibits individual heterogeneity. Free IL-8 has been found in the serum of severe allergic asthma patient, whereas free IL-8 has not been found in normal subjects and patients with mild asthma, which suggest that IL-8 may indicate severe asthma. Another study found that FEV_1 reduction and increased IL-8 secretion by cultured monocytes are negatively correlated with moderate to severe asthma. Our results show that IL-8 was not detected in the serum and BALF in group N, but were significantly higher in group A than in other groups. These results are consistent with those of Shannon et al[12]. After treatment, the IL-8 level in group VD was significantly lower than that group A, whereas that in group L was significantly lower than those in groups A, VD and P have a synergistic effect. Eotaxin and IL-8 were confirmed to be involved in asthma. Thus, eotaxin and IL-8 levels of patients with asthma can be used as key indicators for airway inflammation and for monitoring disease changes and treatment effects.

Asthma is a complex polygenic disease caused by genetic and environmental factors. Inhaled corticosteroids may exert anti-inflammatory effects through a variety of ways and affect multiple aspects of asthma, including inhibition of synthesis and release of inflammatory mediators, inhibition of inflammatory cell chemotaxis effect, reduction of vascular permeability, reducing tissue injury and blocking cytokine and chemokine synthesis, thereby inhibiting chemokine-induced recruitment and migra-

tion of inflammatory cells to the airway. Although the effect of glucocorticoid treatment on asthma is evident, the long-term use of hormones causes endocrine and metabolic disorders and many other side effects, and even some patients with asthma are insensitive to hormone therapy or are hormone-resistant[16]. Therefore, even though hormone therapy is currently the most effective treatment for asthma, it is still unsatisfactory. Thus, some drugs alternatives, or drugs that are synergistic to steroid hormones should be developed to reduce the amount of hormones.

1,25-$(OH)_2D_3$ is the most important and active vitamin D metabolite. Aside from its physiologic function in regulating bone homeostasis and calcium and phosphorus metabolism *in vivo*, studies have mainly focused on the 1,25-$(OH)_2D_3$ regulation of the immune response of asthma and chronic airway inflammation[17,18]. Serum 25-(OH) VD is positively correlated with lung function in children with asthma[19]. Zhou et al[20] found that 1,25-$(OH)_2D_3$ inhibits inducible nitric oxide synthase and relieves asthma symptoms, as well as prevent and treats asthma. VD deficiency causes weakness of cellular immunity and humoural immune function. VD deficiency sufficient to cause rickets increases the risk of asthma in young children[21]. Thus, VD can be used as a novel immunomodulatory agent and used to prevent and treatment of a variety of immune-related diseases. Many of the biologic functions of VD were determined by regulating target gene transcription through VD receptors (VDRs); each cells and tissues contain VDRs. Therefore, VD and the occurrence of a variety of diseases are closely related, including regulating the differentiation of antigen-presenting cells, lymphocyte proliferation and cytokine secretion. VD also affects helper T cells, thereby indirectly inhibiting immunoglobulin synthesis and secretion from B cells and increasing the activity of inhibitory cells. Moreover, some studies confirmed that 1,25-$(OH)_2D_3$ inhibits the Thl immune response, but its function in Th2 response is more complex, and is not yet fully understood. Lai et al[22] confirmed that 1,25-$(OH)_2D_2$ effectively reduces airway remodelling, which provides a new perspective regarding the function of 1,25-$(OH)_2D_2$ in asthma and the correlation between VD and lung function[23,24]. Epidemiological studies[25] have found that VD supplementation significantly reduces the severity of asthma and improves the response to steroid therapy. The present study found that asthmatic rats exhibit typical allergic inflammation, increasing the total number of leukocytes and EOS in BALF. HE staining of lung tissues showed airway inflammation characterised by inflammatory cell infiltration and increased serum IgE and serum BALF eotaxin together with IL-8, which significantly decreased after 1,25-$(OH)_2D_3$ treatment. We also found that budesonide has greater effect on these factors than 1,25-$(OH)_2D_3$, but their combination has a more pronounced inhibitory effect than individual treatments. Moreover, combining 1,25-$(OH)_2D_3$ with glucocorticoids in the treatment of asthma controls inflammation by antagonising chemokines. Glucocorticoids at conventional therapeutic doses combined with 1,25-$(OH)_2D_3$ exhibited a synergistic effect. However, further studies are needed to determine whether the dose of corticosteroids can be reduced, thereby decreasing side effects of hormones. Previous studies have shown that VD increases the incidence of allergic diseases, similar to environmental factors. Bäck et al[26] showed that ingestion of higher doses of VD during infancy increases allergic manifestations. VD3 increases the expression of macrophage-derived chemoattractant protein (MDC), which is associated with Th2 responses. MDC causes Th2 inflammation cell recruitment, thereby aggravating asthma[27].

Conclusions

Although 1,25-$(OH)_2D_3$ has potential therapeutic effects on asthma and autoimmune diseases, its overall effect and dose should be studied further.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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