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Treatment of obese asthma in a mouse model by simvastatin is associated with improving dyslipidemia and decreasing leptin level

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ABSTRACT

Obesity can cause or worsen asthma. Compared with common asthma, obese asthma is difficult to control. Statins are effective serum cholesterol-lowering agents in clinical practice, and they also have anti-inflammatory properties, which in theory are potentially beneficial in asthma. Many studies have shown that simvastatin has good therapeutic effect in animal models of asthma. However, the therapeutic effect and action mechanism of simvastatin for obese asthma remain unclear. Leptin, a satiety hormone, is in positive correlation with total body fat mass and may also play a significant role in the pathogenesis of asthma. In this study, we use the method of high-fat diet and ovalbumin (OVA) sensitization and challenge to establish the mouse model of obesity and asthma, and find that obese asthmatic mice has higher levels of glucose, lipid and leptin in serum, and neutrophil percentage in bronchoalveolar lavage fluid (BALF), and more severe airway inflammation and structural changes in lung tissues than non-obese asthmatic mice, and respond poorly to dexamethasone treatment, which indicates that obese asthma might belong to steroid-resistant (SR) asthma. Simvastatin treatment reduces the levels of glucose, lipid, leptin and neutrophil percentage, and improves airway inflammation and remodeling, which can be as a potential therapeutic target used in the treatment of obese asthma in humans. Correlation analysis shows that there is positive correlation between neutrophil percentage and serum leptin/cholesterol level, which indicates that the therapeutic efficacy of simvastatin on obese asthma might be associated with improving dyslipidemia and decreasing leptin level.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways characterized by reversible obstruction of airway hyper-responsiveness, inflammatory infiltrates in the bronchial walls, and mucus hypersecretion. Glucocorticoids as potent anti-inflammatory therapy drugs are commonly used in the treatment of acute exacerbations of asthma. However, certain asthma patients usually with severe asthma fail to respond to glucocorticoid treatment despite high dose or/and extended the duration of therapy, and this condition is termed “steroid-resistant (SR) asthma” [1]. These SR asthma patients continue their therapy with glucocorticoids despite the onset of severe side reaction and bad clinical outcome. Recent studies

reported that the SR asthma may result from impaired histone deacetylase, cytokine hypersecretion and neutrophilic granulocytosis [2,3].

Obesity is a known risk factor for many human diseases, including asthma, cardiovascular disease, diabetes, and several types of cancer [4,5]. In addition, obesity can worsen the condition of asthma, and alter the efficacy of standard asthma medications, which makes asthma patients respond poorly to the treatment of glucocorticoids [6]. In recent years, mechanisms underlying the relationship between obesity and asthma are widely investigated. Surely obese breathing with a high respiratory rate and a low tidal volume increases the risk of asthma and asthma severity. Furthermore, there is a growing body of evidence that obesity-induced disorders of lipid metabolism and the endocrine function alterations of adipose tissue worsen the condition of asthma [5,7]. For example, we once investigated whether obesity directly led to airway inflammation associated with asthma, and found obesity induced the changes of adipokine secretion, such as leptin, leptin receptor, adiponectin, and vascular endothelial growth factor

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(VEGF), and obesity had no significant direct influence on the development of airway hyperreactivity and lung inflammation in the diet-induced mild obese mice without ovalbumin (OVA) challenge. However, compared with non-obese asthmatic mice, obese asthmatic mice had severe condition of asthma, which suggested that obesity worsening the condition of asthma might be associated with the changes of adipokine secretion. These results were consistent with the studies reported by Saraiva et al. [8] and Jung et al. [9].

Leptin, the product of the *ob* gene, is originally identified as a satiety hormone secreted by adipocytes and is in positive correlation with total body fat mass [10]. As an adipose signal, leptin level increase means the excess of lipid storage, which is implicated in the modulation of the energy balance and restriction of excess accumulation of fat [11]. Leptin is a pleiotropic hormone and also has proinflammatory effect involved in the regulation of immune response. For example, Shirshv et al. [12] demonstrated that the modulation of mononuclear phagocytes by leptin is related to activation of the JAK-STAT pathway, which results in stimulation of phagocytosis, production of reactive species of oxygen and nitrogen, and increased secretion of pro-inflammatory cytokines. Raso et al. [13] also confirmed that leptin can target macrophages and is related to the pathophysiology of inflammation. In addition, Mancuso et al. [14] reported that leptin can enhance leukotriene synthesis in alveolar macrophages, and leukotriene plays the central role in asthma pathophysiology, which implies that leptin might be involved in the occurrence and development of asthma. Further, it has been reported that leptin promotes the release of VEGF by human airway smooth muscle cells, and VEGF is implicated in the severity of asthma [15,16]. Cross-sectional studies in children and adults have shown that plasma leptin level is higher in asthma patients [17,18]. These findings indicate that leptin is associated with asthma, and might serve as a link between obesity and asthma.

Statins, inhibitors of 3-hydroxy-3-methyl-3-glutaryl coenzyme A (HMG-CoA) reductase, are widely used in the treatment of coronary artery disease by decreasing cholesterol [19]. Statins also have several other pharmacological actions, such as anti-inflammatory, anti-oxidant, and immunomodulatory effects, which might be beneficial in airway inflammatory diseases. In fact, simvastatin, one kind of statins, has been widely reported to be effective in the treatment of asthma [20,21]. But for the treatment of obese asthma, its role is still unclear.

Thus in this study, we first established the mouse model of obesity and asthma, and investigated the asthma severity of the obese mice. Furthermore, we investigated the curative effect and action mechanism of simvastatin for obese asthma.

2. Materials and methods

2.1. Experimental animals

Specific pathogen-free (SPF) female C57BL/6J mice (body weight 10.91 ± 0.25 g, 3 weeks old) were purchased from the Experimental Animal Center, Anhui Medical University (Hefei, China). These mice were randomly divided into six groups (15 mice every group): control group, asthma group, obese asthma group, dexamethasone group (treated obese asthmatic mice with dexamethasone), simvastatin group (treated obese asthmatic mice with simvastatin), and combination group (treated obese asthmatic mice with dexamethasone and simvastatin). These mice were raised in the controlled environmental conditions (23 ± 2 °C, $50 \pm 10\%$ humidity, and 12 h light-dark cycle) and allowed free access to water and regular rodent chow in the animal laboratory of Qingdao University of Medical Science for 1 week before initiating the experiments. All

mice used in this study were kept under a protocol approved by the Animal Ethics Community of Qingdao Municipal Hospital.

2.2. Establishment of a mouse model of obesity and asthma, and treatment

To establish a mouse model of obesity and asthma, obese asthma group, dexamethasone group, simvastatin group, and combination group were fed with a high-fat diet (60% kcal from fat) for 15 weeks to induce obesity, while control group and asthma group were fed with standard diet. Obesity was defined as a weight more than 20% over the ideal [22]. Mice were weighted once a week. The start of week 9 was considered as day 1. On days 1, 7, and 14, the mice were sensitized by intraperitoneal injection of 50 µg OVA (Grade V; Sigma Chemical Co., St. Louis, MO, USA) adsorbed on a 1 mg aluminum hydroxide gel as an adjuvant. One week after the final injection, mice were exposed to OVA aerosols (1% in saline) for 30 min once daily for 7 consecutive days from day 21–27 using nebulizer (Pari GmbH, Germany), and then were exposed to OVA aerosols every other day for 3 weeks. The control group was intraperitoneally injected and nebulized with saline at the corresponding time points. The dexamethasone group, simvastatin group, and combination group were given 0.5 mg/kg dexamethasone (Chenxin Pharmaceutical Co., Ltd, Jinan, Shandong, China), 40 mg/kg simvastatin (Hangzhou MSD Pharmaceutical Co., Ltd, Hangzhou, Zhejiang, China), and 0.5 mg/kg dexamethasone combined with 40 mg/kg simvastatin via drinking water, respectively. These treatments were performed daily from day 21 for 4 weeks. The control group, asthma group and obese asthma group were given untreated water.

2.3. Serum collection

The mice were anesthetized with 0.4 ml of 5% chloral hydrate (V/V) 24 h after the final OVA challenge. Blood samples were collected by removing eyeball, kept at room temperature for 30 min, and then centrifuged at $2000 \times g$ for 10 min. The serum was collected and stored at -20 °C.

2.4. Measurement of serum leptin and biochemical indexes

The serum leptin level was determined by Mouse Leptin ELISA kit (R&D system, Minneapolis, MN, USA) according to the instructions of the manufacturer. The levels of glucose (Glu), triacylglycerol (TG), and total cholesterol (TC) in serum were determined by biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA).

2.5. The preparation of the lung tissue and bronchoalveolar lavage fluid (BALF)

Immediately after blood collection, the thoracic cavity was carefully opened. After tying off the right lung at the mainstem bronchus, right lung was removed by dissection, and fixed in 10% neutral-buffered formalin for 24 h. Then BALF was collected by cannulating the trachea and lavaging left lung thrice with 3×0.4 ml of ice-cold PBS (over 85% of the volume inputted was recovered).

2.6. Lung histology

After fixation, lung tissues were embedded in paraffin, cut into 5-µm sections, and stained with H&E, periodic acid-Schiff and Masson's trichrome. Slides were examined under a light microscope (Olympus, Japan), and the images were captured and

analyzed by Axiovision 3.1 software. The degree of inflammatory cell infiltration (0–4+) was determined blind using a 5-point grading system described by Myou et al. [23]. The total airway wall area (WAT), airway smooth muscle area (WAM), and the vascular basement membrane perimeter (PBM) were measured by Axiovision 3.1 software, and the values of WAT/PBM and WAM/PBM were calculated.

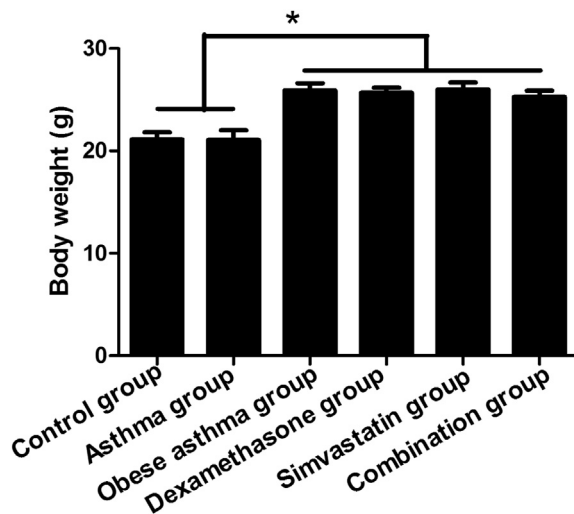


Fig. 1. The body weight of mice in each group. The data are expressed as the mean $\pm$ SEM, $n = 15$ for each group. * $P < 0.05$ versus normal diet-fed mice.

2.7. Inflammatory cell counts in BALF

BALF was centrifuged at 4 °C for 10 min at 300 $\times$ g. The cell pellet was collected and resuspended in normal saline containing 10% Bovine Serum Albumin (BSA, Sigma Chemical Co.). Total white blood cell (WBC) count was determined using a hemocytometer. For cytological examination, cell smears were made on glass slides, fixed, and stained with Wright (Sigma Chemical Co.). Differential cell counts were then performed on 200 WBCs in each cell smear.

2.8. Statistical analysis

The data are presented as the mean $\pm$ SEM. Student's two-tailed t -test or analysis of variance (ANOVA) was performed to analyze significance. P -values of <0.05 were regarded as significant.

3. Results

3.1. Body weight

The body weight of the high-fat diet-fed mice increased significantly more than that of normal diet-fed mice. After 8 weeks, the body weight of the high-fat diet-fed mice was over 20% larger than that of normal diet-fed mice, which indicated that mouse models of obesity are successfully established (Fig. 1). After OVA sensitization, body growth rate and food intake of normal diet-fed and obese mice decreased, and even some normal diet-fed mice had slight loss of body weight. At the end of the study, asthma group had the smallest body weight, followed by control group. The body weight of three treatment groups and obese asthma group are

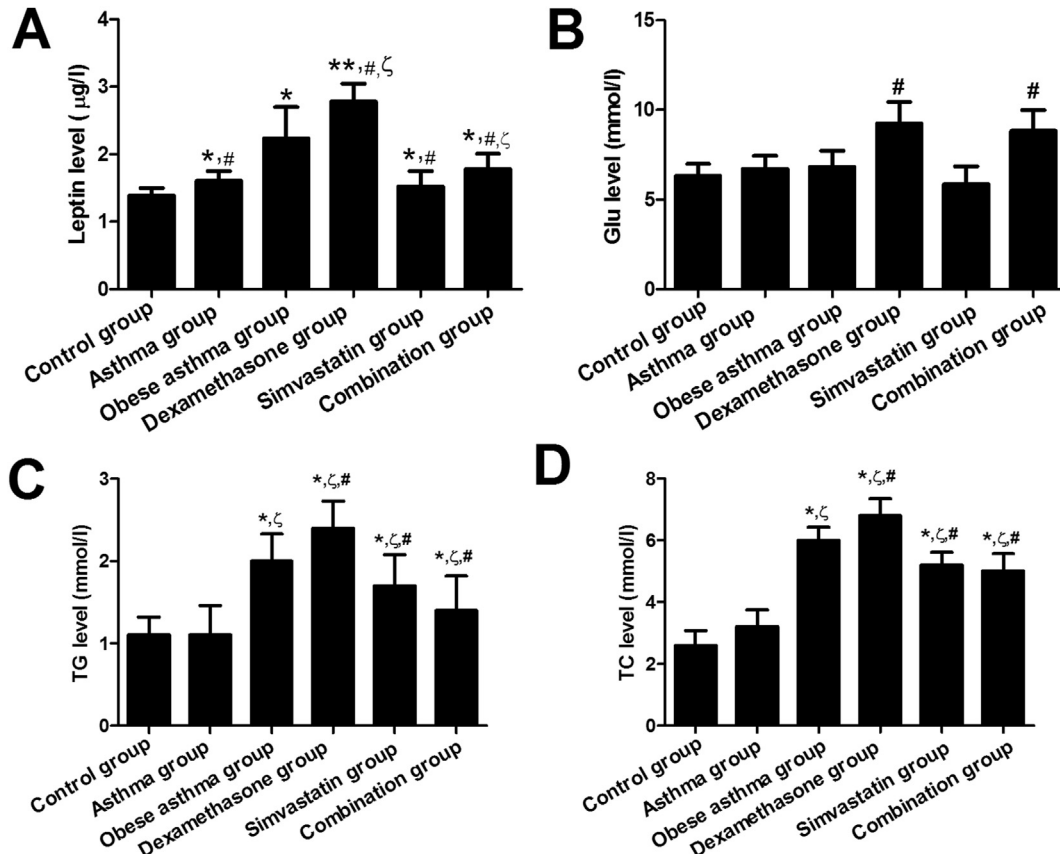


Fig. 2. The serum level of leptin (A), Glu (B), TG (C) and TC (D) in each group. The data are expressed as the mean $\pm$ SEM, $n = 12$ –15 for each group. * $P < 0.05$, ** $P < 0.01$ versus the control group; $^{\zeta}$ $P < 0.05$ versus asthma group; $^{\#}$ $P < 0.05$ versus the obese asthma group, $^{\xi}$ $P < 0.05$ versus simvastatin group. Glu, glucose; TG, triacylglycerol; TC, total cholesterol.

larger than that of control group, while there was nonsignificant difference among three treatment groups and obese asthma group (data not shown).

3.2. Asthmatic attack

During OVA challenge, except the control group, all other groups suffered acute attack of bronchial asthma, such as sneezing, the number of defecations or urinations increasing, and dysphoria. Among these groups, the symptoms of obese asthma group and asthma group were more severe than those of the three treatment groups.

3.3. The level of serum leptin

The results of leptin detection showed that the serum leptin level of all asthmatic groups was higher than that of control group in different degree, which indicated that inflammation and obesity both can cause the increase of leptin level (Fig. 2A). Among them, compared with the obese asthma group, dexamethasone treatment increased leptin level, and simvastatin and combination treatment decreased leptin level. There was nonsignificant difference between simvastatin group and asthma group in the leptin level.

3.4. The level of serum glucose and lipid

The results of biochemical detection showed that Glu level was higher in dexamethasone and combination treatment groups than that in the other groups, which conformed to the effects of glucocorticoid on the blood sugar metabolism (Fig. 2B). The obese asthma group increased the levels of TC and TG compared with the control group and asthma group, which indicated that high-fat diet causes abnormality of blood lipid metabolic (Fig. 2C and D). Simvastatin and combination treatment reduced the levels of TC and TG compared with obese asthma group indicating that simvastatin has anti-lipemic effect (Fig. 2C and D).

3.5. Total and differential WBC count in BALF

We collected the BALF of all groups, and examined the total WBC counts and the percentages of eosinophils, neutrophils, lymphocytes, and macrophages. As shown in Table 1, total WBC counts and the percentages of neutrophils, eosinophils and lymphocytes were significantly increased, and the percentage of macrophages was decreased in BALF of asthmatic animals compared with those of controls. The obese asthma group had higher neutrophil percentage and lower macrophage percentage than asthma group. The three treatment groups significantly reduced WBC count and eosinophil percentage compared to obese asthma group, which indicated that these treatments play an anti-inflammatory role. In

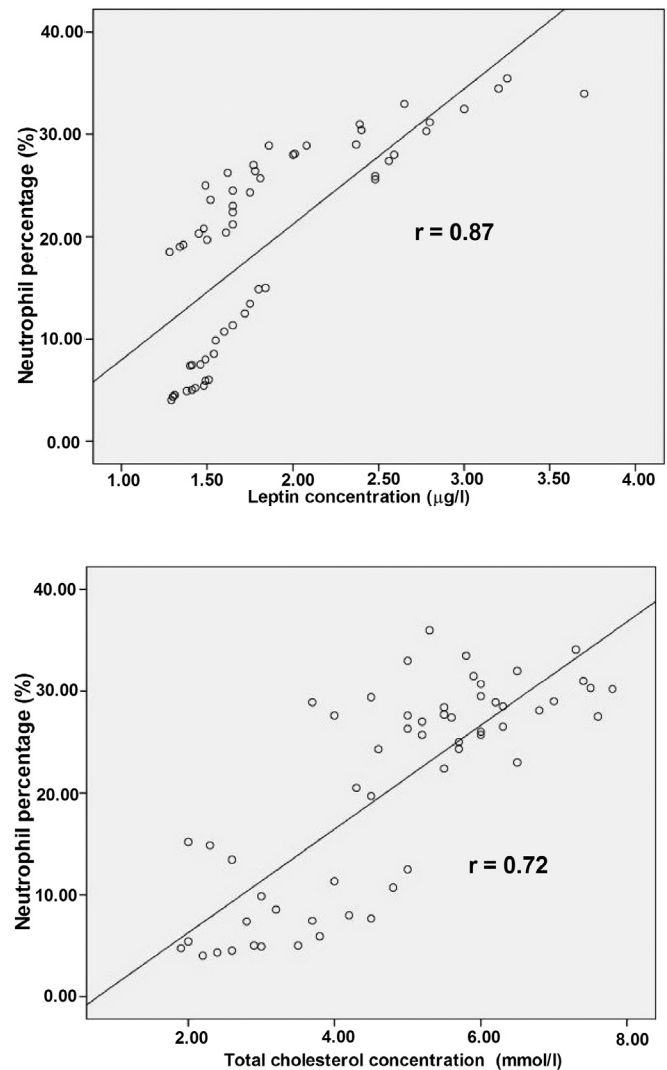


Fig. 3. Correlation analysis between neutrophil percentage and serum leptin (A)/TC (B) level. TC, total cholesterol.

addition, the simvastatin group and combination group also significantly reduced the neutrophil percentage compared with obese asthma group. But there was no significant difference in neutrophil percentage between obese asthma group and dexamethasone group. We further performed correlation analysis and found that there was positive correlation between neutrophil percentage and serum leptin/TC level (Fig. 3A and B).

Table 1
Total and differential white blood cell (WBC) count in bronchoalveolar lavage fluid (BALF).

	Total WBC $\times 10^4/\text{ml}$	Cell percentage (%)			
		Neutrophil	Eosinophil	Macrophage	Lymphocyte
Control group	5.33 $\pm$ 0.76	4.73 $\pm$ 0.40	2.93 $\pm$ 0.17	73.70 $\pm$ 0.80	19.03 $\pm$ 1.37
Asthma group	19.60 $\pm$ 1.35 ^{*#}	10.72 $\pm$ 1.60 ^{*#}	38.02 $\pm$ 2.16 ^{*#}	40.01 $\pm$ 2.52 ^{*#}	21.25 $\pm$ 1.06 [*]
Obese asthma group	23.45 $\pm$ 2.16 [*]	29.26 $\pm$ 2.50 [*]	32.74 $\pm$ 2.06 [*]	12.66 $\pm$ 4.10 ^{*#}	25.34 $\pm$ 2.37 [*]
Dexamethasone group	16.67 $\pm$ 2.43 ^{*#}	32.02 $\pm$ 2.0 ^{*#}	25.24 $\pm$ 2.45 ^{*#}	20.44 $\pm$ 2.57 ^{*#}	23.30 $\pm$ 2.42 [*]
Simvastatin group	18.23 $\pm$ 2.01 ^{*#}	25.02 $\pm$ 2.10 ^{*#}	29.63 $\pm$ 2.09 ^{*#}	21.82 $\pm$ 2.08 ^{*#}	23.53 $\pm$ 2.39 [*]
Combination group	15.11 $\pm$ 2.28 ^{*#}	24.32 $\pm$ 2.17 ^{*#}	22.43 $\pm$ 2.21 ^{*#}	29.28 $\pm$ 2.26 ^{*#}	23.98 $\pm$ 2.81 [*]

The data are expressed as the mean $\pm$ SEM, n = 12–15 for each group.

^{*}P < 0.05 versus the control group; [#]P < 0.05 versus the obese asthma group; ^ξP < 0.05 versus simvastatin group.

3.6. Histopathologic changes in lung tissues

Lung tissue was collected 24 h after the last OVA challenge. The asthma group showed obvious airway inflammation and structural changes, such as marked infiltration of the inflammatory cells into the peribronchiolar and perivascular connective tissues, goblet cell hyperplasia, airway thickening, excess mucus secretion, and apparent collagen deposition compared with the control group (Fig. 4A and B). The obese asthma group had more severe airway inflammation and structural changes than the asthma group, which indicated that obesity increases the severity of asthma (Fig. 4B and C). The three treatment groups attenuated the airway inflammation at different degrees compared with the obese asthma group indicating that dexamethasone and simvastatin both play the anti-inflammatory role (Fig. 4D–F). In the structural changes, simvastatin group and combination group had less collagen deposition than the obese asthma group, while there was no significant difference between dexamethasone group and the obese asthma group, which indicated that simvastatin can suppress the airway remodeling in obese asthmatic mice (Fig. 4D–F). In addition, quantification of airway inflammation and remodeling by inflammation score and the values of WAT/PBM and WAM/PBM confirmed that simvastatin not only has anti-inflammatory effect, but also prevents airway remodeling in obese asthmatic mice, while dexamethasone only suppresses the airway inflammation (Table 2).

3.7. Adverse events

By the end of the experiment, in obese asthma group,

simvastatin group, and combination group, one mouse died, while the control group and asthma group had no death, and dexamethasone group had 3 mice death. Relatively high incidence of mortality in dexamethasone group is probably because systemic treatment with glucocorticoid has strong side-effects on obese asthmatic mice. In fact, the mice had the symptoms of glucocorticoid-like side effects, such as skin damage and wheezing before death in dexamethasone group.

4. Discussion

Obesity is a worldwide epidemic, and its prevalence has increased dramatically in the last two decades, such that about 65% of the US population is classified as overweight or obese. Evidence from recent years has shown that obesity increases the risk of asthma and asthma severity, which make obese asthma difficult to be cured [6,24]. It is now increasingly appreciated that obesity worsens the condition of asthma via induction of lipid metabolism disorders and the endocrine function alterations of adipose tissue [5,7,25]. Thus, the regulation of adipose metabolism might be a potential novel therapeutic strategy for treatment of the obese asthmatic subjects. Simvastatin as one kind of statins has cholesterol-lowering, anti-inflammatory, anti-oxidant, and immunomodulatory effects. Many studies have reported that simvastatin relieves airway inflammation, remodeling and hyper-responsiveness via reduction of secretory cytokines, chemokines, and cell adhesion molecules, and improvement of epithelial cell malfunction in the animal models of asthma [20,26,27]. However, therapeutic effect of simvastatin on obese asthmatics remains unclear. In this study, we aimed to investigate the therapeutical

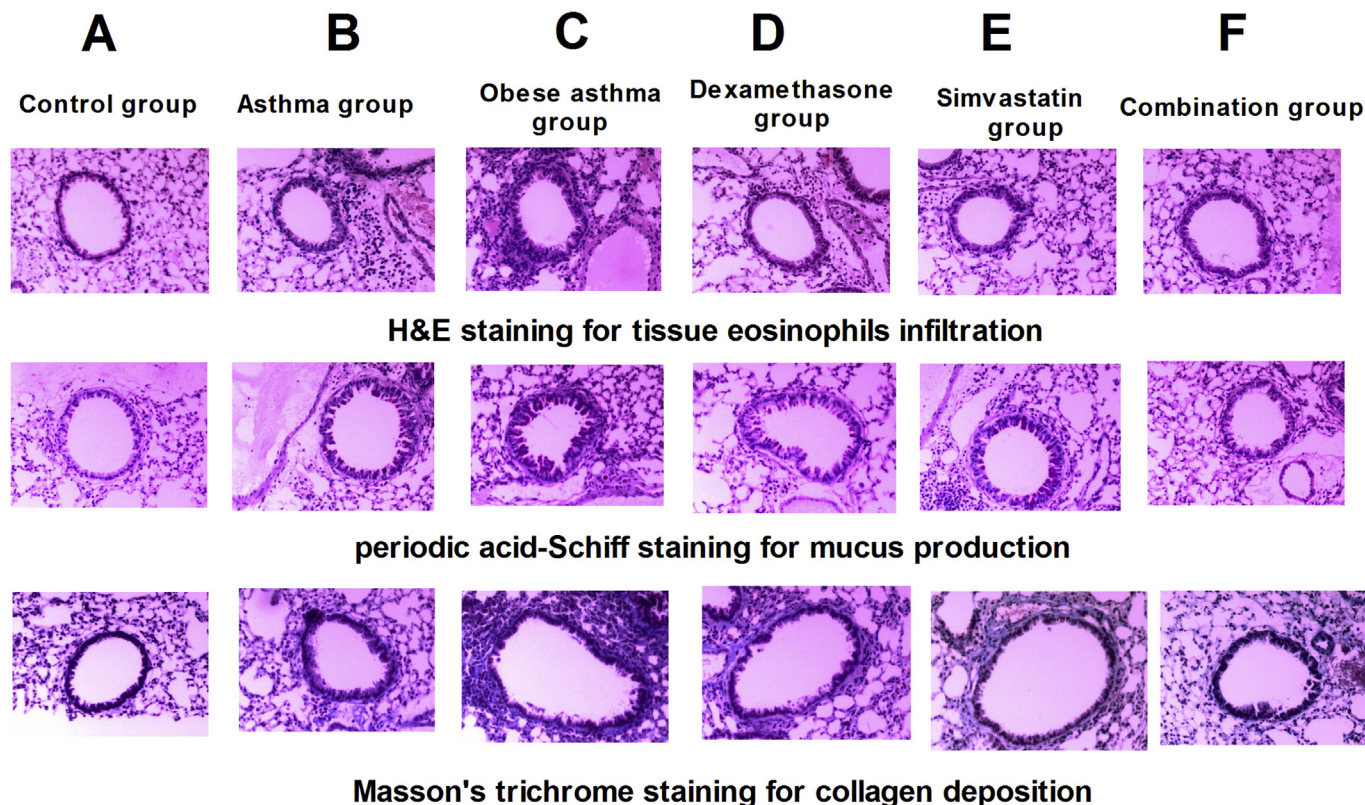


Fig. 4. The conditions of inflammatory infiltration, mucus production and collagen deposition in lungs in each group. The histological examinations of lung tissue are stained with H&E to measure tissue eosinophil infiltration, periodic acid-Schiff to measure mucus production (red are positive for mucus) or Masson's trichrome to measure collagen deposition (blue are positive for collagen deposition). $n = 12-15$ for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

The values of WAT/PBM and WAM/PBM and inflammation score.

	WAT/PBM ($\mu\text{m}^2/\mu\text{m}$)	WAM/PBM ($\mu\text{m}^2/\mu\text{m}$)	Inflammation score
Control group	4.97 ± 0.36	1.84 ± 0.11	0.13 ± 0.35
Asthma group	$12.19 \pm 0.56^{* \#}$	$5.23 \pm 0.67^{* \#}$	$2.8 \pm 0.63^{* \#}$
Obese asthma group	$13.78 \pm 0.45^*$	$6.07 \pm 0.22^*$	$3.40 \pm 0.51^*$
Dexamethasone group	$13.53 \pm 1.84^*$	$5.84 \pm 0.17^*$	$2.87 \pm 0.64^{* \#}$
Simvastatin group	$12.34 \pm 1.80^{* \#}$	$5.57 \pm 0.25^{* \#}$	$2.80 \pm 0.67^{* \#}$
Combination group	$12.26 \pm 0.98^{* \#}$	$5.49 \pm 0.29^{* \#}$	$2.20 \pm 0.42^{* \#}$

The data are expressed as the mean $\pm$ SEM, $n = 12-15$ for each group. $^*P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the obese asthma group. WAT, the total airway wall area; WAM, airway smooth muscle area; PBM, the vascular basement membrane perimeter.

effect and molecular mechanism of simvastatin on obese asthmatics.

We used the method of high-fat diet and OVA sensitization and challenge to establish the mouse model of obesity and asthma. Compared with non-obese asthmatic mice, obese asthmatics have high levels of serum glucose and lipid, which are in line with the features of metabolic syndrome. In addition, obese asthmatics have higher WBC count and neutrophil percentage in BALF and more severe airway inflammation and structural changes in lung tissues than the asthma group. These results indicate that obesity increases the severity of asthma, and obese asthma is a phenotype of refractory asthma, which are consistent with the study reported by Sutherland et al. [28] and Beuther et al. [29]. The patients of refractory asthma are usually treated with systemic glucocorticoid in clinical application. In this study, we also treated obese asthmatic mice with dexamethasone, and found dexamethasone only suppresses the airway inflammation, and has no effect on airway remodeling. In addition, obese asthmatic mice treated by dexamethasone have higher levels of glucose, lipid and neutrophil percentage, and higher mortality than obese asthma group, which indicates that dexamethasone further worsens the metabolic disorders and causes bad side effects, even death in obese asthmatic mice. These findings suggest that obese asthma might be a subtype of SR asthma and responds poorly to glucocorticoids. In contrast, simvastatin can improve the metabolic disorders, and represses airway inflammatory and remodeling. In addition, compared with dexamethasone group, simvastatin treatment reduces neutrophil percentage and mortality. These results indicate that simvastatin is effective in the treatment of obese asthma. Correlation analysis shows that there is positive correlation between neutrophil percentage and serum TC level, which indicates that therapeutic efficacy of simvastatin on obese asthma might be associated with improving dyslipidemia.

Leptin level increase is the physiopathologic basis of metabolic disorders and systemic inflammation reactions [30]. Recent studies have shown that leptin is implicated in the severity of asthma [31]. High concentrations of leptin are reported to worsen airway inflammatory and remodeling for following reasons: induction of eosinophil and neutrophil chemotaxis via activation of ERK1/2 and p38-MAPK signalling pathways; inhibition of neutrophil death via activation of MEK1/2 and NF- κ B signaling pathways; promotion of the inflammatory factor release via activation of natural killer cells and macrophages [32,33]. In this study, we also detected the leptin level, and found that obese asthma group has higher level than asthma group. Compared with the obese asthma group, dexamethasone treatment increased leptin level, and simvastatin decreased leptin level. These results indicated that obesity increases leptin level, while simvastatin can decrease leptin level. Zarkesh-Esfahani et al. reported that leptin can indirectly activate human neutrophils via induction of TNF- α [34]. We performed correlation analysis and found that there is indeed positive correlation between neutrophil percentage and serum leptin level, which indicates that the therapeutic efficacy of simvastatin on

obese asthma might be associated with decreasing leptin level. From therapeutic effect and side reaction showed during the test period, there is no significant difference between combination and simvastatin treatment. In view of potential adverse effects and clinical costs, simvastatin alone has more prospects than combination in the treatment of obese asthma.

In conclusion, our results show that obese asthma might belong to SR asthma and responds poorly to dexamethasone treatment. Simvastatin can effectively cure obese asthma in the mouse model, which might be associated with improving dyslipidemia and decreasing leptin level. In view of good therapeutic effect in the mouse model of obesity and asthma, simvastatin should be considered novel therapeutic targets in the further treatment of obese asthma in humans.

Conflict of interests

All authors declared no competing financial interests.

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Transparency document

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