

ORIGINAL ARTICLE

Effects of Diet-Induced Obesity on Tracheal Responsiveness to Methacholine, Tracheal Visfatin Level, and Lung Histological Changes in Ovalbumin-Sensitized Female Wistar Rats

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Abstract— Many studies have shown a close relationship between obesity and asthma severity. In the present study, the effects of diet-induced obesity were examined on airway responsiveness to methacholine in addition to visfatin level in female Wistar rats' tracheae after sensitization with ovalbumin. The rats were divided into four groups: control with normal diet (ND), ovalbumin (OVA)-sensitized with normal diet (S + ND), high-fat diet (HFD), and OVA-sensitized with a high-fat diet (S + HFD). The animals were fed for 8 weeks with standard pellets or high-fat diet and then sensitized and challenged with OVA or saline for another 4 weeks. At the end of the study, the tracheae were isolated and assessed for airway responsiveness and visfatin protein levels. Diet-induced obesity groups developed increased weight and obesity indices ($p < 0.001$). After sensitization with OVA and diet-induced obesity, there were marked leftward shifts in methacholine concentration-response curves in S + HFD group compared to other groups. Also, maximum response was the highest ($p < 0.05$ to $p < 0.001$), EC_{50} was the lowest ($p < 0.05$ to $p < 0.001$), and visfatin protein level was the highest ($p < 0.05$ to $p < 0.01$) in S + HFD. According to results, diet-induced obesity caused airway hyperresponsiveness to methacholine and enhanced visfatin protein levels in the tracheae of ovalbumin-sensitized female rats. Our results suggested that, in obese ovalbumin-sensitized conditions in female rats, the local production of adipocytokines, such as visfatin, may be increased, resulting in the deterioration of inflammation in lungs. This finding shows a possible mechanism for the altered phenotype in obesity-ovalbumin sensitization conditions in female rats.

KEY WORDS: airway hyperresponsiveness; asthma; obesity; visfatin; female Wistar rats.

INTRODUCTION

Numerous epidemiological studies have shown the role of obesity in the increased prevalence and incidence of asthma in adults and children [1] as well as the deterioration of asthma control [2]. Accordingly, data obtained from animal models support the association between obesity and asthma [3]. Airway inflammation along with airway hyperresponsiveness (AHR) is the main characteristics of bronchial asthma [4]. However, the effects of

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obesity on the airway responsiveness and other characteristics of asthma are not well demonstrated. Obesity has been shown to induce a chronic low-grade systemic inflammation, identified by increased circulating leukocytes and pro-inflammatory cytokines such as IL (interleukin)-1 β , IL-6, TNF (tumor necrosis factor)- α , chemokine, and acute-phase proteins [5]. Moreover, fat tissue is considered as a complex endocrine and immune organ that can modulate biological processes, *e.g.*, insulin sensitivity, appetite, endocrine function, inflammation, immune, and activation of inflammatory signaling pathways [6].

Many of the interactions between the immune system and metabolism appear to be mediated by a complex network of soluble mediators secreted from immune cells and adipocytes, known as adipokines or adipocytokine [7]. Adipokines reportedly enhance airway allergic inflammation and lead to AHR [8]. Several studies have shown that leptin and adiponectin and, most recently, visfatin play important roles in the inflammatory process in asthma [9, 10]. Visfatin, also known as PBEF (pre-B cell colony-enhancing factor) and Nampt (nicotinamide phosphoribosyltransferase), was first discovered in lymphocytes, bone marrow, liver, and muscle [11] and later, in lungs [12]. The main sources of visfatin are granulocytes, monocytes, macrophages, and adipocytes [13]. Human and animal studies have shown that visfatin is a pro-inflammatory cytokine, involved in regulating the inflammation and innate immunity [14]. It is revealed that, in ICU patients, the amount of visfatin significantly increases in acute lung injury conditions [15]. On the other hand, in obese ovalbumin-sensitized male Wistar rats, visfatin mRNA levels are elevated in the trachea [10]. Therefore, we investigated the role of visfatin on airway responsiveness in the obesity associated with asthma. For this purpose, the tracheal responsiveness to methacholine as well as visfatin protein levels in the trachea was evaluated in the diet-induced obesity model and ovalbumin (OVA)-sensitized female rats.

MATERIALS AND METHODS

Animals and Diets

Thirty-two female Wistar rats (8 weeks old, weighing approximately 160 g) were obtained from the animal house of Tabriz University of Medical Science, Iran. The animals were placed in cages under controlled conditions at 22 °C with a 12–12-h light-dark cycle. Food and water were

provided *ad libitum* throughout the accommodation and experimental periods.

After a week of accommodation, the rats were randomly divided into four groups ($n = 8$): control with normal diet (ND), OVA-sensitized with normal diet (S + ND), control with high-fat diet (HFD), and OVA-sensitized with a high-fat diet (S + HFD). To determine the regularity of the estrus cycle, vaginal smear cytology was used to determine the estrous cycle phases [16]. This method predicts the estrous cycle according to the proportion of three cell types observed in the vaginal smear: epithelial cells, cornified cells, and leukocytes. At first, 0.3 mL of normal saline was slowly injected into the animals' vaginas by a Transferpette sampler. Then, 1–2 drops of the injected fluid were taken to perform the smear. Samples were examined by light microscopy. The rats which were in the menstrual cycle during the estrous period were selected for the following steps. At this stage of the cycle, the vaginal smear has more cornified cells than epithelial cells and does not have any leukocytes.

The diet-induced obesity (DIO) model was prepared according to the method reported in previous studies [5, 17]. Briefly, the rats in DIO groups (HFD and S + HFD) were fed high-fat diets (42% energy from fat, 19% energy from protein, and 39% energy from carbohydrate), while other rats (ND and S + ND) were fed standard rat chow (11% energy from fat, 28% energy from protein, and 61% energy from carbohydrate).

After 8 weeks, the sensitization of animals to OVA (Sigma Aldrich Ltd., UK) in S + ND and S + HFD groups was performed with the following protocol and continued for 4 weeks.

Animal Sensitization

S + ND and S + HFD groups were challenged with OVA using the method described previously [18, 19]. Briefly, 1 mg of OVA and 200 mg of aluminum hydroxide (Sigma Aldrich Ltd., UK) were injected intraperitoneally (i.p.) on days 1 and 8. From day 14, the animals were placed in a closed chamber ($30 \times 20 \times 20$ cm³) for exposure to an aerosol of 4% OVA for 18 ± 1 consecutive days, 15 min daily, using a nebulizer (CX3; Omron Co., the Netherlands). In control groups (ND and HFD), saline was administered instead of OVA solution in the same manner. The study was approved by the Ethical Committee of Tabriz University of Medical Sciences, Iran.

Tissue Preparation

The rats were anesthetized by injecting a combination of ketamine (75 mg/kg) and xylazine (3 mg/kg) intraperitoneally. After opening the chests, approximately 2 cm from the tracheae was isolated and immediately put into a cool and oxygenated solution. The tracheae were divided into several 5-mm segments (five to six ring cartilages). Finally, each tracheal segment was immediately placed between two horizontal stainless steel hooks suspended in a 20-mL organ bath (Schuler organ bath type 809, March-Hugstetten, Germany) containing the Krebs-Henseleit solution of the following composition (mM): NaCl 120, NaHCO₃ 25, MgSO₄ 0.5, KH₂PO₄ 1.2, KCl 4.72, CaCl₂ 2.5, and dextrose 11. The Krebs solution was maintained at 37 ± 1 °C and gassed with 95% O₂ and 5% CO₂. The initial tension was 1 g throughout the experiment, and the equilibrium period was 60 min during which the bath solution was replaced with the fresh Krebs solution every 15 min. Responses were measured using an isometric transducer (ADInstruments, Spain) with the sensitivity range of 0–25 g, amplified with an amplifier (ML/118 Quadri Bridge Amp, March-Hugstetten, Germany), and recorded on a power lab (ML-750, four-channel recorder, March-Hugstetten, Germany).

Assessment of Tracheal Responsiveness to Methacholine

The cumulative log concentration-response curve of methacholine hydrochloride (Sigma Chemical Ltd., UK) was obtained in each tracheal segment by adding consecutive concentrations (10^{-8} to 10^{-3} mM) to the organ bath every 2 min. The contraction due to each concentration was recorded after 2 min. The percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine in proportion to the maximum contraction obtained by its final concentration was plotted against the log concentration of methacholine to obtain the curve. The effective concentration of methacholine causing 50% of maximum response (EC₅₀) was measured from the methacholine response curve in each experiment using 50% of maximum response in the *y*-axis and measuring the dose of methacholine causing this response in the *x*-axis. The contractility response to 1 mM methacholine as the magnitude of contraction was also measured. At the end of the experiments, the tissues were weighed. Afterwards, the contraction force induced by M (at the plateau level) was calculated to express as the g force/mg tissue weight (gF/TW) to compared the contraction activity of this spasmogen between groups.

Lung Lavage Fluid and Its White Blood Cell Count

A cannula was located into each remaining trachea coincident with preparing the tracheal segment, and the lungs were lavaged five times with 2 mL of saline (total 10 mL). One milliliters of lung lavage fluid (LLF) was stained with Turk's solution, and the total white blood cell (WBC) number was counted in duplicate in a hemocytometer (in a Burkner chamber). Turk's solution consisted of 1 mL of glacial acetic acid and 1 mL of 1% gentian violet solution in 100 mL of distilled water. The remaining LLF was centrifuged at 2500 g at 4 °C for 10 min, and the supernatant was removed. The smear was prepared from the cells and stained with the Wright-Giemsa. According to staining and morphological criteria, differential cell analysis was carried out under a light microscope by counting 400 cells in each sample, and the percentage of each cell type was calculated.

Body Weight

The animals were weekly weighed on a certain day (at 16:00) during the experimental period. At the end of the study, the rats were anesthetized by the i.p. injection of 75 mg/kg of ketamine and xylazine. Then, they were weighed and the naso-anal length, *i.e.*, the distance between nose and anus, was measured. The final body weight, Lee index, and percentage of body fat (BF%) were also determined to calculate the obesity indices used in rodents. All obesity indices were previously described. Briefly, the following formula shows the calculation of all obesity indices [19]:

Percentage of body weight changes (%)

$$= [(final\ weight - initial\ weight) / initial\ weight] \times 100$$

The Lee index (mg/mm)

$$= Final\ weight^{0.33} / \text{naso-anal length}$$

Percentage of body fat (%)

$$= 0.73 (\text{the Lee index} - 280.8)$$

Lung Histopathological Evaluation

After the rats were anesthetized and sacrificed, their lungs and tracheae were isolated and placed in 10% buffered formalin (37%, Merck, Germany). After 7 days, the tissues were dried by passing through 70–100% ethanol. Then, the tissues were cleared by xylene and embedded in paraffin. Finally, the specimens were cut into 4- μ m sections using an Autotechnicon apparatus and stained with hematoxylin and eosin (H&E stain). The tissues were then evaluated under a light microscope. The pathologic changes in the lung of all groups, including lipid aggregation, emphysema, lymphoid hypertrophy, airway smooth muscle hyperplasia, and edematous changes were recorded and scored according to the following grades: no pathologic changes = 0; patchy changes = 1; local changes = 2; scattered changes = 3; and severe changes = 4 (in the most parts of the lungs).

Tissue Sampling and Protein Measurement

After the rats were anesthetized and sacrificed, tracheal tissues were removed. Following quick freezing with nitrogen, all tissues transferred to -70°C until visfatin measurement. Tissue samples were weighed and the cellular contents were extracted with a Homogenitor (Potter S, Germany) in PBS (pH 7.2–7.4) and centrifuged for 20 min at the speed of 3000 rpm at 4°C . Afterwards, supernatants were removed and visfatin molecules (antigens) were detected by the ELISA method. ELISA assays were performed using commercial ELISA kits (Hangzhou Eastbiopharm Co. Ltd., catalog: CK-E11560). Absorption was determined with a microplate absorbance reader at 450 nm. The lower detection limit was 2 pg/mL for visfatin (coefficients of variation; intra-assay 10%, inter-assay 12%).

STATISTICAL ANALYSES

Results were expressed as mean $\pm$ SEM. The data belonging to different groups were compared using one-way analysis of variance (ANOVA) with the Tukey-Kramer *post hoc* test, and *p* values of <0.05 were considered significant. The correlation between visfatin levels on the one hand and obesity indices, EC50, and maximum response on the other was assessed with Pearson's correlation coefficient.

RESULTS

Body Weight

Figure 1 shows the weight courses in experimental groups. Female rats fed the high-fat diet gained more weight than control groups. This was associated with the raised final body weight, Lee index, and percentage of body fat in both DIO groups (Table 1).

Moreover, the final body weight, Lee index, and percentage of body fat were significantly higher in HFD and S + HFD groups than ND and S + ND ones ($p < 0.001$). However, there were no significant differences in final body weight, Lee index, and percentage of body fat between ND *versus* S + ND and HFD *versus* S + HFD.

Tracheal Response to Methacholine

Based on Fig. 2, there were leftward shifts in methacholine concentration-response curves in groups S + HFD and S + ND compared to groups ND and HFD. Interestingly, after diet-induced obesity in the OVA-sensitized group, this leftward shift became markedly evident. The EC₅₀ in the tracheal segments was significantly lower in S + HFD animals (0.73 ± 0.07) than other groups ($p < 0.05$ to $p < 0.001$) (Fig. 3a). Furthermore, the EC₅₀ in tracheal segments was significantly lower in S + ND (0.93 ± 0.02) than ND (1.52 ± 0.05 , $p < 0.001$) and HFD (1.35 ± 0.07 , $p < 0.001$) groups (Fig. 3a). No significant difference was observed in EC₅₀ between ND and HFD animals ($p = 0.091$).

Contractility results showed that gr force/mg tissue weight (gF/TW) was significantly higher in S + HFD animals (27.59 ± 2.32) than other groups ($p < 0.05$ to $p < 0.001$, Fig. 3b). Also, gF/TW was significantly higher in S + ND animals (20.76 ± 1.01) than ND (13.14 ± 0.88 , $p < 0.01$) and HFD (15.19 ± 0.87 , $p < 0.05$) ones. There was no significant difference in gF/TW between ND and HFD groups (Fig. 3b).

Total WBC Count in LLF

Based on Table 2, the amount of WBC was significantly higher in the S + HFD group than other groups ($p < 0.001$). There also were significant differences in WBC count between S + ND on the one hand and between ND and HFD on the other ($p < 0.001$). No significant difference was seen between ND and HFD groups in leukocyte count.

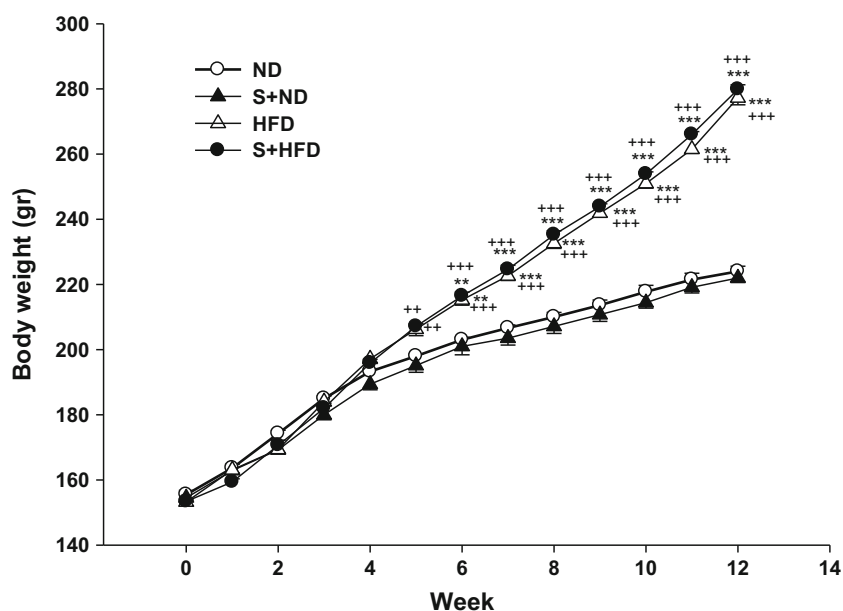


Fig. 1. Weight courses in experimental groups. Values are represented as mean $\pm$ SEM. ND normal diet, S + ND OVA-sensitized with normal diet, HFD high-fat diet, S + HFD OVA-sensitized with a high-fat diet. Differences between the results of ND and those of other groups, $**p < 0.01$ and $***p < 0.001$. Differences between the sensitized normal diet group and obese groups, $++p < 0.01$ and $+++p < 0.001$. For each group, $n = 8$.

Comparison of Differential Inflammatory Cells in LLF

As demonstrated in Fig. 4, the percentage of neutrophils was significantly higher in S + ND and S + HFD groups than ND and HFD ones ($p < 0.001$). Furthermore, the percentage of neutrophils was significantly higher in HFD than ND ($p < 0.01$).

The percentage of mononuclear cells was higher in ND and HFD than other groups ($p < 0.001$). The differences of mononuclear cells in ND group were significantly higher than HFD group ($p < 0.05$). Additionally, the percentage of mononuclear cells was higher in S + HFD group when compared with S + ND group ($p < 0.01$).

The percentages of eosinophil were significantly higher in groups sensitized with OVA (S + ND and S + HFD) than other groups ($p < 0.001$). This value was also significantly higher in S + ND than S + HFD ($p < 0.001$). Nevertheless, there was no significant difference between HFD with ND.

On the other hand, mean ($\pm$ SEM) different inflammatory cells (eosinophil, mononuclear, and neutrophil) were higher in sensitized groups (S + ND and S + HFD) than control groups (ND and HFD) ($p < 0.001$). No significant difference was observed in differential inflammatory cell count between control groups (ND vs. HFD).

Table 1. Weight Changes and Obesity Indices in Experimental Groups

Variables	ND	S + ND	HFD	S + HFD
Initial body weight (g)	155.50 $\pm$ 1.28	154.50 $\pm$ 0.80	153.25 $\pm$ 1.23	153.38 $\pm$ 0.75
Final body weight (g)	224 $\pm$ 1.58	222 $\pm$ 1.43	277.25 $\pm$ 2.13***	279.88 $\pm$ 1.40***
Percentage of body weight change (%)	44.11 $\pm$ 1.75	43.68 $\pm$ 0.66	79.46 $\pm$ 2.09***	82.51 $\pm$ 1.59***
Lee index (mg/mm)	285.61 $\pm$ 0.47	286.99 $\pm$ 1.09	309.56 $\pm$ 0.39***	310.56 $\pm$ 0.79***
Percentage of body fat (%)	3.88 $\pm$ 0.46	4.51 $\pm$ 0.80	20.99 $\pm$ 0.29***	21.71 $\pm$ 0.57***

Values are represented as mean $\pm$ SEM. Differences between the results of ND and those of other groups, $***p < 0.001$. Differences between the results of sensitized normal diet group and obese groups, $+++p < 0.001$. For each group, $n = 8$

ND control with normal diet, S + ND OVA-sensitized with normal diet, HFD control with high-fat diet, S + HFD OVA-sensitized with high-fat diet

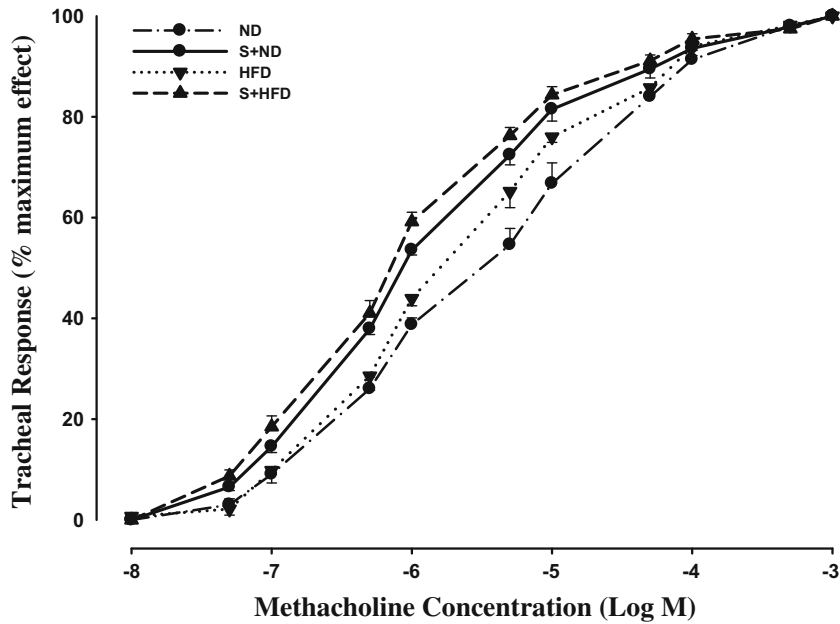


Fig. 2. Cumulative log concentration-response curves of methacholine-induced contraction of isolated tracheae in control with normal diet (ND), OVA-sensitized with normal diet (S + ND), control with high-fat diet (HFD), and OVA-sensitized with high-fat diet (S + HFD) groups (for each group, $n = 8$).

Moreover, mononuclear and neutrophils differences were significantly higher in S + HFD than S + ND ($p < 0.001$) (Table 2).

Lung Histopathology

OVA sensitization caused a significant elevation in lymphoid hypertrophy, airway smooth muscle hyperplasia, and emphysema pathological changes in sensitized groups (S + ND and S + HFD) compared to control groups (ND and HFD) ($p < 0.01$ to $p < 0.001$, Figs. 5 and 6a, d, e). Also, after diet-induced obesity, lipid aggregation in lung tissue was significantly enhanced in obese groups (HFD and S + HFD) in comparison with normal diet groups (ND and S + ND) ($p < 0.001$, Figs. 5 and 6c). Edematous pathological changes were significantly higher in HFD, S + ND, and S + HFD than in ND group ($p < 0.001$ for all cases, Figs. 5 and 6b), but there were no significant differences among other groups. On the other hand, airway smooth muscle pathological changes were significantly increased in S + HFD compared to S + ND ($p < 0.001$, Figs. 5 and 6e), and the emphysema pathological change was significantly higher in S + HFD than S + ND ($p < 0.05$, Figs. 5 and 6a). In addition, the lymphoid hypertrophy pathological change was higher in S + HFD compared with S + ND ($p < 0.001$).

Visfatin Protein Levels in Tracheal Tissue

The protein level of visfatin was higher in S + HFD (1.32 ± 0.09) than ND (0.70 ± 0.03 , $p < 0.001$) and S + ND (0.72 ± 0.03 , $p < 0.001$) groups. In addition, the visfatin protein level was higher in HFD (1.19 ± 0.05) than ND (0.70 ± 0.03 , $p < 0.001$) and S + ND (0.72 ± 0.03 , $p < 0.001$) groups. Although the protein level of visfatin was higher in S + HFD than HFD, this increase was not statistically significant (Fig. 7).

Relationship Between Visfatin Protein Levels and Obesity Indices, EC_{50} , and Maximum Response

Results of the Pearson correlation analysis of visfatin and obesity indices, EC_{50} , and maximum response are depicted in Table 3. There was a significant positive correlation between visfatin protein level on the one hand and obesity indices and maximum response in tracheae on the other. However, the correlation between EC_{50} and visfatin protein level was not statistically significant ($r = -0.325$, $p = 0.069$).

DISCUSSION

In the present study, results revealed increased tracheal responsiveness to methacholine in OVA-sensitized

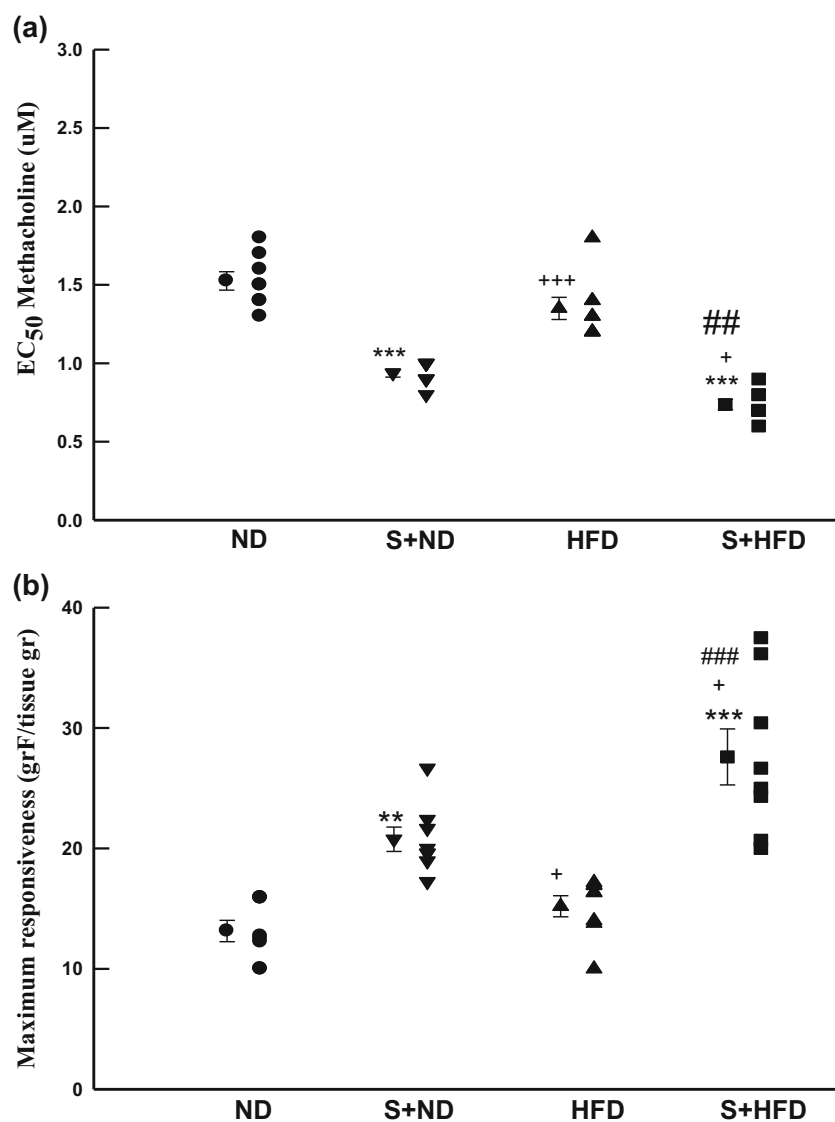


Fig. 3. Individual values and mean + SEM of **a** tracheal response to methacholine (EC_{50} , the effective concentration of methacholine, causing 50% of maximum response) and **b** tracheal contractility to methacholine (gr force/tissue gr) (contractility response to 1 mM methacholine) in control with normal diet (ND), OVA-sensitized with normal diet (S + ND), high-fat diet (HFD), and OVA-sensitized with high-fat diet (S + HFD) groups (for each group, $n = 8$). Statistical differences between ND and other groups, $**p < 0.01$ and $***p < 0.001$. Statistical differences between S + ND and obese groups, $+p < 0.05$ and $+++p < 0.001$. Statistical difference between HFD and S + HFD, $##p < 0.01$ and $###p < 0.001$.

groups, with the greatest increase in obese rats. The maximum response to methacholine was also significantly higher in OVA-sensitized lean and obese rats, with a marked increase observed in the obese rat. Obesity caused an additional increase in the total number of leukocytes, eosinophil, neutrophils, and mononuclear cells in the bronchoalveolar lavage fluid (BALF) of OVA-sensitized rats. Also, the protein level of visfatin in tracheal tissue was

higher in obese OVA-sensitized rat compared with lean OVA-sensitized ones.

The first part of this study investigated the effect of obesity on histological changes in the lungs of OVA-sensitized female rats. The results indicated some aspects of human asthma in both lean and obese sensitized rats (e.g., edema, emphysema, lymphoid hypertrophy, and airway smooth muscle hyperplasia) with marked changes in

Table 2. Bronchoalveolar Lavage Fluid Cellularity

	ND	S + ND	HFD	S + HFD
Leukocytes ($\times 10^5$ /BAL)	1.40 $\pm$ 0.12	5.63 $\pm$ 0.29 ***	1.63 $\pm$ 0.26 ⁺⁺⁺	8.20 $\pm$.41 *** ⁺⁺⁺ , ###
Mononuclear ($\times 10^5$ /BAL)	1.23 $\pm$ 0.10	3.11 $\pm$ 0.17 ***	1.37 $\pm$ 0.24 ⁺⁺⁺	4.94 $\pm$ 0.48 *** ⁺⁺⁺ , ###
Eosinophil ($\times 10^5$ /BAL)	0.02 $\pm$ 0.00	1.40 $\pm$ 0.22 ***	0.02 $\pm$ 0.00 ⁺⁺⁺	1.41 $\pm$ 0.10 *** ⁺⁺⁺ , ###
Neutrophils ($\times 10^5$ /BAL)	0.13 $\pm$ 0.02	1.13 $\pm$ 0.14 ***	0.22 $\pm$ 0.03, ⁺⁺⁺	1.83 $\pm$ 0.13 *** ⁺⁺⁺ , ###

Total and differential cellularity in bronchoalveolar lavage fluid. Values are represented as mean ($\pm$ SEM) of eight animals in each group. Statistical differences between ND and other groups, *** p < 0.001. Statistical differences between S + ND and obese groups, ⁺⁺⁺ p < 0.001. Differences between HFD and S + HFD, ### p < 0.001

ND control with normal diet, S + ND OVA-sensitized with normal diet, HFD control with high-fat diet, S + HFD OVA-sensitized with high-fat diet

the obese OVA-sensitized female group. The results of the present study were in line with those of our previous study on obese OVA-sensitized male Wistar rats [19], with the exception of some histological changes (*e.g.*, higher lipid aggregation in female rats in the present study compared to male obese OVA-sensitized rats in the previous study). This part of the experiment further explored the histological changes due to the high-fat diet-induced obesity condition in the lung tissue of the OVA-sensitized group. Additionally, to evaluate the maximum response of respiratory airways in the obesity condition, we used a diet-induced obesity model instead of genetically obese mice. Genetically, obese mice reportedly have certain complications by co-morbid conditions which may affect the

remodeling of the airway, *e.g.*, small lung size with reduced lung volumes and lung mass [20, 21]. Our results indicated that diet-induced obesity leads to an increase in the body weight and obesity indices (percentage of weight changes, the Lee index, and fat percentage) of obese groups, consistent with the previous study [5]. This study in OVA-sensitized rats also described some aspects of human asthma including AHR, increased maximum response, lymphoid hypertrophy, BALF eosinophilia, smooth muscle hypertrophy, and airway remodeling, which were in line with the previous study [22]. Airway responsiveness to methacholine was increased in sensitized lean and obese rats compared with wild-type controls. Interestingly, notably for AHR, airway responsiveness to methacholine was

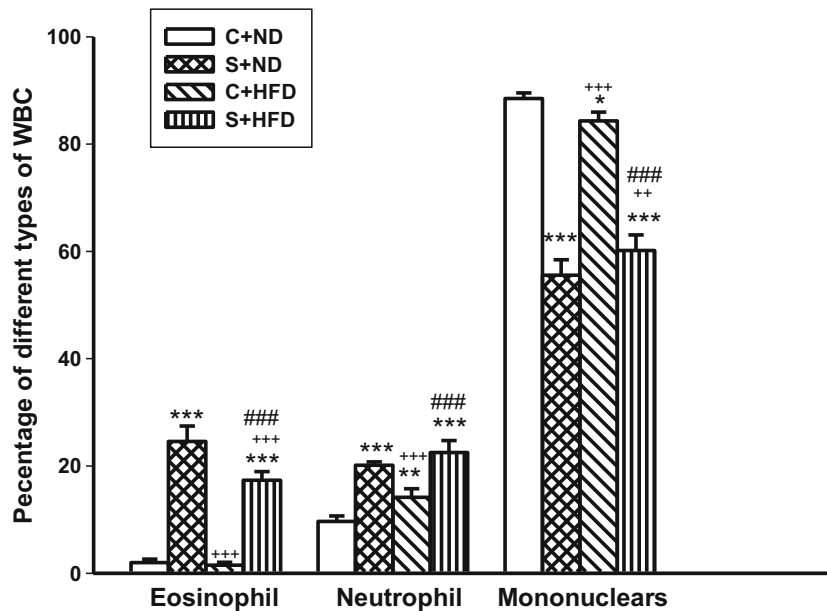


Fig. 4. Percentage of differential WBC count in the bronchoalveolar lavage fluid of control with normal diet (ND), OVA-sensitized with normal diet (S + ND), control with high-fat diet (HFD), and OVA-sensitized with high-fat diet (S + HFD) groups (for each group, $n = 8$). Values are represented as mean $\pm$ SD. Statistical significance for the difference between the data of ND and other groups, * p < 0.05; ** p < 0.01; and *** p < 0.001. Statistical differences between S + ND and obese groups, ++ p < 0.01 and +++ p < 0.001. Difference between the results of HFD and S + HFD, ### p < 0.001.

Table 3. Pearson Correlation Analysis Between Obesity Indices and Visfatin Protein Levels in Tracheal Tissue

Variables	Visfatin protein	
	<i>r</i>	<i>p</i> value
Percentage of body weight changes	0.886	0.000
The Lee index	0.846	0.000
Percentage of body fat	0.849	0.000
EC ₅₀	−0.325	0.069
Maximum response	0.424	0.016

the highest in obese OVA-sensitized female rats, with a significant difference with non-obese sensitized rats. Our results confirmed previous reports on AHR development in obese mice [23].

AHR is a common feature of obese mice, showing an increased airway response to intravenous methacholine. In previous studies which generally used obese mice, lung mechanics measurements were made by artificial ventilation with the same tidal volume and positive end-expiratory pressure (PEEP) after opening the chest [21, 24]. This method reduced functional residual capacity and spontaneous tidal volumes, resulting in innate AHR.

Breathing in low lung volumes is unlikely to cause remodeling in the airway or AHR [23]. In this study, however, we used rat tracheal segments to measure the responsiveness to methacholine. By directly assessing tracheal responses, this method probably minimized the effects of the noted innate AHR, as shown in obese non-sensitized rats. Furthermore, the innate AHR observed in previous studies may have been caused by intravenous injection of methacholine which may have an impact on airway responsiveness [25].

The increased total WBC and eosinophil counts are characterized a feature of sensitized animals and patients

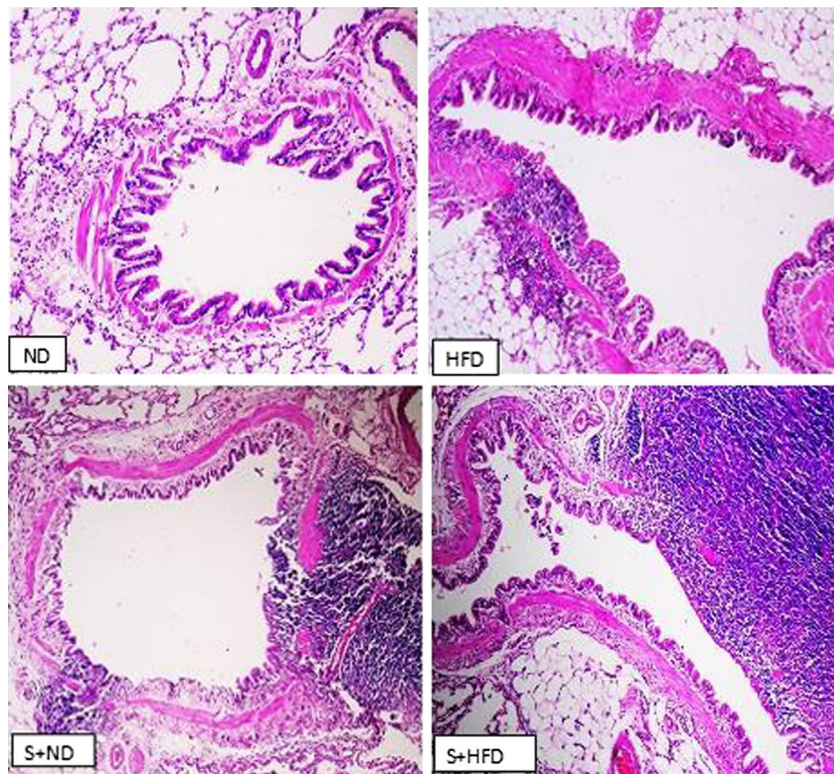


Fig. 5. Photographs of a lung specimen in different groups (magnification for each group, 10×20). ND control with normal diet, S + ND OVA-sensitized with normal diet, HFD control with high-fat diet, and S + HFD OVA-sensitized with high-fat diet.

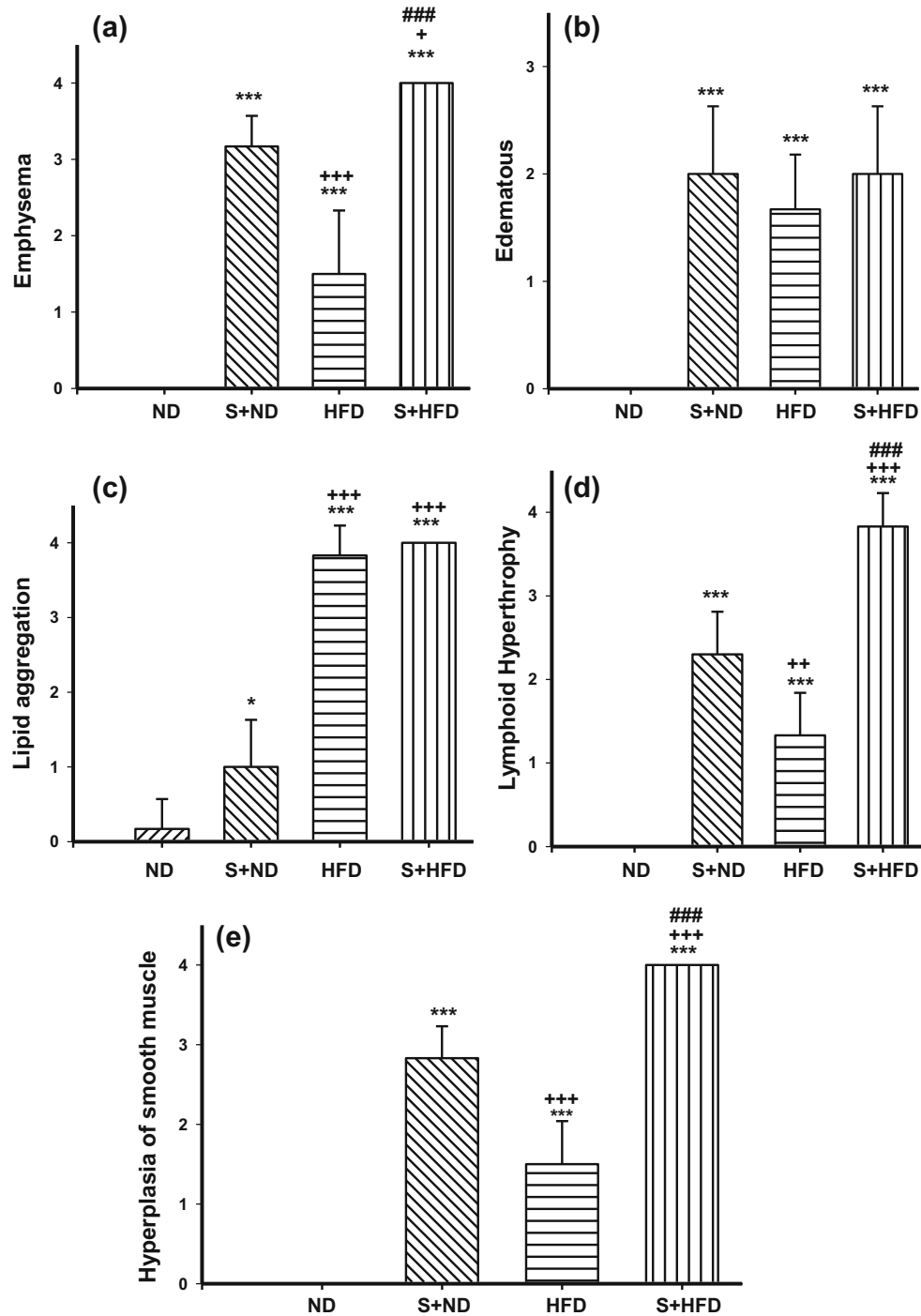


Fig. 6. **a** Emphysema, **b** edematous, **c** lipid aggregation, **d** lymphoid hypertrophy, and **e** airway smooth muscle hyperplasia changes of lungs in control with normal diet (ND), OVA-sensitized with normal diet (S + ND), control with high-fat diet (HFD), and OVA-sensitized with high-fat diet (S + HFD) groups (for each group, $n = 8$). Differences between ND and other groups, $*p < 0.01$ and $***p < 0.001$. Differences between S + ND and control high-fat diet and sensitized high-fat diet groups, $+p < 0.05$; $++p < 0.01$; and $+++p < 0.001$. Difference between HFD and S + HFD, $###p < 0.001$.

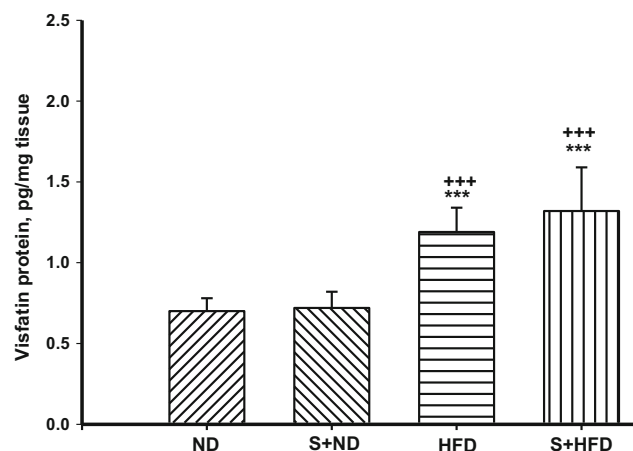


Fig. 7. Visfatin protein levels in the tracheal tissues of experimental groups. Data are represented as mean $\pm$ SD. ND control with normal diet, S + ND OVA-sensitized with normal diet, HFD high-fat diet, and S + HFD OVA-sensitized with high-fat diet group (for each group, $n = 8$). Differences between ND and other groups, *** $p < 0.001$. Differences between S + ND and high-fat diet and sensitized high-fat diet groups, +++ $p < 0.001$.

with asthma [26]. Increased total WBC and eosinophil counts in the lung lavage of sensitized rats were seen in previous studies using the same method of sensitization [4, 26]. Via the release of cationic granule proteins and a variety of inflammatory mediators, eosinophil promotes the pathogenesis of asthma. It is involved in this process with the production of Th2 cytokines and chemokine [27]. Contradictory data are available on the role of eosinophil in the development of AHR in high-fat diet mouse models of allergic asthma. In the present study, sensitized lean and obese rats challenged with OVA showed a markedly increased eosinophil count in BALF, indicating the efficacy of the OVA sensitization and challenge procedure. The percentage of eosinophils was higher in OVA-sensitized obese rats than control ones but was lower compared with sensitized lean rats. Some studies have reported an increase in eosinophil count in asthmatic models of obesity [28]. Nevertheless, other reports have reported no such increase in eosinophil numbers in BALF [23].

Obesity is the state of chronic low-grade systemic inflammation that may affect airway inflammation in obese asthmatic individuals. On the other hand, the effects of obesity on airway inflammation in asthmatic condition have noted Th1 process and increased neutrophil count in BALF and blood [23]. Most studies have noted luminal airway eosinophils, but it is important to consider eosinophil mobilization from airway epithelium to airway luminal. Calixto et al. [28] reported a reduction in eosinophil in the airway lumen in the obese sensitized group, probably due to impaired eosinophil transmission from the airway epithelium to the lumen. The authors suggested that this

delay in the transit of eosinophil to the lumen made these cells remain in the peri-bronchiolar structures of the lungs longer, probably contributing to AHR in obese asthmatic conditions. In the present study, the reason for decreased eosinophil in the obese OVA-sensitized group compared with lean OVA-sensitized rats may be the delayed transit of eosinophil to lumen which needs further histological studies.

In addition, in the present study, we measured the visfatin protein level in tracheal tissue. The results suggested that the protein level of visfatin was significantly increased in the obese OVA-sensitized rats. Fat tissue is considered an endocrine organ which indicates increased inflammatory activity. Obese people typically show increased inflammatory mediators such as adipokines, cytokines, chemokines, acute-phase proteins, and CRP [29, 30]. This condition in obesity represents a chronic low-grade systemic inflammation that is unlikely to lead to AHR. Mouse models of obesity and asthma have illuminated the possible role of leptin, adiponectin, and other inflammatory mediators in the association between obesity and asthma [31]. According to the literature, visfatin plays a role in the pathogenesis of a variety of chronic disorders such as rheumatoid arthritis, type 2 diabetes, acute sepsis, and acute lung injury [15, 32, 33]. Studies have shown that visfatin exerted the pro-inflammatory functions [34]. Indeed, visfatin upregulates the production of IL-1 β , IL-6, IL-10, and TNF- α in human monocytes in an extracellular cytokine manner [34]. Furthermore, visfatin is able to inhibit apoptosis in neutrophils, leading to the perpetuation of inflammation [35]. On the other hand, previous studies

reported that visfatin acts as a potent chemotactic factor, especially for the recruitment of monocytes [36]. Our previous study showed that, in the DIO OVA-sensitized condition, the visfatin protein level was elevated in the lungs of male rats. Interestingly, the noted study showed that the visfatin mRNA level was associated with NF- κ B protein levels in the tracheae of male rats [10]. In the present study, enhanced inflammatory cells were observed in the obese OVA-sensitized group compared with other groups, especially the normal diet asthmatic group. This shows the possibly critical role of adipocytokines in obesity associated with asthma condition. Moreover, our results indicated that the number of neutrophils increased in the obese asthmatic group compared with the normal diet OVA-sensitized group. It is believed that enhanced neutrophil levels in the BALF of the obese asthmatic statue can be due to adipocytokines, *e.g.*, visfatin, and systemically effect of obesity. Also, there was a significant relationship between visfatin and maximum response in respiratory airways which, at least in part, indicates that the elevated level of visfatin protein in the tracheal tissue can result in AHR.

In the animal model of obesity associated with asthma, AHR is a common feature of obese mice [23]. Interestingly, regardless of method of sensitization, obesity *per se* leads to increase in AHR as reported earlier studies [23]. In the present study, despite lack of statistically significant in the shift of EC₅₀ curve in HFD group in compared with ND group, the shift of curve, at least in part, reflects may be the lung inflammation caused by systemic inflammation or localized release of adipocytokines in lung tissue. From the results of our current study as well as our previous study [10, 19], it is clear that under obesity conditions, fat accumulation in the lung is clearly evident. Also, considering that the visfatin level, as an adipocytokine, was higher in the lung of the obese group, it is understood local changes along with systemic inflammation in obesity condition may synergically affect the airway responsiveness.

In conclusion, the results of this study revealed that diet-induced obesity causes AHR and increases the number of inflammatory cells in the airways of female OVA-sensitized rats. In addition, the level of visfatin, as an adipocytokine, was enhanced in the tracheal tissue of obese sensitized rat. Visfatin has a pro-inflammatory and chemotactic function and inhibits apoptosis in neutrophils. Elevated levels of adipocytokines, *e.g.*, visfatin, in obesity associated with the OVA-sensitized condition, may result in the exacerbation of airway inflammation in asthma. It can also alter asthma phenotype in obesity associated with asthma condition.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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