



Ligustrazine improves blood circulation by suppressing Platelet activation in a rat model of allergic asthma

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ABSTRACT

Chuan-xiong (*Ligusticum wallichii*) is a traditional herbal medicine in Eastern Asia, but the effect of its active component ligustrazine remains unclear. We explored its effect and possible mechanism in a well-characterized rat model of allergic asthma. Ligustrazine suppressed bronchial hyper-responsiveness to methacholine, and suppressed lung inflammation in asthmatic rats. Ligustrazine exhibited potent immuno-modulatory and anti-inflammatory effects: it suppressed lymphocyte and eosinophil mobilization, and reduced cytokine IL-5 and IL-13 production significantly in lung tissues from asthmatic rats ($P < 0.05$). Further histological examinations clearly demonstrated that ligustrazine improved blood circulation and ameliorated platelet activation, aggregation and adhesion, which induced sustained infiltration and activation of various inflammatory cells, including lymphocytes and eosinophils, followed by synthesis and release of a variety of pro-inflammatory mediators and cytokines. The present study suggests that ligustrazine is a potent agent for the treatment of allergic asthma due to its strong anti-inflammatory and immuno-modulatory properties.

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1. Introduction

Asthma is a common disease of the respiratory system. It causes the airway to constrict and produce excess mucus, making breathing difficult. Airway obstruction in asthma can be severe, leading to life-threatening narrowing and closure of the airway (Bostantzoglou et al., 2015). Global initiative for an asthma guide reported about 300 million people in the world suffering from asthma, which was predicted to hit 400 million by 2025 according (Bostantzoglou et al., 2015). Asthma is tightly associated with immune dysfunction and characterized by chronic airway inflammation (Riiser, 2015).

Asthma is refractory to current available drug treatments. Recent studies have shown the beneficial effects of traditional Chinese medicine in the treatment of allergic asthma, including Chuan-xiong (*Ligusticum wallichii*) which has been used for the treatment of cardiac-cerebral vascular and pulmonary diseases, including asthma for centuries in Eastern Asia (Guo et al., 2008). Ligustrazine, an alkaloid, was recently identified as the most active component of Chuan-xiong (Guo et al., 2008; Zeng, 1983). It has

been reported that ligustrazine exerted neuroprotective effects possibly through inhibiting the anti-inflammatory response and Bcl-2 dependent neuronal apoptosis (Han et al., 2014; Li et al., 2015; Zheng et al., 2016). However, little is known about the effect of ligustrazine on allergic asthma and its underlying mechanism.

It was reported that the blood of patients with allergic asthma was in a hypercoagulable state, which promoted the formation of platelet-lymphocyte and platelet-eosinophil complex and suppressed the blood circulation (Riiser, 2015). Previous studies suggested that platelets participate in the pathogenesis and development of asthma, while a variety of changes in platelet function have been observed to associate with the disease, such as alterations in platelet secretion, expression aggregation and adhesion of surface molecules (Klinger, 1997; Klinger and Jelkmann, 2002; Pitchford et al., 2008). Moreover, platelets have been found to actively contribute to most characteristic features of asthma, including bronchial hyper-responsiveness, bronchoconstriction, airway inflammation, and airway remodeling (Klinger, 1997; Klinger and Jelkmann, 2002; Pitchford et al., 2008). Therefore inhibiting thrombosis and increasing blood circulation by suppressing platelet activation and aggregation would be beneficial for the treatment of asthma.

In the present study, we have built up a well-characterized rat model of allergic asthma by ovalbumin (OVA) sensitization and challenge. We then took advantage of the rat model and exam-

Abbreviation: (OVA), ovalbumin.

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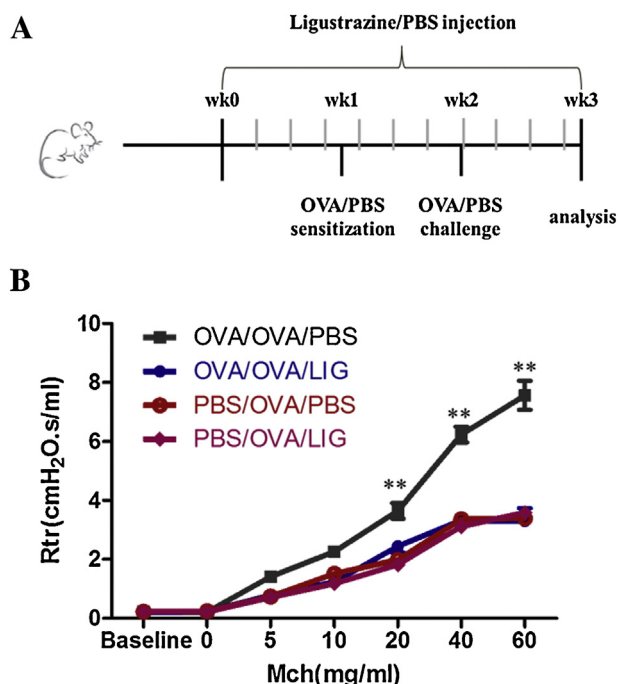


Fig. 1. Ligustrazine suppresses bronchial hyper-responsiveness to methacholine (Mch) in asthmatic rats ($n = 12$ each group). (A) Construction of asthmatic rat model with ligustrazine treatment. (B) Assessment of central airway resistance (Newtonian resistance [Rtr]) as a marker of airway hyper-responsiveness (AHR) after challenge with different doses of MCh. ** $P < 0.01$.

ined the effect of ligustrazine against inflammation, infiltration and mobilization of various inflammatory cells into the lung tissues of asthmatic rats, including lymphocytes and eosinophils. Furthermore, we studied the effect of ligustrazine on the activation, aggregation and adhesion of platelets, which identified a key immuno-modulatory regulator in the pathogenesis of allergic asthma. The present study explored the possible effects and underlying mechanisms of ligustrazine in the treatment of allergic asthma in an asthmatic rat model.

2. Materials and methods

2.1. Allergen sensitization and challenge in rats

Male Sprague-Dawley (SD) rats (220–250 g) were purchased from Shanghai BK Laboratory Animal Limited Company (Shanghai, China). All animals were housed in a room maintained at $23 \pm 2^\circ\text{C}$ with $50\% \pm 10\%$ humidity and a 12 h light 12 h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld for 8 h before the experiments. Rats were immunized with an intraperitoneal injection (1.0 mL/rat) of 10% aluminum hydroxide gel (Beijing Chemical Reagent Factory, Beijing, China) containing 0.2% ovalbumin (OVA, Grade V, Sigma Chemical Co., St. Louis, MO, USA). 15 days after immunization, rats were challenged by exposure to a 1% OVA in phosphate buffer solution (PBS) aerosol once daily for 20 min for 8 consecutive days. The challenge was carried out in a half vented metal chamber (45 cm $\times$ 25 cm $\times$ 15 cm) by an ultrasonic nebulizer, with aerosol flow rate of 3 mL/min. Control group rats were exposed to nebulized sterile saline using the same method. One day after the last challenge the blood and tissue sample were collected (Fig. 1A).

In the present study, all animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). The protocol was approved

by the committee on the Ethics of Animal Experiments of Anhui University of Chinese Medicine. All efforts were made to minimize animal suffering and numbers of animal used.

2.2. Animal grouping and treatment

Rats were divided into six groups with 12 rats in each group. Control group animals were sensitized and challenged with saline; asthmatic model group animals were sensitized with OVA and challenged with OVA; ligustrazine group animals were sensitized and challenged as same to the model group, but were administered with ligustrazine (80 mg/kg) ($\geq 99.95\%$ of purity, Haerbin Sanlian Pharmaceutical Limited Company, Haerbin, China, 79H1505) intraperitoneally once a day for 7 days from day 14–21. The dose of ligustrazine used is based on our preliminary experiments where it has a significant effect against asthma but causes no toxic effect (data not shown), which is consistent with results from other studies (Guo et al., 2008; Han et al., 2014). At day 21, animals were sacrificed, and lung tissues were removed and frozen in liquid nitrogen, and then stored at -80°C for further analysis.

2.3. Histological examination

The lungs were immersed into 10% neutral formalin for 7 days. After tissues were paraffinized, 5 μm sections were prepared and stained with hematoxylin-eosin for observation of inflammatory cell infiltration under a light microscope.

2.4. Rat platelet isolation

Platelets were isolated by differential centrifugation from rat venous blood collected in citrate containing tubes as previously described (Cardenes et al., 2014). Briefly, whole blood was centrifuged at 150g for 10 min in the presence of Prostaglandin I₂ (PGI₂, 1 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich, St. Louis, MO) to obtain platelet rich plasma. Platelets were subsequently pelleted from the platelet rich plasma by centrifugation at 1500g for 10 min. The platelet pellets were washed with Erythrocyte Lysis Buffer (Qiagen, Valencia, CA) and PGI₂. The final samples were re-suspended in modified Tyrode's buffer (20 mM Hepes, 128 mM NaCl, 12 mM bicarbonate, 0.4 mM NaH₂PO₄, 5 mM Glucose, 1 mM MgCl₂, 2.8 mM KCl, pH 7.4) prior to study.

2.5. Western blot

Western blot was performed according to routine protocols. Primary antibodies used were listed as below: IL-5, 1:1000 (BD Biosciences, San Jose, CA, USA); IL-13, 1:1000 (BD Biosciences); GAPDH, 1:2000 (Sigma); vWF, 1:1000 (Cell Signaling, USA); fibronectin, 1:2000 (Sigma). All experiments were repeated for at least three times independently.

2.6. Statistical analysis

The statistical significance of any difference was determined by one-way ANOVA followed by Tukey's protected t -tests when ANOVA was significant. The data are expressed as mean $\pm$ S.E.M. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for the statistical analysis. Values of $P < 0.05$ were considered statistically significant.

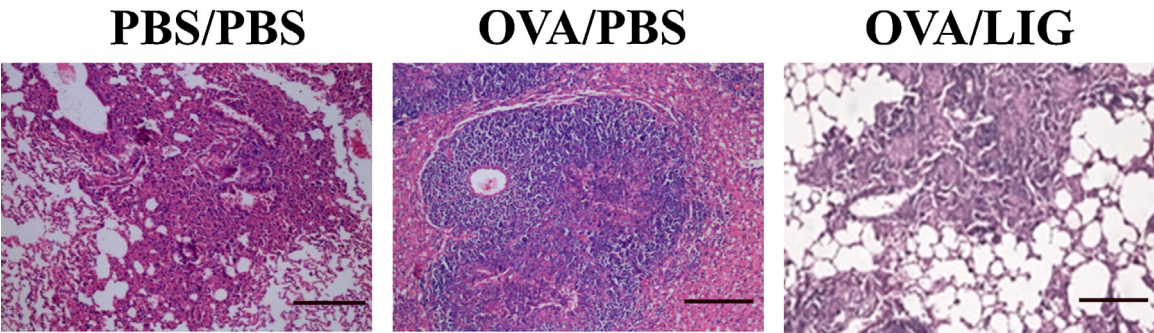


Fig. 2. Ligustrazine suppresses lung inflammation of asthmatic rats (n = 12 each group). Scale bar, 20 μ m.

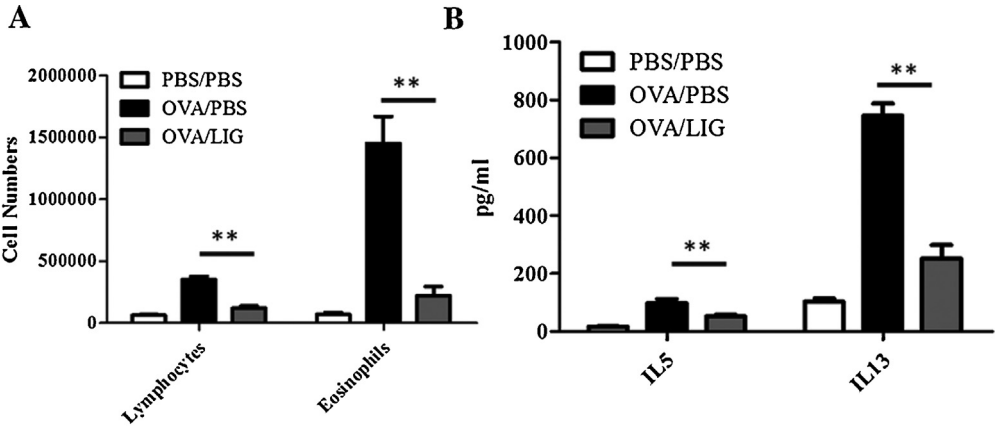


Fig. 3. Ligustrazine suppresses lymphocyte and eosinophil mobilization and cytokine production in the lung tissues from asthmatic rats (n = 12 each group). (A) There are less lymphocytes and eosinophils in lung tissues of ligustrazine protected asthmatic rats. (B) There is less cytokine production in lung tissues of ligustrazine protected asthmatic rats. ** P < 0.01.

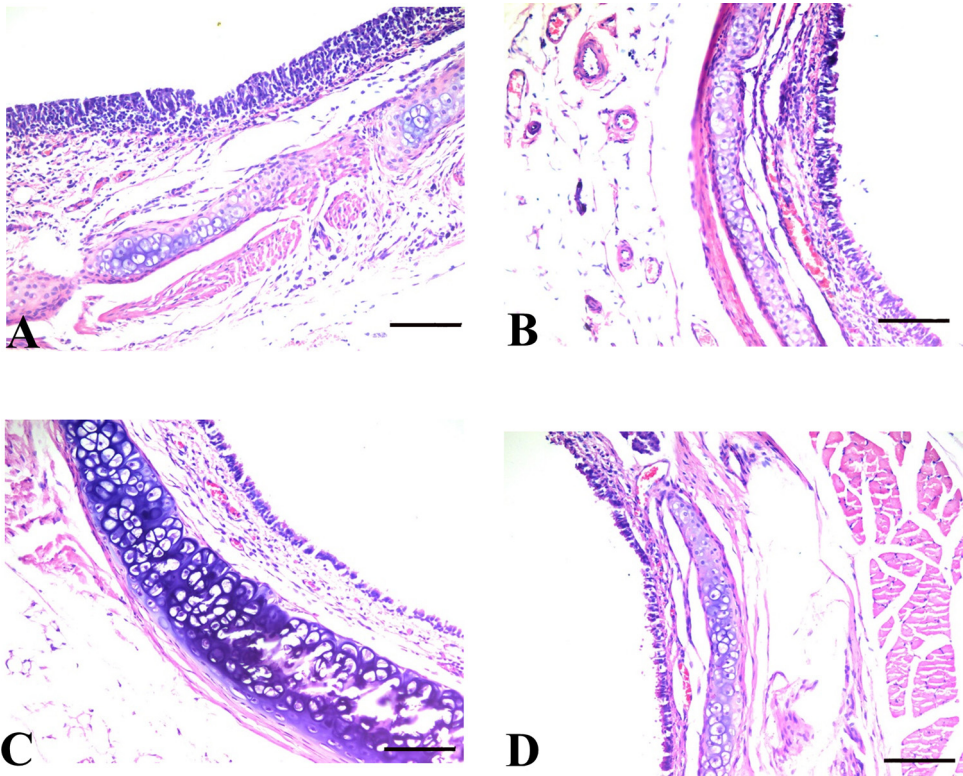


Fig. 4. Ligustrazine improves blood circulation. Histological examination of blood vessels from (A) Normal Rats, (B) Normal Rats treated with ligustrazine, (C) Sham-protected asthmatic rats, and (D) ligustrazine protected asthmatic rats (n = 12 each group). Scale bar, 60 μ m.

3. Results

3.1. Ligustrazine rescued the pulmonary function in rats with allergic asthma

To examine the effect of ligustrazine in the treatment of allergic asthma, a well-characterized rat model of allergic asthma was established by 1% OVA sensitization and challenge (Fig. 1A). Central airway resistance, a marker of airway hyper-responsiveness (AHR), was measured after challenge with different doses of methacholine (MCh), and it was observed that central airway resistance substantially increased in rats sensitized and challenged with OVA (Fig. 1B), suggesting that a rat model of allergic asthma was successfully established. Meanwhile, when asthmatic rats were treated with ligustrazine at 80 mg/kg, such an increase of central airway resistance was markedly suppressed (Fig. 1B), indicating ligustrazine treatment rescued the pulmonary function of those rats with allergic asthma.

3.2. Ligustrazine suppressed lung inflammation of asthmatic rats

Next, we collected and examined the rat lung tissues histologically. It was noticed that the lungs from asthmatic rats (Fig. 2, middle) exhibited stronger airway eosinophilia and lymphocytosis, compared to the rats sham-treated with PBS (Fig. 2, left), suggesting that there existed airway inflammation and increased lymphocyte and eosinophil-mobilization in asthmatic rats. Also, it was clearly demonstrated that such phenomenon in asthmatic rats was markedly ameliorated when treated with ligustrazine (Fig. 2, right), compared to sham-protected asthmatic rats (Fig. 2, middle).

3.3. Ligustrazine suppressed lymphocyte and eosinophil mobilization and cytokine production in lung tissues from asthmatic rats

Further experiments confirmed the effect of ligustrazine to suppress airway inflammation in asthmatic rats, and such effect was significant (Fig. 3A), suggesting ligustrazine inhibited lymphocytosis and eosinophilia, which led to less severe inflammatory damage.

Meanwhile, we collected the bronchoalveolar lavage fluid from the lung tissues, and examined the levels of asthma-related cytokines IL-5 and IL-13 with Western blot. Results showed that ligustrazine treatment significantly suppressed the production of IL-5 and IL-13 (Fig. 3B), indicating that the airway inflammation of the asthmatic rats was suppressed by ligustrazine protection.

3.4. Ligustrazine improved blood circulation in rats with allergic asthma

Blood circulation was also examined in the rats. Histological examination of blood vessels showed that ligustrazine promoted platelet activation and aggregation, hence improved blood circulation in asthmatic rats (Fig. 4).

3.5. Ligustrazine attenuated the platelet adhesion to HUVEC monolayers

Platelet activation and aggregation participate in the pathogenesis and development of asthma. One feature of activated platelets is their increased adhesion to vein endothelial cells. We isolated platelets from asthmatic rats with or without ligustrazine treatment, and tested their adhesion to human umbilical vein endothelial cell (HUVEC) monolayer. It was clearly shown that the platelets from asthmatic rats displayed a higher adhesion to the HUVEC monolayer (Fig. 5), compared to the sham-operated rats. Such an increased adhesion ability of platelets from asthmatic rats

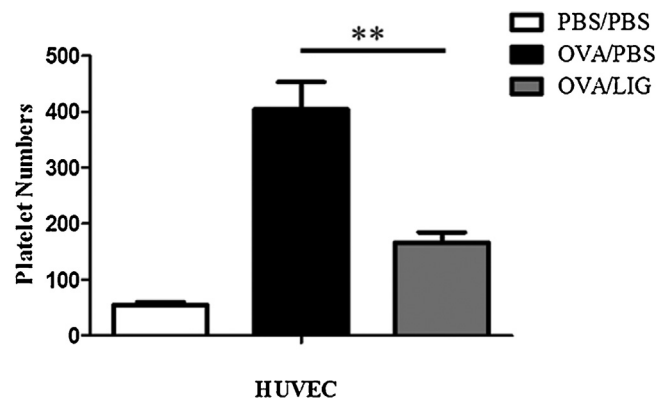


Fig. 5. Ligustrazine attenuates the platelet adhesion to HUVEC monolayer (n = 12 each group). ** P < 0.01.

was suppressed when treated with ligustrazine. 3 independent experiments with different batches of rats confirmed such effect of ligustrazine was significant (Fig. 5, P < 0.01).

3.6. Ligustrazine attenuates the platelets adhesion to adhesion molecules

vWF and fibronectin are two well studied adhesion molecules expressed on the lining endothelial cells, which are recognized by receptors on platelets, leading to platelet adhesion. We examined the levels of vWF or fibronectin adhered on platelets isolated from ligustrazine or sham-protected asthmatic rats by immunostaining (Fig. 6A). It was noticed that the levels of vWF or fibronectin adhered was noticeably higher in the asthmatic rats, compared to the sham control (Fig. 6A and B). However, such an increase was suppressed in the ligustrazine-protected asthmatic rats (Fig. 6A and B).

4. Discussion

Chuan-xiong has been widely used for cardiac-cerebral vascular diseases and pulmonary diseases as a traditional herbal medicine in Eastern Asia. However, the effect of ligustrazine, an active component of Chuan-xiong, on allergic asthma remains unclear. In the present study, pathological analysis clearly demonstrated that 22-day ligustrazine administration effectively reduced central airway resistance in OVA-evoked asthmatic rats (Fig. 1B), decreased the allergic inflammatory infiltration in the lung tissues (Figs. 2 and 3A), and inhibited OVA-induced IL-5 and IL-13 cytokines (Fig. 3B). Collectively, all of these above results demonstrated that ligustrazine was a potent agent in the treatment of asthma due to its anti-inflammatory property.

Asthma is associated with immune dysfunction and characterized by chronic airway inflammation. Inhibition of the regional inflammatory response through the reduction of inflammatory cytokines production is an effective therapeutic method in the treatment of asthma. The present study clearly demonstrated that inflammatory cytokines IL-5 and IL-13 were significantly reduced in asthmatic rats (Fig. 3B) when treated with ligustrazine, compared to the sham-protected rats. IL-5 uniquely and specifically participates in the control of eosinophil production and differentiation (Klein Wolterink et al., 2012; Lee et al., 2001). Attenuated synthesis of IL-5 could therefore relieve allergic responses caused by eosinophils. IL-13 was reported to be a critical mediator of allergic response in asthma (Agrawal and Townley, 2014; Heinzmann et al., 2000). Overproduction of IL-13 induced many common features of the allergic diathesis, such as airway hyper-responsiveness, eosinophilic inflammation, IgE production, mucus hypersecretion, and subepithelial fibrosis (Wills-Karp, 2001).

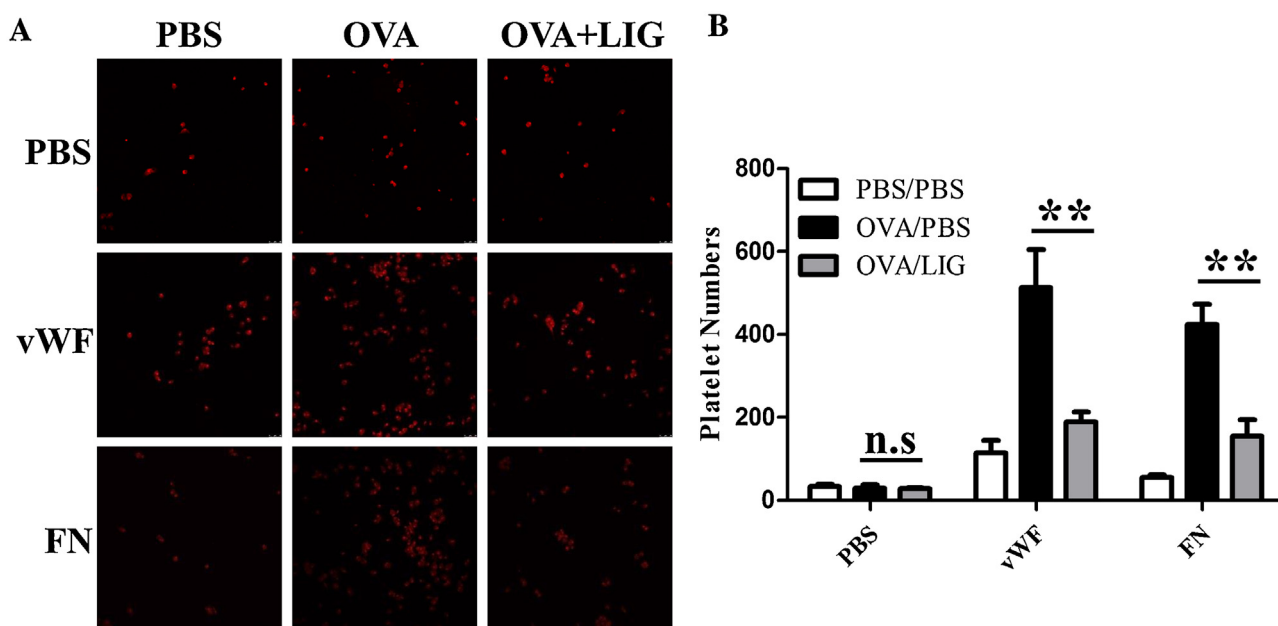


Fig. 6. Ligustrazine attenuates the platelet adhesion to adhesion molecules. (A) Rodamine-Phalloidin staining for vWF or fibronectin adhered platelets isolated from ligustrazine or sham protected asthmatic rats ($n = 12$ each group). Bar = 5 μM . (B) Total numbers of platelets of ten fields shown in (A). ** $P < 0.01$.

Interestingly, further mechanistic study revealed that ligustrazine markedly inhibited platelets activation and aggregation, and promoted blood circulation (Figs. 4 and 5), a process during which platelet adhesion was significantly attenuated (Figs. 5 and 6), inhibiting the infiltration and mobilization of lymphocytes and eosinophils (Fig. 3A). Circulating platelets played an integral role in the modulation of airway inflammation (Averill et al., 1992; Klinger, 1997; Sullivan et al., 2000). It has been reported that the activation and aggregation of platelets were increased in asthmatic patients, and circulating platelet aggregates have been identified in humans after an asthma attack, where bronchoalveolar lavage fluid from asthmatic subjects contained activated platelet products (Pitchford et al., 2008). Platelet activation was considered to be associated with bronchial hyper-responsiveness (Provotorov et al., 2015).

More importantly, platelets could directly interact with eosinophils, forming circulating complexes that facilitate inter-cellular signaling, resulting in the priming and activation of eosinophils (Kornerup et al., 2010). Complex formation induced the surface expression of integrins on the eosinophil, priming it for recruitment and adhesion to the inflamed lung (Johansson et al., 2012; Kornerup et al., 2010), consistent with results in the present study (Figs. 2 and 3A). Since ligustrazine ameliorated platelet activation and aggregation in asthmatic rats, the circulating complex of platelets and eosinophils was decreased, and the recruitment and adhesion of eosinophils to the asthmatic lung were also reduced (Figs. 2 and 3A). The eosinophil was regarded as a key mediator of the pathology and abnormal physiology of bronchial asthma (Kay, 2005; Seminario and Gleich, 1994). Eosinophils contributed to tissue damage (Seminario and Gleich, 1994; Uhm et al., 2012) and airway inflammation. In allergic asthma, there existed an increase in eosinophils in the lung, which correlated with the disease severity (Johansson et al., 2012; Seminario and Gleich, 1994; Uhm et al., 2012).

Also, platelets were reported to facilitate lymphocyte recruitment and infiltration (Fukuda et al., 1993; Pahl et al., 2002). In animal models of allergic asthma, platelet depletion reduced the severity of lung inflammation, including lymphocyte and eosinophil recruitment (Pitchford et al., 2003). Clearly, ligustrazine exhibited a significant immuno-modulatory effect by inhibiting

the activation, aggregation and adhesion of platelets and immune response that followed, for example, eosinophil and lymphocyte recruitment in the lung as observed in the present study (Figs. 2 and 3A).

In conclusion, ligustrazine suppressed airway inflammation in the rat model of OVA-induced allergic asthma due to its potent anti-inflammatory and immuno-modulatory properties, and it may be further investigated as a potential agent for the treatment of asthma.

Conflict of interest

The authors declare that they have no conflict of interest.

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