Japanese Loquat (*Eriobotrya Japonica*) Seed Extract, a Rich Source of Beta-Sitosterol Inhibits Airway Hyperresponsiveness in BALB/C Mice

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Abstract: *Seed extract of Loquat fruit (Eriobotrya japonica) is abundant in anti-oxidant components, such as beta sitosterol, which may have beneficial effects on asthmatic inflammation. We investigated preventative effects of diluted loquat seed extract (DLSE) on mouse asthmatic airway inflammation. BALB/c mice sensitized by exposure to Dermatophagoides farinae (Der f) with diesel exhaust particles (DEPs) were given DLSE ad libitum. Serum Der f -specific IgG1 titers and cytokine profiles in bronchoalveolar lavage fluids (BALF) were analyzed.*

We found that DLSE suppressed Der f-specific IgG1 serum levels and airway hyperresponsiveness (AHR) in allergen-induced mice. DLSE-treated mice had significantly decreased secretions of IL-5, IL-13, TGF-β1 and MCP-1 and up-regulated IFN-γ secretion.

The number of inflammatory cells in BALF was considerably reduced in DLSE-treated Der f + DEP induced (DD) group as compared with asthmatic group (Tap water/ Der f + DEP (TD)). Regarding inflammatory cells in BALF, a significant reduction of eosinophils was observed in DD group as compared with TD group (p<0.05). In TD group, the intensity of airway resistance increased simultaneously with the increase in acetylcholine concentration in a dose-dependent way. AHR was significantly inhibited in DD groups as compared with the TD group (p<0.05). Regarding serum specific-IgG1, significantly lower levels of this antibody were observed in DD groups as compared with the TD group (p<0.05).

DLSE treatment attenuated Th2 cytokines production and AHR in Der f + DEP -induced asthmatic mice. Our results strongly suggest that DLSE may have significant therapeutic benefits for the control of allergic airway disorders.

Keywords: diesel exhaust particles, allergen, airway hyperresponsiveness, eosinophil, Loquat extract

1. INTRODUCTION

Bronchial asthma is a chronic inflammatory airway disease that is characterized by eosinophilic inflammation, bronchial hyperresponsiveness and airway remodeling [1, 2]. Many clinical and experimental studies suggested that Th2 cells that secrete IL-4, IL-5 and IL-13 play important roles in directing and maintaining airway inflammatory processes [3].

While the prevalence of asthma was lower than it in Western countries (20.7%), asthma was found to be also relatively common in Japan (10.1%) [4]. Evidence clearly indicates that household and other environmental allergen (dust mites, cockroaches, pets and pollens) in disease development in older children and adults. Air pollutants, such as diesel exhaust particles (DEPs), formaldehyde, fine particles and oxidant gases, have not been definitively linked to disease development, although these agents can trigger asthma exacerbations. Recently, reactive oxygen species (ROS) were found to play an important role in bronchial asthma [5-9]. Our previous study reported that administration of the anti-oxidant reagent N-acetyl-L-cysteine (NAC) decreased DEPs-induced eosinophil chemotaxis [10].

Natural products from dietary components /foods with anti-oxidant properties have been studied to prevent or control diseases, and there is much epidemiologic evidence that has been reported.

Green tea consumption reduces the risk of gastric cancer [11], colorectal cancer [12] and breast cancer [13, 14]. Cocoa and tea reduces high blood pressure [15].

Loquat fruit (*Eriobotrya japonica*) is considered as one of the favorite fruit in Japan. Loquat has been used as a traditional Chinese medicine, 'Dai-yaku-oh-jyu' in Japan, for over a thousand years. Many Japanese people have used loquat syrup as a cough drop and its aged leaves have been used on skin as an analgesic and anti-edemic agent. Zhou et al. reported that loquat leaf contained amygdalin, urusolic acid, oleanolic acid, nerolidol and farnesol [16].

Recently, Yokota and her colleague identified amygdalin, unsaturated fatty acids, -sitosterol, caffeic acid, chlorogenic acid and various essential amino acids [17]. They previously reported that Eriobotrya japonica seed extract (ESE) is a strong reactive oxygen species scavenger [17] and effective for prevention and treatment of disorders, such as itch feeling of allergic disease [18] and allergic contact dermatitis [19].

Possible mechanism of anti-allergic effect of ESE is as follows: oral administration of ESE attenuated production of Th1 cytokines such as interferon-gamma (IFN-) and interleukin-2 (IL-2), induction of Th2 cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10) in the lesional skin. Serum immunoglobulin E (IgE) levels were decreased by attenuated IL-4. As a result development of ear thickness of allergic dermatitis was decreased. Finally allergic inflammation reduced by treatment of ESE. However, any beneficial effects of Loquat seeds have not been systematically studied.

In this study, we examined possible preventative effects of administering an extract of loquat seeds on bronchial asthma in mice.

2. MATERIALS AND METHODS

2.1. Preparation of Loquat Seeds Extract

Loquat fruit (*Eriobotrya japonica*) was obtained from Kochi and Wakayama in 2007. Dried seeds of Loquat (1kg) were crushed with a homogenizer (1,000 rpm) at 4 ℃, and after getting seed extract, the extract was stirred with 70% ethanol (2 L) for 1 week. After filtration, the extract was concentrated with a rotary evaporator and added to pure water (200 ml total). Loquat seeds extract was stored at 4 ℃ prior to use and then diluted to 100-fold with pure water (DLSE) prior to administration to animal. Finally, the 50 mg/ml solution of DLSE was used throughout the experiment. In the following experiment, according to the daily water intake of mouse strain used (4 ml), each mouse was received 200 mg of DLSE daily for the DLSE /*Dermatophagoides farinae*plus + DEP and DLSE /*Der f* + phosphate buffered saline groups. Five of mice group received tap water or DLSE solutions ad libitum according to mice group from day 1 to day 36.
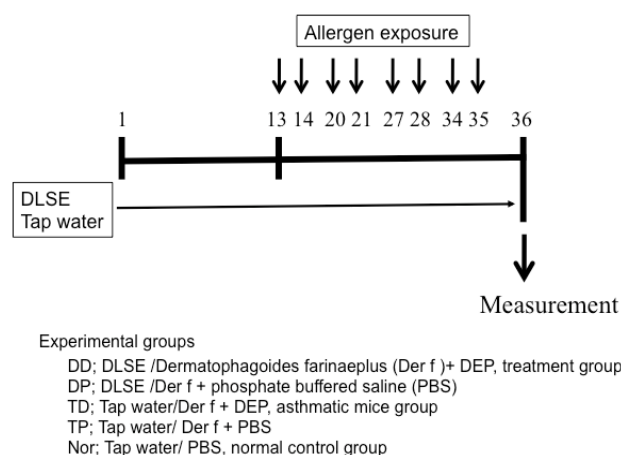

Experimental groups
DD; DLSE /Dermatophagoides farinaeplus (Der f)+ DEP, treatment group
DP; DLSE /Der f + phosphate buffered saline (PBS)
TD; Tap water/Der f + DEP, asthmatic mice group
TP; Tap water/ Der f + PBS
Nor; Tap water/ PBS, normal control group

Fig1. *Experimental Protocol*

2.2. Dermatophagoides Farinae Antigen

Dermatophagoides farinae (*Der f*) crude extract (mite antigen, lyophilized, Torii, Tokyo, Japan) was used as an antigen [20]. *Der f* was dissolved in phosphate buffered saline (PBS), pH 7.4 (200 µg /ml). Before use, aliquots (100 µl) were stored at -20 ℃.

2.3. Preparation of Diesel Exhaust Particles (DEP) Suspensions

Diesel exhaust particles (DEP) (4.6 g) were extracted using Soxhlet extraction for 24 h with dichloromethane. An extract (0.5 g) was suspended in 2.5 ml of dimethyl sulfoxide (DMSO) and then diluted with PBS (1840 μg / ml) and filtered to remove particulate matter. Before use, aliquots (100 μl) were stored at −20 ℃.
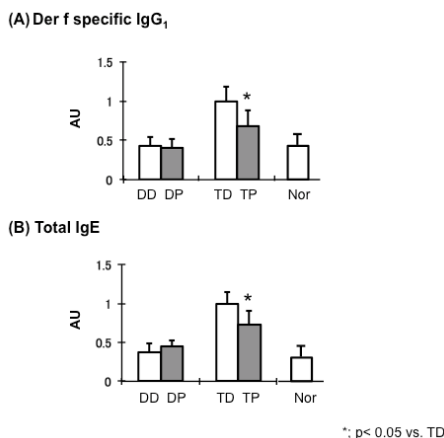

(A) Der f specific IgG$_1$

(B) Total IgE

*; p< 0.05 vs. TD

Figure2. *Effects of DLSE on serum levels of Der f-specific IgG$_1$ (A) and total IgE (B) in allergen-sensitized mice. Blood was collected and serum was isolated on day 36. The figure 2A shows that the serum level of Der f specific-IgG$_1$ was significantly lower in the DD group (p<0.01) as compared with the TD group. The figure 2B shows that the serum level of total IgE in the DD group was significantly lower than that in the DP group (p<0.05). Serum antibodies in mice were measured according to the methods. Results are means ± SD of data from 6 mice in each group. *P < 0.05 vs. TD group.*

2.4. Mice

Thirty BALB/c mice, aged five weeks, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Animals were housed under conventional conditions at the animal facility of Kochi Medical School in filter-topped macrolon cages with a bedding of wood chips, at a temperature of 23°C, 50–60% relative humidity, and a 12-h light/dark cycle. They received standard lab chow ad libitum. Animals were kept under conventional conditions until they were 7 weeks old (~ 20-24 g body wt) at the time of sensitization. They were divided into six groups of five mice each according to allergen exposure and treatment, **DD**; DLSE /*Dermatophagoides farinae*plus *(Der f)*+ DEP, **DP**; DLSE /*Der f* + phosphate buffered saline (PBS), **TD**; Tap water/*Der f* + DEP, **TP**; Tap water/ *Der f* + PBS and **Nor**; normal control. The Nor group received only Tap water and exposed to PBS during this experiment. All research and experimental protocol adhered to the animal facility guidelines of Kochi Medical School (A000586).

2.5. Allergen and AHR Induction

Allergen-exposed mice were actively challenged with an intratracheal instillation of 4 μg of *Der f* + 62.5 mg DEP solution on days 13-14-20-21-27-28-34-35, for a total of eight times as shown on the experimental protocol (Figure 1). Measurement of AHR to intravenous acetylcholine (ACh) was performed as previously described [21]. Briefly, to measure AHR, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of ACh. The mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration and then ventilated with a rodent ventilator (New England Medical Instruments, Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. Changes in respiratory overflow volume were measured using an increasing dose of ACh. The increase in respiratory overflow volume induced by ACh was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. This experiment was performed with two independent experiments and, given the fact that results were similar, the data from the second experiment was included in the present paper.

The area under the curve (AUC) calculated from dose–response curves for ACh was used to express the magnitude of AHR. Briefly, AHR chart was saved as bmp format file; then AUC was

selected and calculated using Image J software 1.44p (National Institutes of Health, USA) with each value of doses converted logarithmically and represented as arbitrary units.
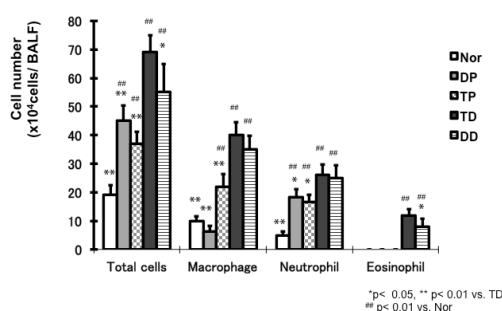

Figure3. *DLSE reduces BALF leukocytes. The figure shows that the number of Total cells (A), Macrophages (B), Neutrophils (C) and Eosinophils (D) in BALF were significantly higher in the TD group as compared with the Nor group ($p< 0.01$, $p< 0.01$, $p<0.01$, $p<0.01$, respectively). However, in the DD group, number of eosinophils in BALF were low as compare with the TD group ($p<0.05$). Mice were intraperitoneally administered with 500 mg/kg of pentobarbital solution in 1.5 ml of 0.9% saline solution, and 80% (1.2 ml) of the solution were recovered. The number of total cells, macrophages, neutrophils and eosinophils were measured. Results are mean $\pm$ SD of data from 6 mice in each group. [##] $p< 0.01$ vs. Nor group; *p <0.05, ** p< 0.01 vs. TD group.*

2.6. Collection of Blood Samples, Measurement of Total IgE, Allergen-Specific IgG1

Blood samples were drawn from which mice allergen exposed mice on day 36 in order to determine the level of serum IgE, allergen-specific IgG_1. Samples were kept at $-80°C$ in the freezer until analyses were performed with the use of specific ELISA kits for mice (mouse IgE kit from Morinaga Co. Ltd., Yokohama, Japan). The mouse IgG_1 kit was prepared by our laboratory. Briefly, serum IgG_1 was bound with coated *Der f* antigen, and then it was detected with horse radish peroxidase conjugated antibody. Animals were sacrificed using a high dose of pentobarbital on day 36.

2.7. Bronchoalveolar Lavage (BAL), Cells Count and Cytokine Assays

To perform the bronchoalveolar lavage (BAL) on day 36, animals were intraperitoneally administered 500 mg/kg of pentobarbital solution. BAL was performed with the use of 1.5 ml of saline solution and 80% (1.2 ml) of the 0.9% saline solution were recovered. The remaining sample was centrifuged, and the supernatant was stored at $-70°C$ until cytokine assays were performed. The pellet was re suspended in PBS, and a cytospin preparation in BAL fluid (BALF) cells was stained with Diff-Quik (International reagents Corp., Kobe, Japan). Number of inflammatory cells such as eosinophils, neutrophils, lymphocytes and macrophages in the BALF was recorded. Interferon gamma (IFN-□,. IL-5, IL-13, eotaxin, TGF-□$_1$ and MCP-1 levels in BALF were determined by ELISA (R&D Systems, Minneapolis, MN, USA). The lower limits of detection for the cytokines were as follows: IFN-γ (> 2 pg/ml), IL-5 (>5 pg/ml), IL-13 (>1.5 pg/ml), eotaxin (>3 pg/ml), TGF-□$_1$ (>1.7 pg/ml) and MCP-1 (>2 pg/ml).

2.8. Histopathological Analysis of Lung Specimens

On day 36, after sacrificing animals, left lungs were taken then fixed with 10% buffered formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 μm, placed on glass slides, and deparaffinized. For light microscopy and morphometry, the lung sections were stained with hematoxylin and eosin (HE) to assess the eosinophils infiltrate, Periodic Acid-Schiff (PAS) staining to detection of mucin in the lungs. Morphometry was performed by individuals blinded to the protocol design. Cell counts were performed with the computerized image analyzer program (BX50, Olympus, Tokyo, Japan). A minimum of 10 fields throughout the upper and lower right lung tissue were randomly examined for the morphometric analyses.

2.9. Statistical Analyses

Results were represented as the mean ± standard deviation (SD). Statistical comparison among the treatment groups were performed by one-way ANOVA, followed by nonparametric Tukey test, with the use of PASW statistics 17.0 (SPSS Inc., Chicago, Illinois). Results were considered to be statistically significant when p-value was less than 0.05.
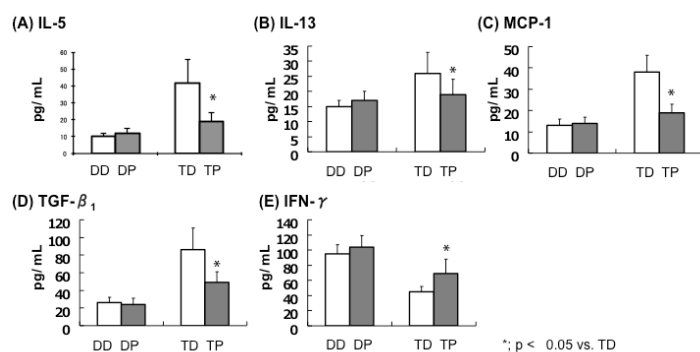
Figure4. *DLSE reduces a Th2 cytokine production in BALF. BALF was collected from BALB/c mice 48 h after the last Der f -challenge as described in Figure 3. Levels of IL-5 (A), IL-13 (B), MCP-1 (C), TGF-β_1 (D) in the BALF were significantly lower in the DD group as compared with the DP group (p< 0.05, p< 0.05, p<0.05, p<0.05, respectively). However, IFN-γ (E) level in the DD group was significantly higher as compared with the DP group (p>0.05). Results are means ± SD (n = 6 per group).*p < 0.05 vs. TD group.*

3. RESULTS

3.1. DLSE Suppress the Immune- Response on Serum Levels of Antigen-Specific IgG$_1$ and IgE

It is well-known that airways exposure to allergens such as *Der f* in sensitive mice species induced an increased level of allergen specific IgG$_1$ in serum. In this experiment, significantly lower levels of *Der f* specific-IgG$_1$ were observed in DD groups as compared with the TD group (p<0.05) (Figure 2A). Significantly lower levels of total IgE were observed in DD groups as compared with the TD group (p<0.05) (Figure 2B).

3.2. DLSE Suppresses the Recruitment of Inflammatory Cells into the Airway

To examine the effect of DLSE on chemotaxis, that is, recruitment of inflammatory cells into the airway, total and differential cell counts were performed in BALF. The TD group showed a significant increase of number of total cells, macrophages, neutrophils and eosinophils as compared with the Nor group (p<0.01, p<0.01, p<0.01, p<0.01, respectively) (Figure 3). However, the DD group significantly reduced the number of eosinophils in BALF as compared with the TD group (p<0.05). The observed reduction in eosinophils chemotaxis into the airway was well-correlated with the histological changes of lung parenchyma. No eosinophils were observed in the BALF of the groups without DEP.
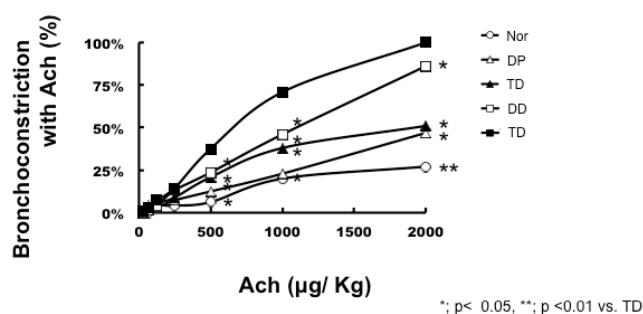

Figure5. *Treatment of DLSE attenuates AHR. The figure shows that the increase in AHR, following ACh challenges, was significantly inhibited in DD group as compared with the TD group (p<0.05). Both DEP non-exposed groups (TP, DP) showed lower sensitivity to ACh throughout the experiment. To measure AHR to ACh, changes in respiratory overflow volume were measured using an increasing dose of ACh. The area under the curve (AUC) calculated from dose–response curves for ACh was used to express the magnitude of AHR. Briefly, each dose was converted logarithmically; AUC was calculated and represented as arbitrary units. Nor; open circle, DP; open triangle, TP; solid triangle, DD; open square, TD; solid square. Results are means ± SD (n = 6 per group). *P < 0.05, **P < 0.01, vs. TD group.*

3.3. DLSE Attenuates a T-Helper Type 2 Cytokine Response in Allergen-Sensitized Mice

To examine the effect of DLSE on cytokine production, the level of cytokines were measured in BALF (Figure 4). Cells from TD and DD groups released significantly higher levels of IL-5, IL-13, MCP-1 and TGF-$\square_1$ compared with TP group ($p<0.05$). In contrast, cells from TD and DD groups released significantly lower levels of IFN-$\square$ compared with TP group ($p<0.05$). Cells from DD group released significantly lower levels of IL-5, IL-13, MCP-1 and TGF-$\square_1$ compared with TD group ($p<0.05$), whereas cells from DD group released significantly higher levels of IFN-γ compared with TD group ($p<0.05$).

3.4. DLSE Attenuates AHR

Repeated challenges to mice lung with ACh induced AHR in mice. As shown in Figure 5, the TD group developed AHR in response to inhaled ACh. However, DLSE-treatment reduced this process in the DD group by approximately 76 % ($p < 0.05$). Both DEP non-exposed groups (TP, DP) showed very low sensitivity to ACh throughout the experiment.

3.5. Histological Evaluation of Lung Specimens from DLSE-Treated Mouse and Controls

After sacrificing mice using high dose of pentobarbital, full lung specimens were taken from animals for histological analysis. As shown in Figure 6, sections from the control mice displayed normal structure and no pathologic changes under a light microscope (A). TD group showed markedly perivascular and peribronchial infiltration of eosinophils into the lungs of BALB/ c mice (B), a trait of allergic airway inflammation. Such infiltration of inflammatory cells into the airways of the DD group was markedly reduced in mice treated with DLSE (D). To evaluate whether DLSE affects mucin production from bronchial goblet cells, lung specimens were stained with PAS (E-H). There was a marked goblet cells hyperplasia in lung specimens from the TD group (F), while the number of those cells was reduced in the DD group (H). Lung specimens in DP showed similar to TP (data not shown).

4. DISCUSSION

A recent study reported that anti-oxidant components of DLSE, such as $\square$-sitosterol [22], had the potential to inhibit histamine release by mast cells [18]. However, no previous study has reported on possible in vivo effects of DLSE on airways inflammation, our aim in this study was to evaluate its potential therapeutic effects on asthma and to determine possible mechanisms for affecting asthma. Th2 type cells, which can produce several pro-inflammatory cytokines after activation, are activated in greater proportions in asthmatics [23]. Th2 cells play a central role in the pathogenesis of allergic bronchial asthma, as each of their characteristic cytokines like IL-4, IL-5, IL-9 and IL-13 contributes to hallmarks of this disease, including airway eosinophilia, increased mucus production, production of allergen-specific IgE and development of airway hyper-responsiveness [24]. Decreasing allergic airway inflammation by treatments with anti-oxidant agents during sensitization is in agreement with the previous study that removing oxidative stress is protective against excessive Th2 sensitization, as has also been shown in experimental asthma [21, 25-27].

In the present study, we have demonstrated a beneficial effect of DSLE on airway inflammation in a mouse model of experimental asthma. In this model, mice were given repeated instillations of both *Der f* and DEP into the airways, after which these mice showed airway hyperresponsiveness, eosinophilia and remodeling. Treating them during the sensitization phase improved the typical features of allergic asthma, AHR, bronchial eosinophilia, Th2 cytokine production, and titers of allergen specific IgG$_1$ and total IgE. DD mice had significantly decreased AHR compared with TD mice. DD mice also showed decreased levels of *Der f* specific IgG$_1$ and total IgE compared with TD mice. Cytokines in BALF from mice given DLSE had significantly inhibited secretions of IL-5, IL-13, MCP-1 and TGF-β_1 compared with TD mice, while IFN-γ secretion was significantly higher. These results indicated that treatment with DLSE suppressed Der f-DEPs induced Th2 cytokines in mice.

Clinical investigations have shown correlations between the presence of inflammatory cells and histological changes in pulmonary tissues and the severity of airway hyperactivity [28, 29]. The levels of pro-inflammatory cytokines are indicators for the severity of airway inflammation [30]. Recent studies suggest that chemoattractant cytokines may be important for eosinophil influx, as

shown by increased levels of MCP-1, MIP-1α, RANTES and eotaxin in asthma [31]. We demonstrated that administering DLSE during mouse sensitization significantly decreased IL-5 and MCP-1 compared with TD mice.

IL-5 can support the in vitro growth and differentiation of mouse B cells and eosinophils [32] and recruit eosinophils into the lung. MCP-1 is spontaneously produced by eosinophils [33], which stimulates collagen synthesis and production of the pro-fibrotic factor TGF-β by fibroblasts [34]. TGF-$β_1$, which plays a central role in the pathogenesis of a variety of fibrotic disorders, stimulates the production of extracellular matrix proteins and inhibits the formation of extracellular proteases [35]. Our previous study showed that MCP-1 production by eosinophils was enhanced when treated with DEPs and that eosinophil chemotaxis was significantly increased [10]. Therefore, these results suggested that DLSE treatment can attenuate local inflammation by decreasing the levels of IL-5 and MCP-1.

In histological studies, eosinophil infiltrations in the DD mice were decreased compared with TD mice and goblet cells were significantly decreased. Further, edema in mice given DLSE was significantly decreased compared with TD mice. IL-13 is a link to mucus hypersecretion by hyperplasic goblet cells that create airway mucous plugs, especially in the peripheral airways of asthmatics [36] and is also linked to AHR [37]. In this research, the production of IL-13 was significantly lower in DLSE treated mice. Therefore, DLSE treatment attenuated both IL-13 production and AHR in *Der f*-DEPs induced asthmatic mice.
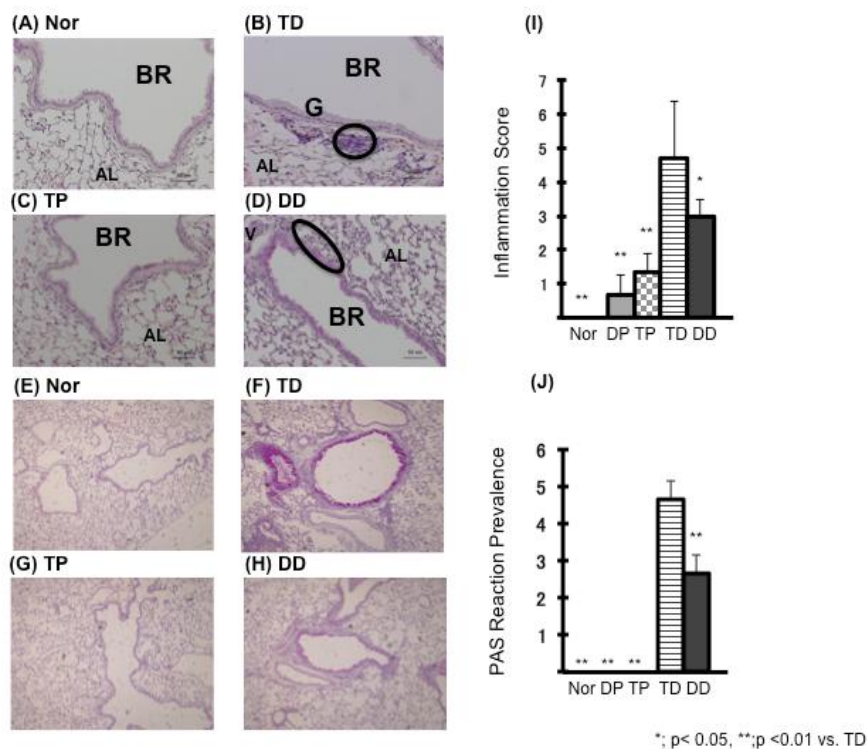

Figure6. *DLSE reduces eosinophilia, the number of PAS positive cells of the lung specimens in Der-f + DEP induced BALB /c mice. The left lungs were removed after the cessation of the final allergen challenge and then fixed with 10% buffered formalin. Tissues were sliced and embedded in paraffin, and 5-μm sections were stained with HE (A-D) and PAS (E-H). Sections from the control mice displayed normal structure and no pathologic changes under a light microscope (A). Der f-sensitization and challenge induced a clear and marked perivascular and peribronchial infiltration of eosinophils into the lungs of BALB/ c mice (B), a trait of allergic airway inflammation. Such infiltration of inflammatory cells into the airways of the DD group was markedly reduced in mice treated with DLSE (D). To evaluate whether DLSE affects mucin production from bronchial goblet cells, lung specimens were stained with PAS (E-H). There was a marked goblet cells hyperplasia in lung specimens from the TD group (F), while the number of those cells was reduced in the DD group (H). Representative photomicrographs in a 10 times magnification (inset) of HE (histology) (A-D), PAS (mucin) (E-H). The black circle indicates representative eosinophils in the infiltrate. *p <0.05, ** p< 0.01 vs. TD group, bars = 50 μm. Abbreviations: AL, alveolus; BR, bronchiole; V, blood vessel; G, goblet cell hyperplasia.*

5. CONCLUSIONS

In order to evaluate the preventive effects of DLSE on asthmatic inflammation in *Der f* + DEP-induced mice, serum *Der f* -specific IgG_1 titers, cytokine profiles in BALF and AHR were analyzed. We found that DLSE suppressed *Der f*-specific IgG_1 in serum and attenuated AHR in *Der f* + DEP -sensitized mice. BALF from mice given DLSE had significantly decreased secretions of IL-5, IL-13, TGF-β_1 and MCP-1 and up-regulated IFN-γ secretion. Therefore, DLSE treatment attenuated Th2 cytokines production and AHR in *Der f* + DEP -induced asthmatic mice. Our results strongly suggest that DLSE may have significant therapeutic benefits for the control of allergic airway disorders.

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