

Grape Seed Proanthocyanidin Extract Attenuates Allergic Inflammation in Murine Models of Asthma

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Abstract

Background Antioxidants have been suggested to alleviate the pathophysiological features of asthma, and grape seed proanthocyanidin extract (GSPE) has been reported to have powerful antioxidant activity.

Purpose This study was performed to determine whether GSPE has a therapeutic effect on allergic airway inflammation in both acute and chronic murine model of asthma.

Methods Acute asthma model was generated by intraperitoneal sensitization of ovalbumin (OVA) with alum followed by aerosolized OVA challenges, whereas chronic asthma model was induced by repeated intranasal challenges of OVA with fungal protease twice a week for 8 weeks. GSPE was administered by either intraperitoneal injection or oral gavage before OVA challenges. Airway hyperresponsiveness (AHR) was measured, and airway inflammation was evaluated by bronchoalveolar lavage (BAL) fluid analysis and histopathological examination of lung tissue. Lung tissue levels of various cytokines, chemokines, and growth factors were analyzed by quantitative polymerase chain

reaction and ELISA. Glutathione assay was done to measure oxidative burden in lung tissue.

Results Compared to untreated asthmatic mice, mice treated with GSPE showed significantly reduced AHR, decreased inflammatory cells in the BAL fluid, reduced lung inflammation, and decreased IL-4, IL-5, IL-13, and eotaxin-1 expression in both acute and chronic asthma models. Moreover, airway subepithelial fibrosis was reduced in the lung tissue of GSPE-treated chronic asthmatic mice compared to untreated asthmatic mice. Reduced to oxidized glutathione (GSH/GSSG) ratio was increased after GSPE treatment in acute asthmatic lung tissue.

Conclusion GSPE effectively suppressed inflammation in both acute and chronic mouse models of asthma, suggesting a potential role of GSPE as a therapeutic agent for asthma.

Keywords Proanthocyanidins · grape seed extract · asthma · antioxidants · airway remodeling

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Introduction

Asthma, characterized by chronic airway inflammation and airway hyperresponsiveness (AHR) [1, 2], is a prime example of a multifactorial disease. Various factors, including genetic and environmental factors, are known to be involved in its development. Despite the continued debate about whether the enhanced oxidative stress observed in asthmatic patients is a simple consequence of inflammation or a causative factor of the disease, many recent reports have supported the critical role of oxidative stress in the pathogenesis of asthma.

Although the determination of the precise underlying mechanism of oxidative stress in the pathogenesis of asthma needs to be addressed, enhanced oxidative stress is known to contribute to the progression of existing airway inflammation

through enhancement of AHR, stimulation of mucin secretion, and induction of various pro-inflammatory chemical mediators [3–7]. Furthermore, factors such as steroid insensitivity, persistence of inflammation, and development of airway remodeling causing decreased lung function are all related to severe asthma and closely linked to enhanced oxidative stress [8–10].

In clinical practice, the emphasis in asthma treatment is usually placed on anti-inflammatory therapies, with glucocorticoids remaining the first choice of treatment because the early use of inhaled corticosteroids is remarkably effective in attenuating airway inflammation and thus managing asthma symptoms [1, 2]. However, there is concern about the life-long use of corticosteroids in the management of asthma. Furthermore, the symptoms of some asthmatic patients, especially those with severe asthma, are not well controlled by inhaled corticosteroid therapy [11, 12]. Recently, there has been a substantial increase in the number of severe asthmatic patients who need to be treated. Thus, a novel alternative drug is urgently required for the better management of asthma.

The previous human studies that have examined the therapeutic effect of various antioxidants, such as vitamin C, vitamin E, flavonoids, and selenium on asthma have not been impressive [13–18]. The disappointing efficacy of antioxidants as anti-asthmatic drugs may be due to relatively weak antioxidant capacity of the antioxidant used, inability to induce glutathione synthesis, lack of intrinsic anti-inflammatory effects, and inter-individual variability in terms of the susceptibility to oxidative stress. These limitations may be overcome by searching for new antioxidants that have strong antioxidant capacity and the development of novel antioxidant agents that also target a critical process of the underlying functional mechanism of the oxidative stress in asthma.

Proanthocyanidins are the major phenolic compounds in grape seed and skin of grape which possesses strong antioxidative capacity. Grape seed proanthocyanidin extract (GSPE) is known to have greater antioxidant activity than several well-known antioxidants, including vitamin C, vitamin E, and gallic acid [19]. GSPE has been shown in animal models to prevent development of many diseases such as diabetic nephropathy, drug-induced renal toxicity, cancer metastasis, fungal infection, and ischemic cardiomyopathy [20–24]. Moreover, GSPE was shown to attenuate inflammation in collagen-induced arthritis in mice suggesting its therapeutic role in inflammatory disorders [25]. GSPE is currently used in the treatment of human veno-lymphatic diseases [26]. Many of these effects are believed to be the result of strong free radical scavenging activity of GSPE [27–30]. A recent study has evaluated the effect of GSPE on the airway eosinophilic inflammation of asthma in mice [31]. However, its precise role in chronic asthma and therapeutic potential has not been thoroughly investigated.

In the current study, we used both acute and chronic murine model of asthma to evaluate the inhibitory effects and therapeutic role of GSPE on airway inflammation, AHR, and airway remodeling.

Materials and Methods

Animal Models

In this study, both acute and chronic murine model of asthma were used. Six- to eight-week-old female BALB/c mice were maintained in a specific pathogen-free area and administered OVA-free feed and water. To generate a conventional acute mouse model of asthma that is characterized by typical pathobiology of allergic asthma, including prominent eosinophilic airway inflammation and AHR, each mouse was immunized via intraperitoneal (IP) injection of 20 µg chicken OVA (Grade V; Sigma Chemical Co., St Louis, MO, USA) with 100 µL alum (Imject, Thermo Scientific, Waltham, MA, USA) on days 0 and 14. The immunized mice underwent an aerosolized antigen challenge of 1 % OVA (wt/vol in PBS) on days 21, 22, and 23 for 30 min each day. GSPE (Entelon®; produced by Hanlim Pharmaceuticals, Seoul, Korea) was administered via intraperitoneal injection (50 and 100 mg/kg) 30 min before the OVA challenge. Phosphate-buffered saline (PBS) was intraperitoneally injected into control mice on days 0 and 14, and these injections were followed by 3 consecutive day challenges of inhaled PBS on days 21, 22, and 23.

Chronic asthma model, which possibly represents chronic airway inflammation and airway remodeling, was generated by intranasally (IN) challenging mice with 20 µg chicken OVA (Grade V; Sigma) mixed with 5 µg fungal protease (protease from *Aspergillus melleus*, Sigma) twice a week for 8 weeks [32]. In control mice, PBS was administered IN twice a week. GSPE was administered by oral gavage (50 and 100 mg/kg) 30 min before each IN challenge.

In some experiments, to show therapeutic effect of GSPE in established asthmatic mice, we modified aforementioned acute asthma model. In brief, asthmatic mice were first made by challenging with OVA and alum just as described above but without GSPE. Then, AHR was measured on day 24 to confirm the asthmatic feature, and finally treated with GSPE before repeated antigen challenges on days 28, 29, and 30. Therapeutic effects were evaluated on day 31.

Animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Medical Center, Korea.

Determination of Airway Hyperresponsiveness

In both models, 24 h after the final inhalational challenge of OVA, AHR was assessed in conscious and unrestrained mice by means of whole-body plethysmography with an

OCP-3000 (Allmedicus, Seoul, Korea). Each mouse was placed in a plastic chamber and exposed to increasing concentrations of aerosolized methacholine (Sigma-Aldrich) for 3 min. Beginning 10 s after each aerosol exposure, enhanced pause (Penh) readings, which were calculated automatically based on the mean pressure generated in the plethysmography chamber during inspiration and expiration combined with the time of each phase, were recorded for 3 min.

Analysis of the Bronchoalveolar Lavage Fluid

After measurement of the AHR, mice were anesthetized, the trachea was cannulated, and bronchoalveolar lavage (BAL) was performed with 2 mL PBS. The recovered fluid was centrifuged to separate the cells from the supernatant. The BAL cell pellet was resuspended in PBS, and the total number of cells was determined. Slides for differential cell counts were prepared with cytopspin and stained with Diff-Quik (Sysmex, Hyogo, Japan). Three hundred cells were counted in each sample for the differential cell counts.

Histopathologic Examination

For the histopathologic assessment, lungs were fixed overnight in 10 % neutral-buffered formalin, embedded in paraffin, sectioned into 4- μ m-thick sections, and stained with hematoxylin and eosin. The degree of lung inflammation was evaluated by a previously described method [33]. Briefly, the degree of inflammation in different pulmonary regions, including the alveolar septal, perivascular, and peribronchial areas, was graded on a numeric scale of 1 to 4 in terms of severity (1 = minimal, 2 = mild, 3 = moderate, and 4 = marked) by two independent pathologists blinded to the treatments. The overall histopathologic scores were generated from the sum of the individual lesion scores of each mouse. In addition, periodic acid-Schiff and Masson's trichrome staining were performed on sections from mice in the chronic asthma experiment in order to evaluate mucus secretion and lung fibrosis, respectively. The mass of airway smooth muscle was also estimated from the measurement of the area of α -smooth muscle actin (α -SMA) immunoreactivity (anti- α -SMA antibody; Sigma-Aldrich) by using fluorescence microscopy and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Results are expressed as the area of α -SMA staining per μ m length of basement membrane of bronchioles with an internal diameter of 150–200 μ m. At least 10 bronchioles were measured for statistical analysis.

Glutathione Assay

Oxidative stress in lung tissue was evaluated by using a commercially available glutathione assay kit (Cayman Chemical, Ann Arbor, MI). The kit measures GSH by an enzymatic

recycling method using glutathione reductase for the quantification of total tissue glutathione. Fifty mg of lung tissue was homogenized in MES buffer (MES, 0.1 M phosphate, and 2 mM EDTA, pH 6.0) and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was removed and deproteinized for analysis (Centrifugal Filter Units, 10,000 MW Cut-Off, R&D Systems, MN). We tested both total glutathione in lung homogenates after reducing GSSG to GSH as well as GSSG alone according to the manual instructions and calculated the ratio of reduced GSH to oxidized GSSG (GSH/GSSG).

Measurement of Cytokine Production

Total RNA was prepared from lung tissue with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA with the Transcriptor first strand cDNA synthesis kit (Roche, Upper Bavaria, Germany). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was done in triplicate with LightCycler® DNA Master SYBR Green I (Roche) and a Lightcycler LC 480 (Roche). In some of the acute asthma model experiments, semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR) analysis was done with reverse transcriptase (Roche) in an automated thermal cycler (BioRad, Hercules, CA, USA).

The primer sequences used were as follows.

For the sqRT-PCR in the acute asthma experiments: IL4, 5'-GTCATCCTG CTCTTCTTTCTCG-3' and 5'-ATGGCGTCCCTTCTCCTGT-3'; IL5, 5'-ATGTCCTG TAGT CAGTTAAACC-3' and 5'-AAGTTCAGTTACACG GAGAAGT-3'; IL13, 5'-CAGTTCTAC AGCTCCCTGGT-3' and 5'-CAAAGCTGAGGCATCTCCCT-3'; interferon- γ , 5'-GCCATCG GCTGACCTAGAG-3' and 5'-CGAATCAG CAGCGACTCCTT-3'

For the qRT-PCR in the chronic asthma experiments: IL4, 5'-CATCGGCATTTTGAACGAG-3' AND 5'-CGAGCT CACTCTCT GTGGTG-3'; IL5, 5'-ACA TTGACCGCCAAAAGAG-3' and 5'-CACCATGGAG CAGC TCAG-3'; IL13, 5'-CCTCT GACCCTTAAGGA GCTTAT-3' and 5'-CGTTGCACAGGGGA GTCT-3'; interferon- γ , 5'-ATC TGGAGGAAGTGGCAAAA-3' and 5'-TTCAAGACTTCA AAGAGTCTGAGGTA-3'; eotaxin-1, 5'-AGAGCTCCACAGCGCTTCT-3' and 5'-GCAGGAA GTTGGGATGGAG-3'; TGF- β 1, 5'-TGGAGCAA CATGTGGAAGTC-3' and 5'-CAGCAGCCGGTTAC CAAG-3'; PDGFB, 5'-CGGCCTGTGACTAGAAGTCC-3' and 5'-GAGCTTGAGGCGTCTTGG-3'; FGF2, 5'-CGG CTCTACTGCAAGAACG-3' and 5'-TGCTTGGAGTTG TAGTTTGACG-3'

Eotaxin-1 protein level was measured in BAL fluid supernatant from chronic asthma model with enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

Detection of OVA-Specific IgE

Serum was separated from the blood clot by centrifugation at 3,000 rpm for 10 min at 4 °C and stored at -70 °C until

assayed. OVA-specific serum IgE were measured as previously described with some modifications [31]. Briefly, 96-well plates were coated with 100 µg OVA diluted in PBS and then washed extensively with PBS containing 0.05 % Tween-20.

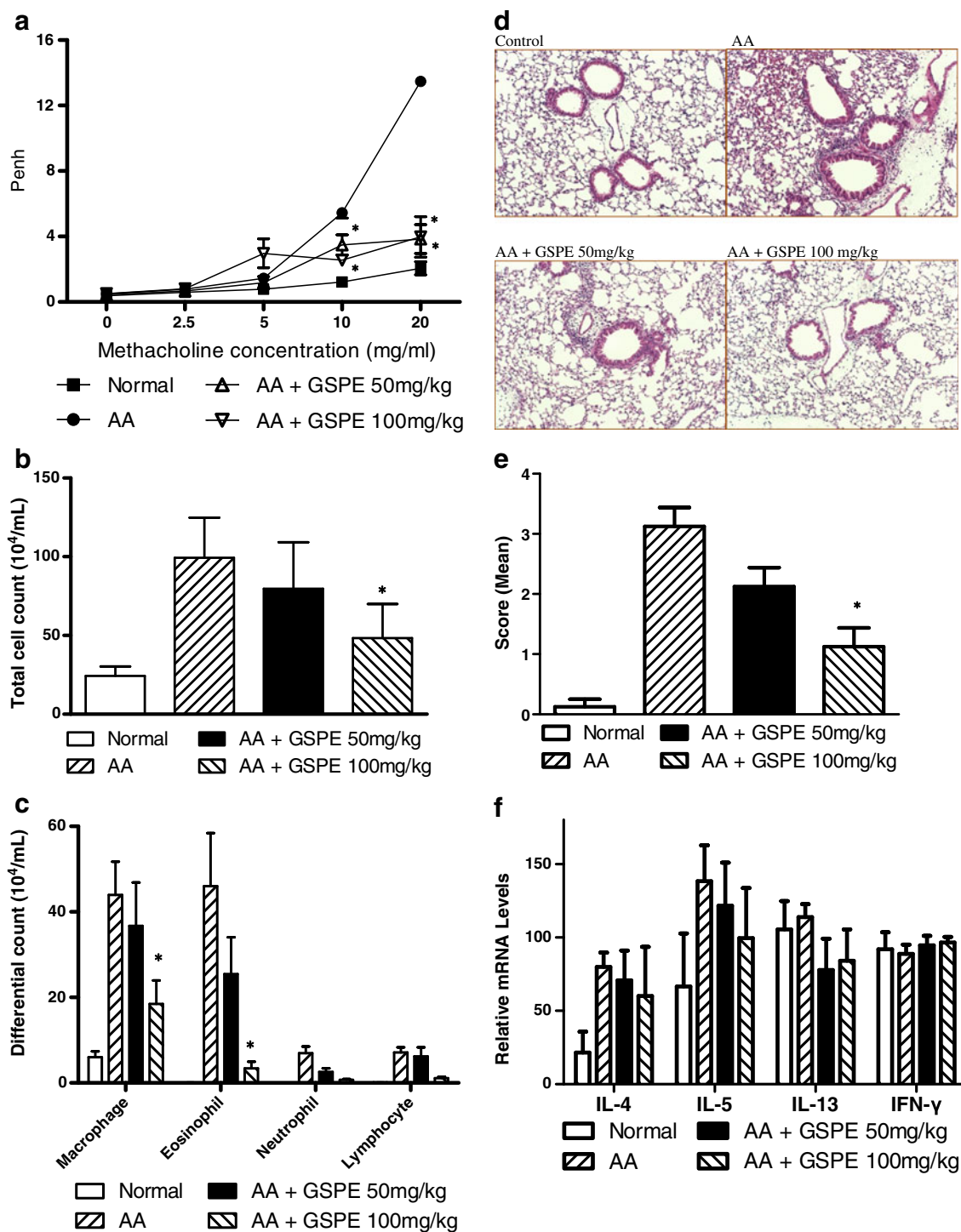


Fig. 1 The effect of GSPE on allergic airway inflammation in an acute asthma model. AA, acute asthma. * $p < 0.05$ compared to the untreated asthmatic group. **a** Airway hyperresponsiveness to inhaled methacholine. **b** Total number of inflammatory cells in bronchoalveolar lavage

fluid. **c** Differential count of cells from bronchoalveolar lavage fluid. **d** Hematoxylin and eosin staining of lung tissue (original magnification, $\times 200$). **e** Degree of lung inflammation. **f** Semiquantitative RT-PCR of lung tissue

Sera (1:50 dilution) and anti-ovalbumin mouse monoclonal antibody (clone: 2C6, Lifespan Bioscience, Seattle, WA, USA), which was used as a standard, were diluted in PBS containing 1 % BSA and 0.05 % Tween 20 and incubated for 2 h at room temperature. This step was followed by incubation with an anti-mouse IgE antibody (Pharmingen, USA, 1:1,000 in PBS containing 1 % BSA and 0.05 % Tween 20). After the addition of HRP-conjugated antibodies (Chemicon, Korea, 1:50,000 in PBS containing 1 % BSA and 0.05 % Tween 20), reactivity was detected with TMB.

Statistical Analysis

Statistical significance was defined as a *p* value of less than 0.05, and all calculations were carried out with SPSS software (version 18; SPSS, Inc., Chicago, IL, USA). All data are expressed as means $\pm$ SEM. For comparison between groups, the Mann-Whitney test was used.

Results

The Effect of GSPE on Allergic Airway Inflammation in an Acute Asthma Model

First, to evaluate the effect of GSPE on AHR, the average Penh values of the groups were compared. The Penh values of mice treated with GSPE were significantly lower than those of untreated asthmatic mice ($p < 0.05$, Fig. 1a), which demonstrated that GSPE could effectively suppress AHR. Next, the anti-inflammatory effect of GSPE on airway inflammation was evaluated in acute asthmatic mice. The examination of the inflammatory cells in the BAL fluid revealed that the administration of 100 mg/kg GSPE induced a remarkable decrease in not only the total cell counts but also in the number of eosinophils compared to those observed in untreated asthmatic mice ($p < 0.05$) (Fig. 1b and c). On histopathological examination, intense inflammation with infiltrating eosinophils and mononuclear cells surrounding the bronchi was evident in the lungs of asthmatic mice, and this was greatly diminished by the administration of GSPE ($p < 0.05$ between the untreated asthma and GSPE 100 mg/kg groups) (Fig. 1d and e). Furthermore, the expression of various cytokines was evaluated in the lungs by sqRT-PCR. IL-4 and IL-5 expression were enhanced in the untreated asthmatic mice but tended to be suppressed by GSPE treatment, whereas IL-13 and IFN- γ expression did not significantly differ between groups (Fig. 1f).

To assess antioxidative effect of GSPE in acute asthma model, glutathione assay was done to measure GSH/GSSG ratio. Asthmatic lung tissues showed decreased GSH/GSSG ratio in relation to controls (Fig. 2). However, GSPE treatment restored GSH/GSSG ratio in asthmatic mice toward normal.

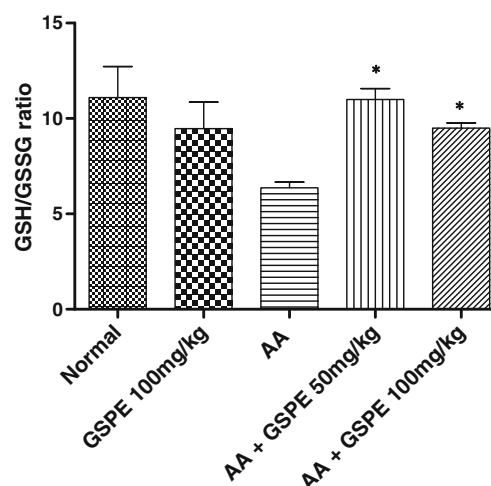


Fig. 2 Glutathione assay of lung tissue. Ratio of reduced GSH to oxidized GSSG measured from lung tissue homogenates are shown

The Effect of GSPE on Airway Inflammation and Remodeling in a Chronic Asthma Model

As with the acute asthma model, GSPE treatment was able to reduce AHR to inhaled methacholine in the chronic asthmatic mice (both at 50 and 100 mg/kg, $p < 0.05$ compared to untreated asthmatic mice, Fig. 3a). In the analysis of the BAL fluid, the chronic asthmatic mice showed significantly increased total cells and eosinophil counts compared to the untreated asthmatic mice. Treatment with 100 mg/kg GSPE led to significant decreases in total cells and eosinophils in the BAL fluid of chronic asthmatic mice ($p < 0.05$, Fig. 3b and c). Histopathological examination of lung tissue stained with hematoxylin and eosin revealed significantly less inflammation in the GSPE-treated asthmatic mice compared to the untreated asthmatic mice (Fig. 3d and e). Furthermore, there was a significant decrease in OVA-specific IgE in GSPE-treated mice (both 50 and 100 mg/kg) compared to untreated asthmatic mice ($p < 0.05$) (Fig. 3f).

In the chronic model, the effect of GSPE on airway remodeling was investigated by assessing the magnitude of subepithelial fibrosis and mucin hypersecretion. Airway fibrosis was significantly ameliorated by the administration of GSPE, as GSPE-treated mice showed significantly less fibrosis than untreated asthmatic mice ($p < 0.05$, Fig. 4a and b). Immunofluorescence staining of peribronchial α -SMA showed that the area of α -SMA expressing cells were increased in chronic asthma model but decreased with GSPE treatment (Fig. 4c and d). However, PAS staining showed no difference in the hyperplasia of airway mucin secretory cells between the GSPE-treated mice and the untreated asthmatic mice (Fig. 4e and f).

In the cytokine analysis, the results of the qRT-PCR analysis of the lungs showed that the administration of GSPE led

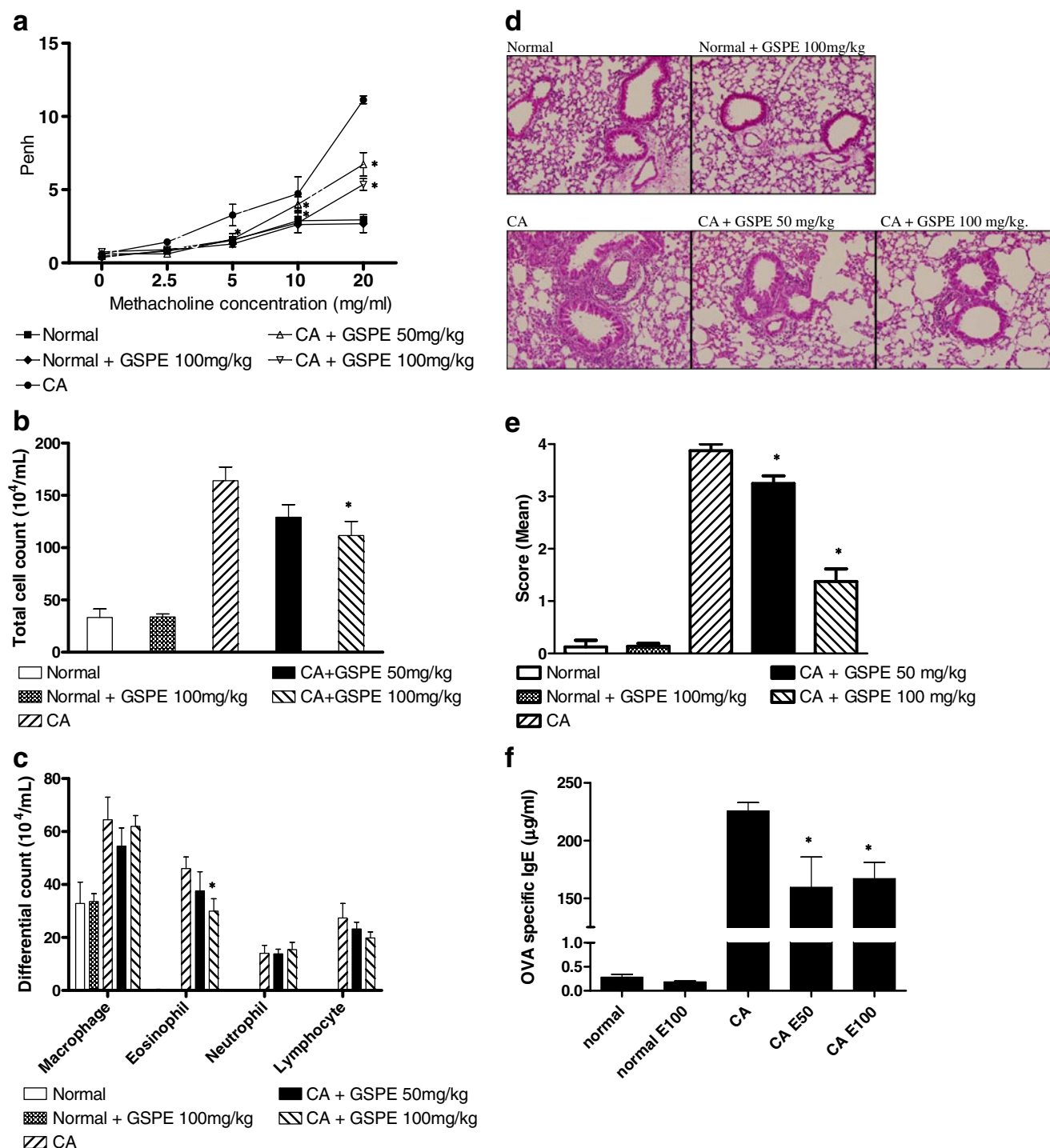


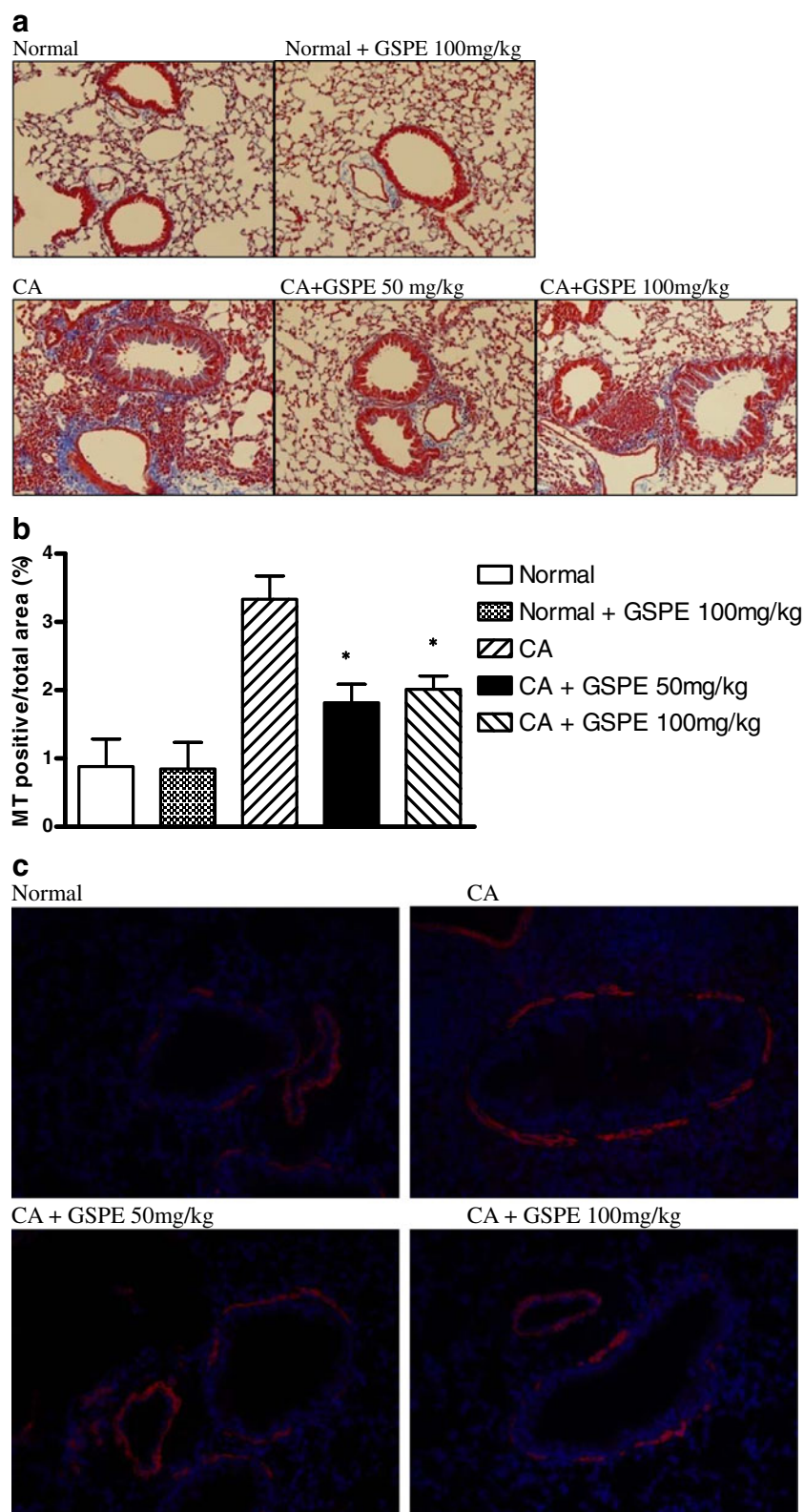
Fig. 3 The role of GSPE in a chronic asthma model. CA, chronic asthma. * $p < 0.05$ compared to the untreated asthmatic group. **a** Airway hyperresponsiveness to inhaled methacholine. **b** Total number of inflammatory cells in bronchoalveolar lavage fluid. **c** Differential count

of cells from bronchoalveolar lavage fluid. **d** Hematoxylin and eosin staining of lung tissue (original magnification, $\times 200$). **e** Degree of lung inflammation. **f** Serum OVA-specific IgE

to marked reductions in IL-5 and IL-13, and a marked increase in interferon- γ in chronic asthmatic mice, whereas it had no effect on the level of IL-4 (Fig. 5a–d). In addition, both qRT-PCR and ELISA demonstrated a marked reduction in the expression of eotaxin-1 in the GSPE-treated asthmatic mice

compared to the untreated asthmatic mice ($p < 0.05$, Fig. 6a and b). Various growth factors associated with airway remodeling, including TGF- β , FGF, EGF, and PDGF, were also evaluated in the lungs, but no significant differences were found (data not shown).

Fig. 4 The effect of GSPE on lung fibrosis and mucin production in a murine model of chronic asthma. CA, chronic asthma. $*p<0.05$ compared with untreated asthmatic group. **a** Masson's trichrome staining (lung tissue, original magnification, $\times 200$). **b** Degree of fibrosis measured from Masson's trichrome staining. **c** α -Smooth muscle actin staining (lung tissue, fluorescence microscopy). **d** Calculated area of α -smooth muscle actin staining per μm length of basement membrane of bronchioles. **e** PAS staining (lung tissue, original magnification, $\times 400$). **f** Degree of mucin production measured from PAS staining

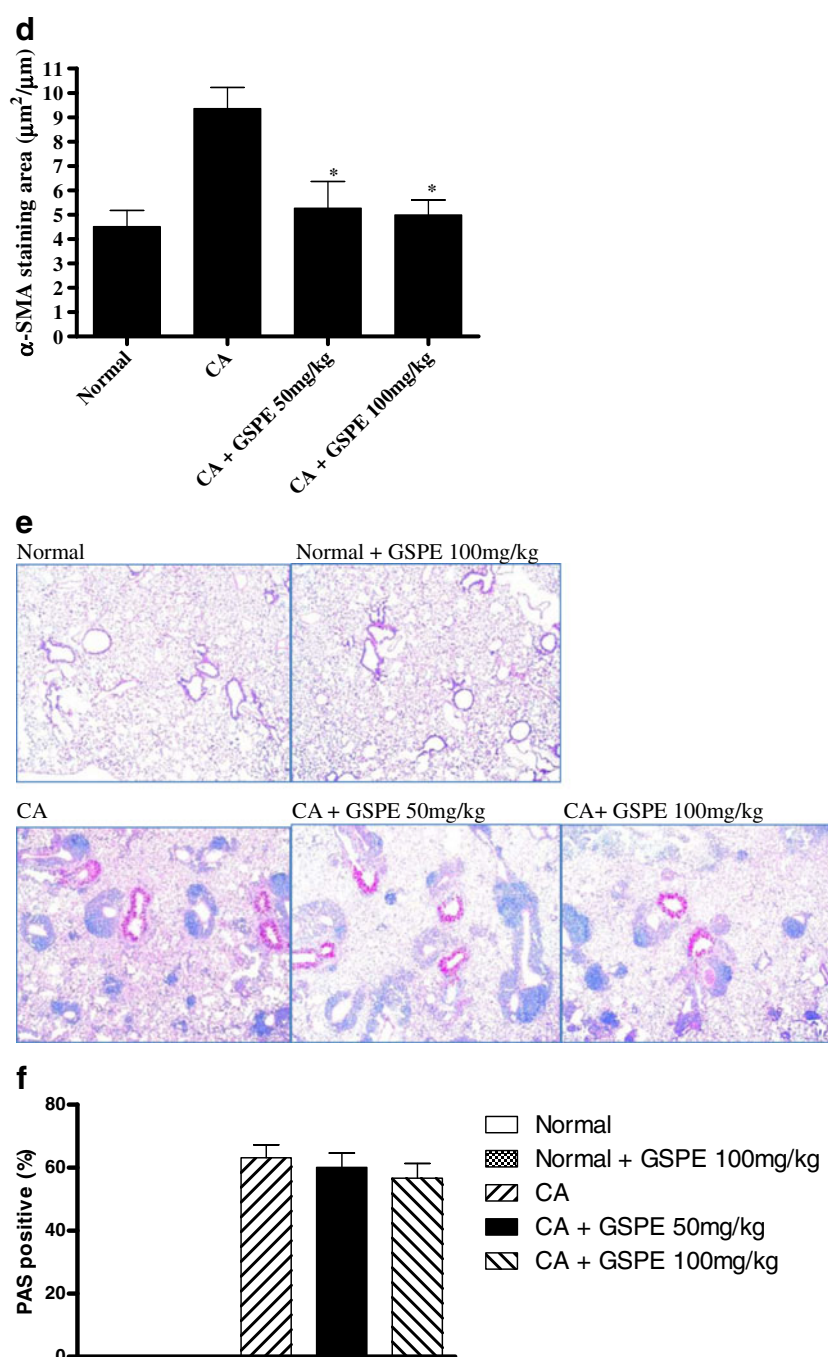


The Therapeutic Effect of GSPE in Established Asthmatic Mice

The therapeutic effect of GSPE in established asthmatic mice with acute inflammation and airway resistance was

evaluated. At day 24, acute asthma model was well established in the experimental groups with increased AHR (Fig. 7a). When these asthmatic mice were treated with GSPE with additional OVA challenges, AHR was reduced

Fig. 4 (continued)



significantly compared to untreated asthmatic mice ($p < 0.05$, Fig. 7b). Moreover, total inflammatory cells in the BAL fluid were also reduced with GSPE treatment in the mice with acute asthma ($p < 0.05$, Fig. 7c).

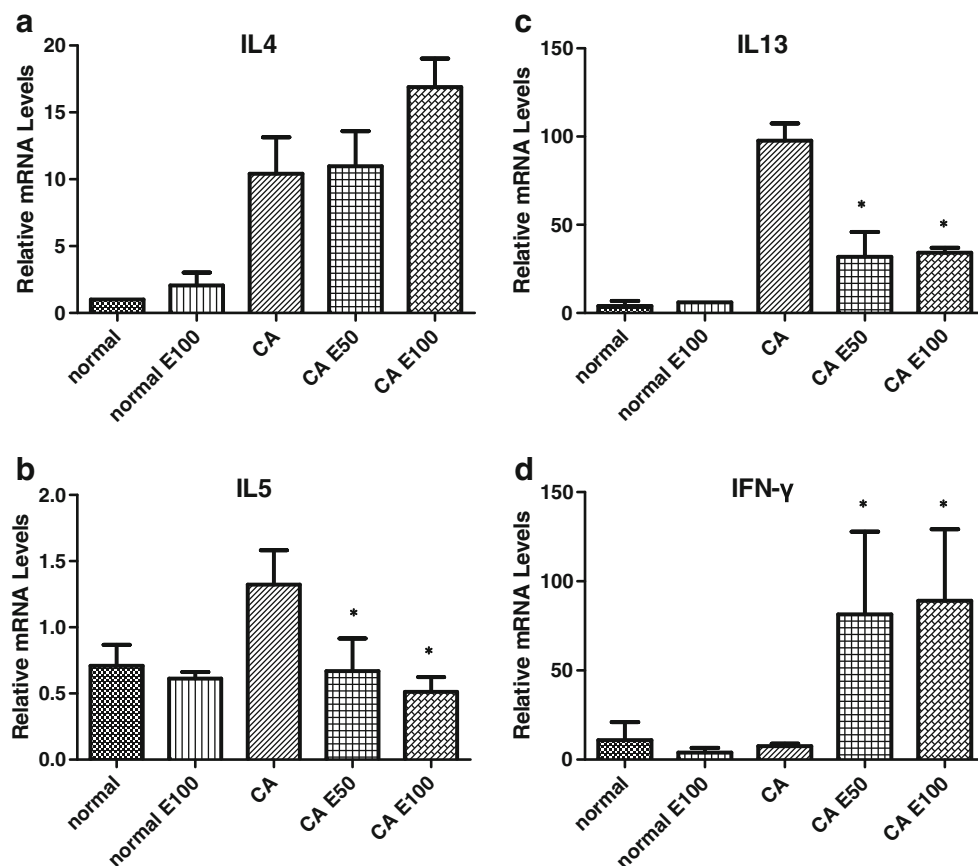
Discussion

In the present study, we evaluated the effects of GSPE on OVA-induced airway inflammation in both acute and chronic asthma model. The results revealed that GSPE treatment

reduced airway inflammation, AHR, and oxidative stress. Furthermore, airway fibrosis was also attenuated in the chronic asthma model. These results suggest that GSPE may be effective in both reducing the inflammation and alleviating the airway remodeling in asthma and could be a useful adjuvant therapy for patients with chronic asthma.

Although a Th2-dominant immune response is believed to be critical for the development of asthma, the precise pathogenesis of asthma is not yet clearly defined. Given that the clinical features and nature of the airway inflammation in asthmatic patients are greatly heterogeneous [34], it

Fig. 5 The effect of GSPE on cytokine production (quantitative RT-PCR in lung tissue) in chronic asthmatic mice. CA, chronic asthma. E50, GSPE 50 mg/kg. E100, GSPE 100 mg/kg. * $p < 0.05$ compared with untreated asthmatic group. **a** IL-4. **b** IL-5. **c** IL-13. **d** IFN- γ



is conceivable that diverse underlying mechanisms are involved in the pathogenesis of asthma. Oxidative stress is one of the important factors suggested to play a role in the pathogenesis of asthma. In fact, plenty of recent studies have supported the role of oxidative stress in asthma. Epidemiological studies revealed a higher incidence of bronchial asthma in areas with greater air pollution, a known exogenous oxidant [35, 36]. The reduced intake of foods containing antioxidants has also been reported to be associated with increased incidence of asthma [37, 38]. Asthmatic subjects show higher levels of oxidative stress in various biological samples, including plasma, blood cells, and BAL fluid [39–44]. In contrast, the concentrations of natural antioxidants, including glutathione peroxidase, superoxide dismutase, glutathione, and vitamins C and E, are reduced in the biological fluids of asthmatic patients [40, 43, 45–50].

Furthermore, enhanced oxidative stress is particularly associated with the development of severe asthma that is not very well controlled by the currently available anti-asthma medications [8, 51]. In fact, air pollution was suggested to not only cause more severe asthma but also more frequent asthma attacks [52], and increased oxidative stress in asthmatic patients has been known to be linked to reduced pulmonary function [53].

On the basis of these facts, it would be quite reasonable to employ a therapeutic strategy for asthma in which

antioxidants are used to control oxidative stress. However, previous human studies examining the effects of antioxidants on asthma have shown only modest effects at best [13–18]. Although various mechanisms have been proposed to explain this lack of antioxidant effectiveness, these shortcomings may be overcome by the discovery of novel drugs with stronger antioxidant capacity and different mechanisms of function.

In this study, GSPE was employed as a novel antioxidant, and its anti-asthmatic effect was evaluated. GSPE has been reported to have greater antioxidant activity than well-known antioxidants and the antioxidant properties of proanthocyanidins stem from several antioxidant mechanisms, such as radical scavenging, singlet oxygen quenching, chelating, and the inhibition of oxidases [19]. Furthermore, proanthocyanidins has been reported to have various biological functions, including antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilatory actions [27], many of which have been attributed to the antioxidant activity [27–30]. All of these facts indicate that GSPE could provide great benefit as an antioxidant treatment for asthma.

Glutathione (GSH) is one of the most abundant non-protein thiol that buffers reactive oxygen species (ROS) in the human cells. It eliminates H_2O_2 and organic peroxides by glutathione peroxidase (GPx) [54]. During detoxification, ROS are reduced by GPx at the expense of GSH to form

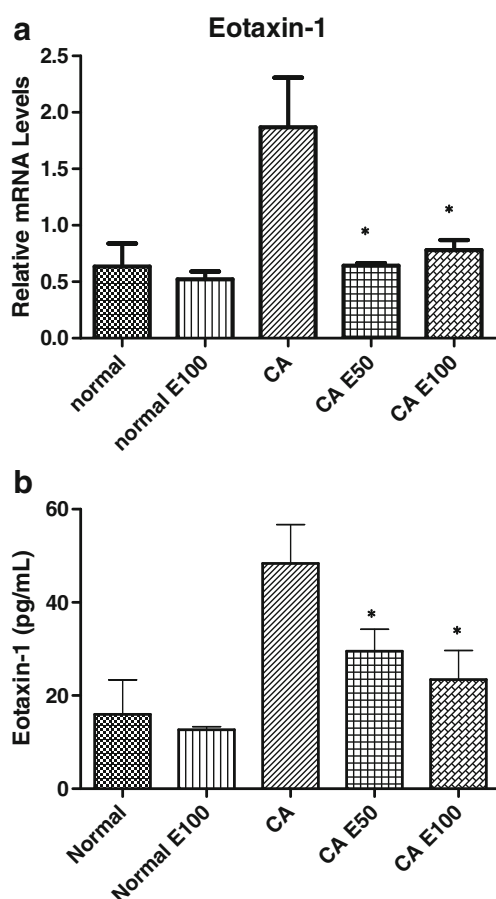


Fig. 6 The effect of GSPE on eotaxin-1 production in chronic asthmatic mice. CA, chronic asthma. E50, GSPE 50 mg/kg. E100, GSPE 100 mg/kg. * $p < 0.05$ compared with untreated asthmatic group. **a** Quantitative RT-PCR of eotaxin-1 in lung tissue. **b** ELISA of eotaxin-1 in BAL fluid supernatant

glutathione disulfide (GSSG). GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by glutathione reductase with a consumption of one NADPH. The ratio of GSH and GSSG has been widely-used as an index of intracellular oxidative stress. In this study, asthmatic lung tissues were characterized by the decrease in GSH/GSSG ratio (Fig. 2) in relation to controls, indicating increased ROS in asthma model. GSPE treatment restored GSH/GSSG ratio in asthmatic mice lung tissue toward normal, demonstrating reduction in ROS. These results suggests that GSPE could have been both anti-inflammatory and anti-fibrotic in murine asthma model due to its antioxidant properties.

There have been several studies supporting GSPE's effectiveness against diseases, such as coronary artery disease, hepatic diseases, infectious diseases, neurological diseases, rheumatoid arthritis, and malignant diseases, in animal models [25, 29, 30]. Several functions of GSPE, including its cholesterol-lowering effects, nitric oxide-synthesizing effects, pro-apoptotic functions, and antiproliferative effects, have been suggested to be the underlying biological mechanisms

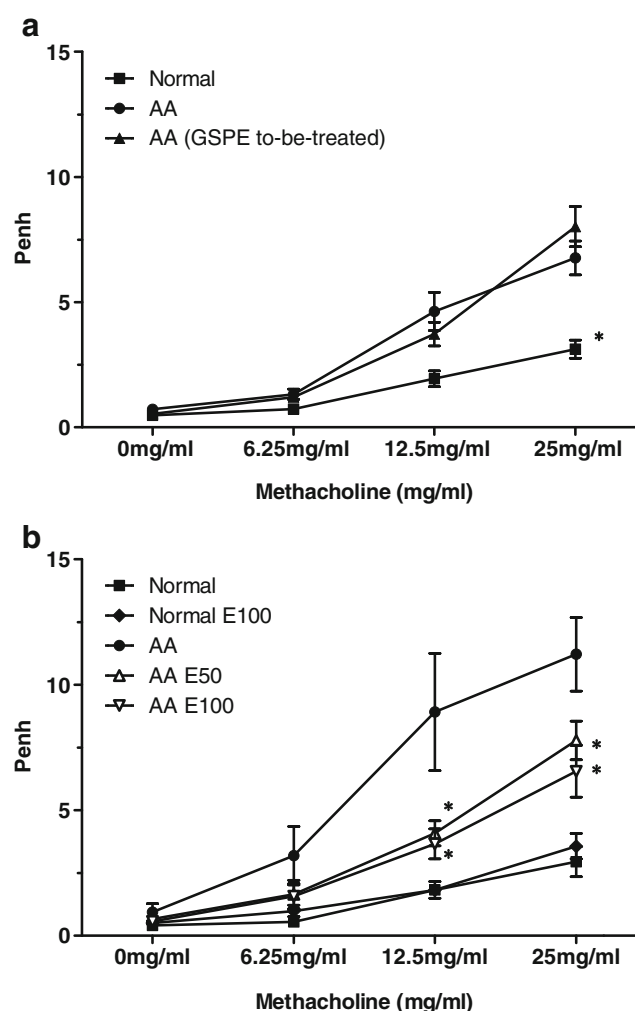


Fig. 7 The therapeutic role of GSPE in established asthmatic mice. AA, acute asthma. E50, GSPE 50 mg/kg. E100, GSPE 100 mg/kg. * $p < 0.05$ compared with untreated asthmatic group. **a** Airway hyperresponsiveness at day 24 after OVA challenge but before GSPE treatment. **b** Airway hyperresponsiveness at day 31 after additional OVA challenge and concomitant GSPE treatment in established asthmatic mice. **c** Total number of inflammatory cells in bronchoalveolar lavage fluid at day 31 after additional OVA challenge and concomitant GSPE treatment in established asthmatic mice

for the effectiveness of GSPE in ameliorating these diseases [30]. Although the fundamental mechanism of the effects of GSPE has not been clearly identified, the reduction of oxidative stress has been suggested to be a critical mechanism [29].

The effectiveness of GSPE as a therapeutic agent has not been thoroughly investigated with regard to chronic airway inflammatory diseases such as asthma, although a recent report demonstrated the beneficial effects of GSPE in preventing acute allergic airway inflammation in an acute asthma model [31]. In our study, we have additionally shown the therapeutic effect of GSPE in already established acute asthmatic mice. Moreover, we evaluated the role of GSPE not only in acute asthma model but also in chronic asthma model. The chronic asthma model used in this study is

considered to be more clinically relevant, because unlike the acute model, the route of allergen sensitization is natural and features of human chronic asthma with airway remodeling such as subepithelial fibrosis are present [32]. Thus, the evaluation of possible therapeutic effects of novel drugs might be clinically more valid when executed in the chronic model employed in this study.

Consistent with the results of a previous study [31], we observed significant amelioration of airway inflammation and AHR in the groups given GSPE in the acute asthma model experiment. In the chronic asthma model utilized in our study, GSPE treatment also attenuated airway inflammation. In both asthma models used in this study, inflammatory cell, particularly eosinophils, infiltration was reduced by GSPE. This reduction in eosinophilic infiltration could be closely linked to the decreased expression of crucial chemical mediators for eosinophilic inflammation such as IL-5, IL-13, and eotaxin, all of which were clearly diminished in the lungs of the chronic asthmatic mice treated with GSPE. There was also a tendency for the expression of these cytokines to be decreased by GSPE in the acute asthma model, but there were no significant differences. AHR was also found to be decreased by GSPE treatment, and this too may be attributed to the reduction of these chemical mediators as IL-13 and eosinophils which are well known to play important roles in the development of AHR [55]. In addition, OVA-specific IgE production was inhibited by GSPE, which may be particularly associated with the reduced IL-4 expression. Accordingly, the results of the current study strongly indicate that the amelioration of all the characteristic features of asthma by GSPE could be critically associated with a suppressed Th2 immune response, which may be induced by a GSPE-mediated reduction in oxidative stress. The precise underlying mechanism of GSPE's anti-eosinophilic inflammation effects, however, is not defined in this study. Further research is necessary to clarify that mechanism.

Interestingly, our study revealed that GSPE treatment in the chronic asthma model could lead to a significant improvement in the peribronchial fibrosis, one of the most important components of airway remodeling. To evaluate the mechanism of the anti-fibrotic function of GSPE, the expression of several growth factors known to be involved with tissue remodeling, including FGF, EGF, TGF, and PDGF [56–59], were investigated (data not shown). However, no significant differences were found between GSPE-treated and untreated asthmatic mice in terms of the expression of these growth factors. Although the exact mechanism of how GSPE suppressed airway fibrosis in the current study is not clear, it may be assumed that a reduced level of reactive oxygen species in the airway tissue would directly inhibit the activation of myofibroblasts, leading to decreased extracellular matrix production [60]. Furthermore, epithelial-mesenchymal transition (EMT)

may be inhibited in GSPE-treated airways by decreased oxidative stress, either directly or through a decrease in other chemical mediators associated with the EMT process that was caused by a GSPE-mediated reduction in airway inflammation [61]. Nevertheless, as the precise role of oxidative stress in the development of airway fibrosis has not yet been clarified in asthmatic patients, further investigation is needed.

Airway remodeling in asthmatic patients is critically related with fixed airway obstruction and the progressive reduction of pulmonary function, which is especially important in the development of severe asthma [1, 2, 62]. Considering the increasing burden and significance of severe asthma, a therapeutic strategy to modulate airway remodeling is urgently needed in clinical practice. However, to date, few preventive or therapeutic agents for asthmatic airway remodeling have been reported. Based on the findings of our study, GSPE might be a candidate drug to control airway remodeling. Indeed, GSPE treatment in established asthma decreased AHR as well as lung inflammation (Fig. 7). These results illustrate novel therapeutic potential of GSPE in asthma. Further animal studies are needed to confirm its therapeutic role in chronic asthma and clinical studies are required for human applications.

In terms of clinical applications, GSPE has some advantages as it is currently used in clinical practice for the treatment of human veno-lymphatic disease [26]. Thus, if the clinical efficacy of GSPE for asthmatic patients is proven, GSPE can be immediately employed for the treatment of asthma. However, as many other antioxidants have not shown impressive therapeutic efficacy in the treatment of asthma, well-designed clinical trials with GSPE as a therapeutic agent against asthma should be performed in the near future.

In conclusion, we have shown that GSPE decreases oxidative stress in mice airway and effectively suppresses allergic inflammation and AHR in both acute and chronic murine models of asthma. These results suggest that oxidative stress plays an important role in the asthmatic airway and that GSPE may be useful as an adjunctive therapeutic agent for asthmatic patients.

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References

1. Bateman and Boulet, Global strategy for asthma management and prevention (2010 update), www.ginaasthma.com. 2010.
2. Expert Panel Report 3 (EPR-3). Guidelines for the diagnosis and management of asthma-summary report 2007. *J Allergy Clin Immunol*. 2007;120(5 Suppl):S94–138.

3. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Radic Biol Med*. 1990;9(3):235–43.
4. Bowler RP, Crapo JD. Oxidative stress in airways: is there a role for extracellular superoxide dismutase? *Am J Respir Crit Care Med*. 2002;166(12 Pt 2):S38–43.
5. Henderson WR, et al. A small molecule inhibitor of redox-regulated NF-kappa B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. *J Immunol*. 2002;169(9):5294–9.
6. Rahman I. Oxidative stress, chromatin remodeling and gene transcription in inflammation and chronic lung diseases. *J Biochem Mol Biol*. 2003;36(1):95–109.
7. Rahman I, Yang SR, Biswas SK. Current concepts of redox signaling in the lungs. *Antioxid Redox Signal*. 2006;8(3–4):681–9.
8. Fitzpatrick AM, et al. Airway glutathione homeostasis is altered in children with severe asthma: evidence for oxidant stress. *J Allergy Clin Immunol*. 2009;123(1):146–152.e8.
9. Fitzpatrick AM, et al. Levels of nitric oxide oxidation products are increased in the epithelial lining fluid of children with persistent asthma. *J Allergy Clin Immunol*. 2009;124(5):990–6.e1–9.
10. Comhair SA, et al. Correlation of systemic superoxide dismutase deficiency to airflow obstruction in asthma. *Am J Respir Crit Care Med*. 2005;172(3):306–13.
11. Lasmar L, et al. Adherence rate to inhaled corticosteroids and their impact on asthma control. *Allergy*. 2009;64(5):784–9.
12. Rachelefsky G. Inhaled corticosteroids and asthma control in children: assessing impairment and risk. *Pediatrics*. 2009;123(1):353–66.
13. Riedl MA, Nel AE. Importance of oxidative stress in the pathogenesis and treatment of asthma. *Curr Opin Allergy Clin Immunol*. 2008;8(1):49–56.
14. Shaheen SO, et al. Randomised, double blind, placebo-controlled trial of selenium supplementation in adult asthma. *Thorax*. 2007;62(6):483–90.
15. Garcia V, et al. Dietary intake of flavonoids and asthma in adults. *Eur Respir J*. 2005;26(3):449–52.
16. Ram FS, Rowe BH, Kaur B. Vitamin C supplementation for asthma. *Cochrane Database Syst Rev*. 2004;3:CD000993.
17. Pearson PJ, et al. Vitamin E supplements in asthma: a parallel group randomised placebo controlled trial. *Thorax*. 2004;59(8):652–6.
18. Fogarty A, et al. Oral magnesium and vitamin C supplements in asthma: a parallel group randomized placebo-controlled trial. *Clin Exp Allergy*. 2003;33(10):1355–9.
19. Ariga T. The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors*. 2004;21(1–4):197–201.
20. Li BY, et al. Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. *J Cell Biochem*. 2008;104(2):668–79.
21. Gulor A, et al. Proanthocyanidin prevents myocardial ischemic injury in adult rats. *Med Sci Monit*. 2011;17(11):BR326–331.
22. Ulusoy S, et al. Anti-apoptotic and anti-oxidant effects of grape seed proanthocyanidin extract in preventing cyclosporine A-induced nephropathy. *Nephrol (Carlton)*. 2012;17(4):372–9.
23. Mantena SK, Baliga MS, Katiyar SK. Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis*. 2006;27(8):1682–91.
24. Han Y. Synergic effect of grape seed extract with amphotericin B against disseminated candidiasis due to *Candida albicans*. *Phytomedicine*. 2007;14(11):733–8.
25. Cho ML, et al. Grape seed proanthocyanidin extract (GSPE) attenuates collagen-induced arthritis. *Immunol Lett*. 2009;124(2):102–10.
26. Henriët JP. Veno-lymphatic insufficiency. 4,729 patients undergoing hormonal and procyanidol oligomer therapy. *Phlebologie*. 1993;46(2):313–25.
27. Bagchi D, et al. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicology*. 2000;148(2–3):187–97.
28. Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*. 2000;52(4):673–751.
29. Xia EQ, et al. Biological activities of polyphenols from grapes. *Int J Mol Sci*. 2010;11(2):622–46.
30. Nassiri-Asl M, Hosseinzadeh H. Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive compounds. *Phytother Res*. 2009;23(9):1197–204.
31. Zhou DY, et al. Grape seed proanthocyanidin extract attenuates airway inflammation and hyperresponsiveness in a murine model of asthma by downregulating inducible nitric oxide synthase. *Planta Med*. 2011;77(14):1575–81.
32. Kheradmand F, et al. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J Immunol*. 2002;169(10):5904–11.
33. Barrett EG, et al. Cigarette smoke-induced airway hyperresponsiveness is not dependent on elevated immunoglobulin and eosinophilic inflammation in a mouse model of allergic airway disease. *Am J Respir Crit Care Med*. 2002;165(10):1410–8.
34. Kim HY, DeKruyff RH, Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol*. 2010;11(7):577–84.
35. D'Amato G, et al. Urban air pollution and climate change as environmental risk factors of respiratory allergy: an update. *J Investig Allergol Clin Immunol*. 2010;20(2):95–102. quiz following 102.
36. D'Amato G, et al. Outdoor air pollution, climatic changes and allergic bronchial asthma. *Eur Respir J*. 2002;20(3):763–76.
37. Fogarty A, et al. Dietary vitamin E, IgE concentrations, and atopy. *Lancet*. 2000;356(9241):1573–4.
38. Grievink L, et al. Dietary intake of antioxidant (pro)-vitamins, respiratory symptoms and pulmonary function: the MORGEN study. *Thorax*. 1998;53(3):166–71.
39. Wood LG, et al. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. *Lipids*. 2000;35(9):967–74.
40. Rahman I, et al. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med*. 1996;154(4 Pt 1):1055–60.
41. Vachier I, et al. Increased oxygen species generation in blood monocytes of asthmatic patients. *Am Rev Respir Dis*. 1992;146(5 Pt 1):1161–6.
42. Chanez P, et al. Generation of oxygen free radicals from blood eosinophils from asthma patients after stimulation with PAF or phorbol ester. *Eur Respir J*. 1990;3(9):1002–7.
43. Kelly FJ, et al. Altered lung antioxidant status in patients with mild asthma. *Lancet*. 1999;354(9177):482–3.
44. Kharitonov SA, et al. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet*. 1994;343(8890):133–5.
45. Nadeem A, et al. Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol*. 2003;111(1):72–8.
46. Kongerud J, et al. Ascorbic acid is decreased in induced sputum of mild asthmatics. *Inhal Toxicol*. 2003;15(2):101–9.
47. Hasselmark L, et al. Lowered platelet glutathione peroxidase activity in patients with intrinsic asthma. *Allergy*. 1990;45(7):523–7.
48. Pearson DJ, et al. Selenium status in relation to reduced glutathione peroxidase activity in aspirin-sensitive asthma. *Clin Exp Allergy*. 1991;21(2):203–8.
49. Smith LJ, et al. Reduced superoxide dismutase in lung cells of patients with asthma. *Free Radic Biol Med*. 1997;22(7):1301–7.
50. Picado C, et al. Dietary micronutrients/antioxidants and their relationship with bronchial asthma severity. *Allergy*. 2001;56(1):43–9.
51. Ercan H, et al. Oxidative stress and genetic and epidemiologic determinants of oxidant injury in childhood asthma. *J Allergy Clin Immunol*. 2006;118(5):1097–104.

52. Rusznak C, Devalia JL, Davies RJ. The impact of pollution on allergic disease. *Allergy*. 1994;49(18 Suppl):21–7.
53. Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. *Eur Respir J*. 2000;16(3):534–54.
54. Meister A. Glutathione metabolism and its selective modification. *J Biol Chem*. 1988;263(33):17205–8.
55. Yang G, et al. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine*. 2004;28(6):224–32.
56. Cang CX, Luan B. Expression of basic fibroblast growth factor and nuclear factor-kappaB and the effect of budesonide on their expression in rats with asthma. *Zhongguo Dang Dai Er Ke Za Zhi*. 2009;11(5):393–6.
57. Lewis CC, et al. Airway fibroblasts exhibit a synthetic phenotype in severe asthma. *J Allergy Clin Immunol*. 2005;115(3):534–40.
58. Puddicombe SM, et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J*. 2000;14(10):1362–74.
59. Vignola AM, et al. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med*. 1997;156(2 Pt 1):591–9.
60. Kabuyama Y, et al. Involvement of selenoprotein P in the regulation of redox balance and myofibroblast viability in idiopathic pulmonary fibrosis. *Genes Cells*. 2007;12(11):1235–44.
61. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opin Allergy Clin Immunol*. 2012;12(1):53–9.
62. Lee T, et al. Smoking, longer disease duration and absence of rhinosinusitis are related to fixed airway obstruction in Koreans with severe asthma: findings from the COREA study. *Respir Res*. 2011;12:1.