

Grape Seed Proanthocyanidin Extract Attenuates Airway Inflammation and Hyperresponsiveness in a Murine Model of Asthma by Downregulating Inducible Nitric Oxide Synthase

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Key words

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Abstract

▼ Allergic asthma is characterized by hyperresponsiveness and inflammation of the airway with increased expression of inducible nitric oxide synthase (iNOS) and overproduction of nitric oxide (NO). Grape seed proanthocyanidin extract (GSPE) has been proved to have antioxidant, anti-tumor, anti-inflammatory, and other pharmacological effects. The purpose of this study was to examine the role of GSPE on airway inflammation and hyperresponsiveness in a mouse model of allergic asthma. BALB/c mice, sensitized and challenged with ovalbumin (OVA), were intraperitoneally injected with GSPE. Administration of GSPE remarkably suppressed airway resistance and reduced the total inflammatory cell and eosinophil

counts in BALF. Treatment with GSPE significantly enhanced the interferon (IFN)- γ level and decreased interleukin (IL)-4 and IL-13 levels in BALF and total IgE levels in serum. GSPE also attenuated allergen-induced lung eosinophilic inflammation and mucus-producing goblet cells in the airway. The elevated iNOS expression observed in the OVA mice was significantly inhibited by GSPE. In conclusion, GSPE decreases the progression of airway inflammation and hyperresponsiveness by downregulating the iNOS expression, promising to have a potential in the treatment of allergic asthma.

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

Introduction

▼ Allergic asthma is an airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) [1]. The elevated level of exhaled nitric oxide (eNO) is also a marker of asthmatic airway inflammation [2]. NO is generated via oxidation of L-arginine that is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [3]. iNOS is responsible for prolonged production of larger amounts of NO [4]. NO plays an important role in the pathophysiology of asthma, particularly in the late airway responses. Increased NO level is found to be positively related to asthmatic airway inflammation and hyperresponsiveness [5]. iNOS is also increased in asthmatic tissues, representing airway eosinophilic cells, T helper (Th) 2 cells, and mast cell infiltration [6]. Clinically, emphasis is usually placed on the anti-inflammatory therapies for asthma, with glucocorticoids remaining the first

choice of treatment. Although glucocorticoids are efficacious in the prevention of allergic inflammation, an extended period of inhaling glucocorticoids may pose serious side effects, and no significant curative effect is observed among those with severe asthma or steroidal resistance [7–10]. In addition, years of steroid therapy cannot guarantee that asthmatic patients regain normal bronchial reactivity. It is therefore safe to say that an alternative anti-inflammatory treatment of asthma is badly needed.

Recent years have seen growing interest in the use of naturally available botanicals as chemopreventive or chemotherapeutic agents for some diseases, mostly dietary polyphenolic or flavonoids substances [11]. Grape seed proanthocyanidin extract (GSPE), a natural occurring polyphenol rich extract, is known to exhibit a broad spectrum of antioxidant, antitumor, and other pharmacological effects on human health, becoming a hot-button issue in medical research [12–14]. However, no data to date is available on the inhibitory effect of GSPE on allergic asthma. The aim of the present

study was to investigate the effect of GSPE on airway inflammation and hyperresponsiveness in a mouse model of allergic asthma.

Materials and Methods



Chemicals

GSPE was purchased from Jianfeng, Inc. Gas chromatography-mass spectrometry analyses in conjunction with high-performance liquid chromatography showed that it contained 56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins, and small amounts of monomeric and high-molecular-weight oligomeric proanthocyanidins and flavonoids; lot No. GO50412 (**Fig. 1S**, Supporting Information).

Experimental animals

Forty-eight 6-week-old female BALB/c mice, 18–22 g each, were purchased from the Shanghai Laboratory Animals Center and acclimatized to the animal facility for 14 days before experiments. All animals were treated according to a protocol approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (No. 2009032; Dec. 2009).

Antigen sensitization, challenge, and GSPE treatment

Experimental animals were randomly and averagely divided into six groups with eight mice in each, i.e., the low- ($20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), the middle- ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), and the high-dose ($60 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) GSPE groups; the ovalbumin (OVA), the budesonide, and the control groups. Mice in the first five groups were sensitized on days 0, 7, and 14 by intraperitoneal injection of $100 \mu\text{g}$ OVA (Grade V; Sigma) emulsified in 1 mg aluminum hydroxide (Pierce Chemical Co.) with a total volume of 0.2 mL. Two weeks after the last sensitization, mice were challenged with 1% aerosolized OVA daily for 30 min over a period of four consecutive days. Intraperitoneal injection of GSPE was consecutively conducted in the mice of the three treatment groups, beginning at 1 h before every airway challenge with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, and $60 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, respectively. Mice in the budesonide group were treated with aerosolized budesonide (99.1% purity, Pulmicort Respules®; AstraZeneca AB), 2 mg/4 mL for 30 min per day from days 29 to 32, 1 hour before exposure. Mice in the control group received the same schedule for sensitization and challenge with an equivalent amount of 0.9% sterile saline instead of OVA.

Measurement of airway responsiveness

Airway responsiveness to acetylcholine chloride (ACh) was evaluated by using an AniRes 2005 animal lung function analysis system (SYNOL High-Tech) as previously described [15]. Briefly, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) 24 h after the last OVA challenge. The mice, with a tracheal cannula (2 mm in diameter), were then placed in a whole-body plethysmography chamber and mechanically ventilated at a rate of 90 breaths/min with a tidal volume of 6 mL/kg. With stable airway pressure recording established, ACh was administered via the caudal vein by using a microinfusion pump at a rate of 36 mL/h with a progressive increase of doses (10, 30, 90, and 270 $\mu\text{g/kg}$). After each dose was administered, data were continuously collected from 5 s to 1 min, and the maximum values of lung resistance (RL) were taken to express changes in murine airway function.

Analysis of bronchoalveolar lavage fluid (BALF) and serum

After airway responsiveness had been measured, mice were bled by retro-orbital puncture using heparinized capillary tubes. Blood samples were centrifuged at $2000 \times g$ for 10 min at 4°C , and plasma was stored at -70°C . With blood removed, airway lumina were washed three times with 0.4 mL, 0.3 mL, and 0.3 mL sterile saline respectively. BALF was centrifuged ($1000 \times g$ for 10 min at 4°C), and the total number of inflammatory cells was counted. Differential cell counting was done using cyto-spin techniques and Wright's staining, which was carried out by two independent investigators in a single-blind study that analyzed at least 200 cells each of four different random locations using a microscope. Commercially available ELISA kits were used to determine interleukin (IL)-4 (Jingmei Biotech), interferon (IFN)- γ , and IL-13 (R&D Systems) levels in BALF and total serum immunoglobulin E (IgE) (BPD Biomedicals). The limits of detection were 7 pg/mL for IL-4, 4 pg/mL for IL-13, 7 pg/mL for IFN- γ , and 1 ng/mL for total serum IgE.

Histological examination

The left lung was immersed in 10% neutral buffered formalin overnight, embedded in paraffin, and sectioned (5 μm thickness) before staining with either hematoxylin-eosin (HE) or periodic acid-Schiff (PAS). The histological analyses were conducted by using a light microscope attached to an image-analysis system (Image-Pro Plus; Media Cybernetics). Data were expressed as the area of staining per μm length of the basement membrane of bronchi with an internal diameter of 150–200 μm by counting at least 10 bronchi in each slide. As described previously, the number of PAS-negative and PAS-positive cells (goblet cells) in individual bronchus was counted to quantify the level of mucus expression in the airway [16]. According to the number of PAS-positive cells per bronchus divided by the total number of epithelial cells in each bronchus, we calculated the percentage of PAS-positive cells per bronchus [17].

Immunohistochemistry for iNOS

Immunohistochemistry was performed for the detection of iNOS proteins in the lung tissue according to a modified procedure as described elsewhere [18]. Briefly, formalin-fixed and paraffin-embedded sections (5 μm thickness) were deparaffinized and rehydrated with xylene and graduated alcohol solutions, respectively, and the endogenous peroxidase activity was blocked by incubating sections with 3% H_2O_2 . After flooding with 5% normal goat serum to avoid nonspecific absorption of immunoglobulin, the specimens were incubated for 90 min with rabbit anti-mouse iNOS polyclonal antibody (Abcam) in a dilution of 1:50. Slides were incubated overnight at 4°C , rinsed three times with phosphate-buffered saline (PBS), incubated with peroxidase-labeled goat anti-rabbit IgG for 30 min at 37°C and then washed again with PBS, which was followed by staining with diaminobenzidine (Santa Cruz Biotechnology). The sections were counterstained with hematoxylin, dehydrated and then observed under a light microscope. Negative and positive controls were used simultaneously to ensure specificity and reliability of the staining process. The primary antibody was replaced with normal rabbit IgG as the negative control, and a positive section supplied by the manufacturer of the iNOS polyclonal antibody was taken as the positive control. Positive staining with iNOS was defined by brown staining of the cytoplasm. The percentages of the positive staining regions were quantitatively calculated using image anal-

ysis software Image-Pro Plus 5.0 (Media Cybernetics, Inc.). Results were expressed as the area of iNOS immunostaining per μm length of the basement membrane of bronchi with an internal diameter of 150–200 μm .

Western blot analysis

Right lung tissues from each group were added with lysis buffer and then homogenized. With absolute schizolysis and centrifugation at $12\,000 \times g$ for 15 min at 4°C , we collected the resulting supernatants as the total protein samples and used the method of bicinchoninic acid to determine the protein concentrations. The protein from each mouse in all the six groups was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, which was incubated in blocking solution (5% skimmed milk in Tris-buffered saline) for 2 h at room temperature and then exposed to polyclonal antibody directed against iNOS (Abcam) or anti- β -actin antibody (Santa Cruz Biotechnology) overnight at 4°C with gentle shaking. Then the membrane was incubated with peroxidase-conjugated secondary antibody (1:5000) for 1 h at 37°C . Protein quantitation was detected by the ECL Plus (Amersham Biosciences). Densitometric analysis was carried out by Image densitometry software after the scanning of the Kodak X-Omat LS X-ray films.

Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM). Differences among each group were evaluated using one-way analysis of variance and least significant difference test. A 2-sided p value < 0.05 was considered significant, and all calculations were carried out with SPSS software (SPSS).

Supporting information

The fingerprint chromatogram showing the composition of GSPE and color figures (on how treatment with GSPE attenuates airway inflammation and antigen-induced mucus production in a murine model of asthma as well as of the qualitative measure of iNOS expression in the lungs via immunostaining) are available as Supporting Information.

Results

To detect the effect of GSPE on airway responsiveness, we assessed airway resistance of anesthetized mice in response to increasing concentrations of Ach. No significant differences were identified in baseline airway resistance among the six groups. There was an obvious increase of airway resistance in the OVA, budesonide, and GSPE groups in a dose-dependent manner by administration of Ach (Fig. 1). In the OVA group the airway resistance was significantly greater than that in the control group ($p < 0.01$). Administration of GSPE resulted in a sharp reduction in airway resistance in contrast with the OVA group ($p < 0.05$). Very few inflammatory cells were detected in the control mice except for macrophages. The total cell and eosinophil counts in BALF increased markedly via sensitization and challenge with OVA, followed by a significant decrease by treatment with GSPE or budesonide (Fig. 2). An infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues was clearly observed in the OVA group. The mice administered with GSPE showed a marked reduction in inflammatory cell infiltration within the peribronchiolar and perivascular regions (see

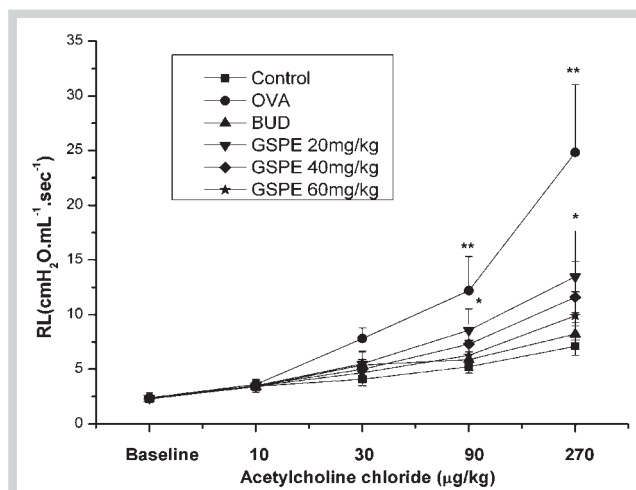


Fig. 1 Effects of GSPE on the development of airway hyperresponsiveness. Data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group. RL: lung resistance.

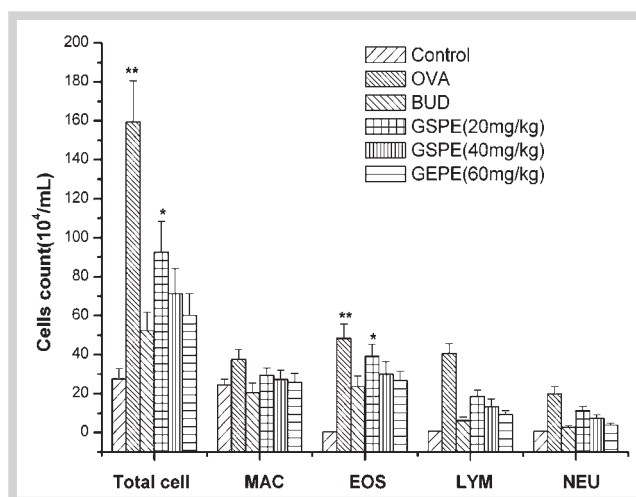


Fig. 2 Effects of GSPE on inflammatory cell accumulation in BALF. Data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group. MAC: macrophage, EOS: eosinophilia, LYM: lymphocyte, NEU: neutrophil.

Fig. 3A, Fig. 2S Supporting Information). Furthermore, the OVA-challenged mice, but not the saline-challenged mice, suffered obvious goblet cell hyperplasia and mucus hypersecretion in the bronchi, which was significantly reduced by GSPE administration (see Fig. 3A, 3B). Thus, evident anti-inflammatory effects of GSPE were revealed.

OVA sensitization and challenge significantly induced the production of IL-4 and IL-13 in BALF. Treatment with GSPE greatly reduced the levels of these Th2 cytokines in BALF compared with the OVA group ($p < 0.05$; Table 1). In contrast, the IFN- γ level in the OVA group was less than that in the control group, and GSPE administration significantly promoted the IFN- γ level in a dose-dependent manner ($p < 0.05$; Table 1). GSPE also dropped total

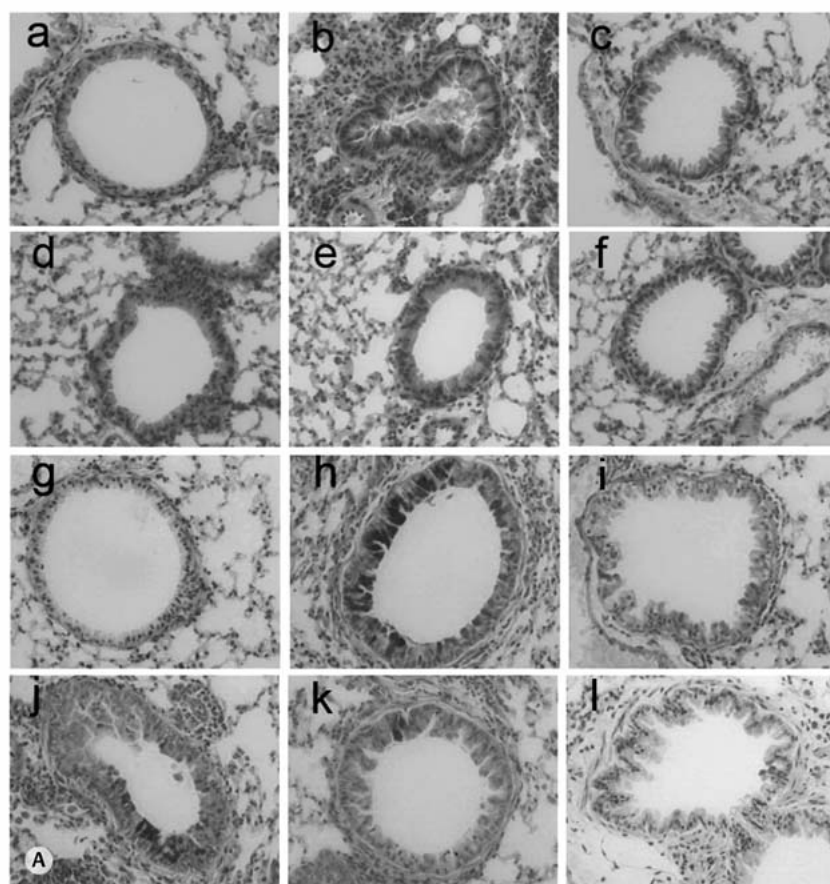


Fig. 3A Treatment with GSPE attenuates airway inflammation and antigen-induced mucus production in a murine model of asthma. **A** (a–g) Haematoxylin and eosin staining (original magnification $\times 400$) of lung tissue from (a) control, (b) OVA-sensitized and -challenged, (c) BUD-treated mice, (d) GSPE1 (20 mg/kg)-treated mice, (e) GSPE2 (40 mg/kg)-treated mice, and (f) GSPE3 (60 mg/kg)-treated mice. (g–l) Periodic acid-Schiff (PAS) staining (original magnification $\times 400$) of lung tissue from (g) control, (h) OVA, (i) BUD-treated mice, (j) GSPE1-treated mice, (k) GSPE2-treated mice, and (l) GSPE3-treated mice.

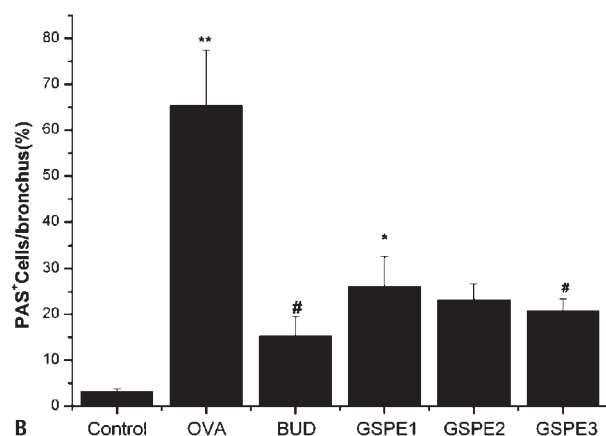


Fig. 3B Treatment with GSPE attenuates airway inflammation and antigen-induced mucus production in a murine model of asthma. **B** The bar chart shows percentage of PAS-positive cells per bronchiole. Data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group; # $p < 0.01$ compared with the OVA group.

serum IgE levels to near baseline as compared with the OVA group ($p < 0.05$; **Table 1**).

To further ascertain whether iNOS was related to the GSPE-induced antiasthmatic effects, we investigated the iNOS expression in the lungs. The protein expression of iNOS was evaluated by im-

Table 1 Serum total IgE and cytokines levels in BALF measured by ELISA.

Group	BALF (pg/mL)			Serum IgE (ng/mL)
	IL-4	IL-13	IFN- γ	
Control	23.7 $\pm$ 4.5	21.6 $\pm$ 4.1	29.7 $\pm$ 4.5	19.03 $\pm$ 4.9
BUD	29.7 $\pm$ 3.8	36.5 $\pm$ 4.5	27.2 $\pm$ 5.2	45.2 $\pm$ 9.2
OVA	128.5 $\pm$ 27.4**	145.6 $\pm$ 8.5**	18.2 $\pm$ 3.4**	198.8 $\pm$ 18.2**
GSPE (20 mg/kg)	57.6 $\pm$ 5.4*	67.2 $\pm$ 12.9*	21.7 $\pm$ 4.2*	78.3 $\pm$ 10.1*
GSPE (40 mg/kg)	49.7 $\pm$ 3.5	58.3 $\pm$ 12.3	23.9 $\pm$ 6.5	69.8 $\pm$ 12.3
GSPE (60 mg/kg)	38.8 $\pm$ 4.3	42.3 $\pm$ 9.7	25.4 $\pm$ 6.2	53.8 $\pm$ 10.8

Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group

munohistochemical staining (see **Fig. 4A**, **Fig. 3S** Supporting Information). Strong cytoplasmic staining was mainly localized to bronchial epithelial cells, bronchial smooth muscular cells, and alveolar epithelial cells. In addition, the infiltrating macrophages and neutrophils around the bronchi also showed positive expression. The iNOS-stained cells were predominant in the OVA-induced inflammatory lung tissue compared with those of the control mice. GSPE treatment dramatically decreased them (**Fig. 4B**). Western blot analysis was used to further quantify the iNOS expression (**Fig. 5A**). The relative amount of targeted protein was quantified as the density of the targeted protein versus β -actin. After normalization of the data according to the ex-

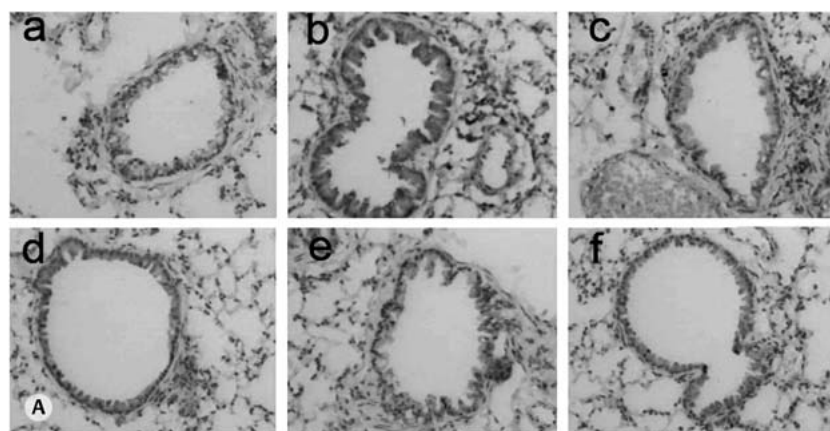


Fig. 4A Qualitative measure of iNOS expression in the lungs via immunostaining. **A** (a–f) Immunostaining (original magnification $\times 400$) of lung tissue from (a) control, (b) OVA-sensitized and -challenged, (c) budesonide, (d) GSPE1 (20 mg/kg), (e) GSPE2 (40 mg/kg), and (f) GSPE3 (60 mg/kg)-treated mice.

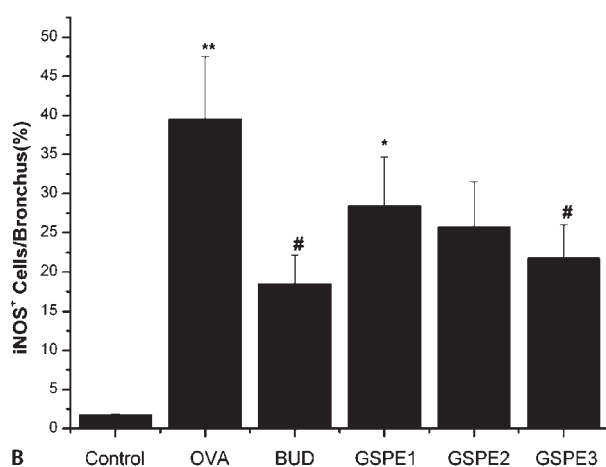


Fig. 4B Qualitative measure of iNOS expression in the lungs via immunostaining. **B** The bar chart demonstrates the percentage of iNOS-positive cells per bronchus. Data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group; # $p < 0.01$ compared with the OVA group.

pression of β -actin protein, the relative density of iNOS is presented as fold numbers. As demonstrated in **Fig. 5B**, the elevated iNOS expression observed in the OVA mice was significantly inhibited by GSPE.

Discussion

In the present study, we evaluated the effects of GSPE on OVA-induced airway inflammation in an acute experimental model of asthma. Our results revealed that GSPE attenuated the airway inflammation, including infiltration of inflammatory cells, expression of inflammatory cytokines, and mucus secretion, as well as AHR in allergic mice. To our knowledge, it is the first study to demonstrate that GSPE decreases the expression of iNOS in the lung tissues of an OVA-induced airway inflammation model. GSPE, a natural occurring polyphenol rich extract, contains mainly dimers, trimers, and highly polymerized oligomers of monomeric catechins [19]. In recent years, mounting evidence indicates that GSPE can counter oxidative stress, protect circula-

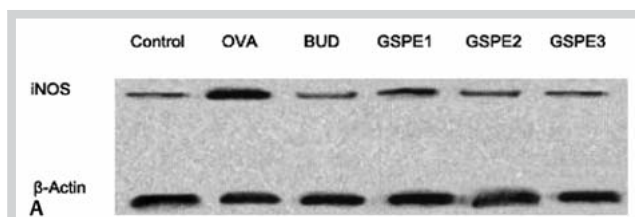


Fig. 5A Treatment with GSPE decreases the iNOS expression. **A** Antibodies were anti-iNOS and anti- β -actin. Lane 1: Control; Lane 2: OVA; Lane 3: BUD (budesonide); Lane 4: GSPE1 (20 mg/kg); Lane 5: GSPE2 (40 mg/kg); Lane 6: GSPE3 (60 mg/kg).

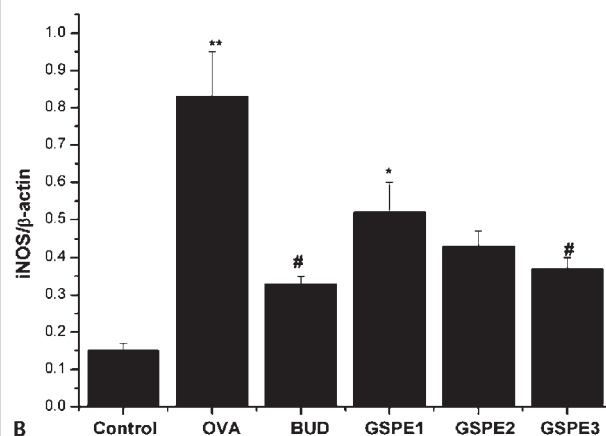


Fig. 5B Treatment with GSPE decreases the iNOS expression. **B** The relative amount of targeted protein was quantified as the density of the targeted protein versus β -actin. Data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with the OVA group; ** $p < 0.01$ compared with the control group; # $p < 0.01$ compared with the OVA group.

tion and has anti-inflammatory and anticarcinogenic activity. Reportedly, GSPE is also involved in the immunoregulatory activity *in vitro* and *in vivo* [20]. In the case of asthma, Th2 cytokines are a crucial contributing factor of allergic airway inflammation and AHR [21, 22]. IL-4, IL-5, and IL-13 stem from various lung resident cells, i.e., bronchial epithelial cells, tissue mast cells, and alveolar

macrophages, as well as infiltrated inflammatory cells. Our present results showed that GSPE not only significantly reduced the levels of IL-4 and IL-13, but also increased the level of IFN- γ in BALF from the OVA-challenged mice. We also demonstrated a dramatic reduction in airway mucus production and serum IgE in GSPE-treated mice. Administration of GSPE significantly inhibited leukocyte recruitment into the lung and regulated the Th1/Th2 balance to suppress OVA-induced airway inflammation. Furthermore, GSPE inhibited OVA-induced AHR, which may be attributed to the reduction of IL-13 production and tissue eosinophilia because IL-13 and eosinophils play an important role in the development of AHR [23]. In our study, the mice treated with GSPE showed a marked reduction in inflammatory cell (especially the eosinophils) infiltration within the peribronchiolar and perivascular regions. Previous studies suggested that IL-4 could selectively stimulate eosinophils adherence to endothelium and transendothelial migration by inducing endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1) [24, 25]. As GSPE can downregulate VCAM-1 expression in human endothelial cells [26, 27], it is reasonable to believe that GSPE reduces the eosinophils infiltration by suppressing the local release of IL-4 and expression of VCAM-1.

It is well known that NO, produced by iNOS, has an important role in inducing airway diseases including asthma [28, 29]. As a short-lived, easily diffusible molecule with high biological activity, endogenous NO is believed to be a major marker of physiological aggravation of airway functions and is closely implicated in airway diseases like asthma [30]. Airway inflammation is attributed to an enhanced expression and activity of iNOS, which increases NO production and contributes to higher levels of NO gas in exhaled air [31]. Sourcing NO in asthma may be of help in the elucidation of its pathogenic role and providing an alternative therapy. The dual effects of NO in many pathological conditions are probably related to its source, concentration, and site of action. In this process, excessive NO may increase eosinophils in the airway and shift the immune balance toward Th2 cells, thus exacerbating inflammation in the airway. Inhibiting iNOS can reduce resistance of the respiratory system response, eosinophilic and mononuclear cell recruitment, and collagen and elastic fibers content in airways [32]. In the present study, we found that the expression of iNOS was significantly higher in the OVA group than that in the control group, and GSPE administration suppressed the expression of iNOS in a dose-dependent manner. Our results are in accordance with a previous study performed by Houde et al. that GSPE strongly decreased NO and iNOS expression by LPS-stimulated macrophages [33]. Therefore, we assumed that the suppression of iNOS was one of the many anti-inflammatory effects of GSPE in asthma.

Reportedly, necropsy and blood testing as well as histological examination showed that a dietary concentration of 2.5% grape seed extract caused no adverse effects [34]. Japanese researchers also evaluated the toxicity of grape seed extract using 344 rats, and no evidence showed acute oral toxicity at dosages of 2 and 4 g/kg [35]. However, high-dose GSPE could cause apoptotic cell injury via effector caspase-3 activation and subsequent induction of reactive oxygen species generation [36]. Therefore, the dosages of GSPE should be optimized to avoid potential harmful pro-oxidant effects. In our study, no significant cytotoxicity was observed in GSPE-treated mice.

Taken together, this study is the first to demonstrate that GSPE modulates the inflammatory response in OVA-induced allergic airway disease by suppression of iNOS expression and activity.

With anti-inflammatory and immunomodulatory roles, GSPE may be useful as an adjuvant therapy for asthmatic patients in the future.

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