

Glycyrrhizin alleviates experimental allergic asthma in mice

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Abstract

Asthma is a chronic respiratory disease, the incidence of which is increasing globally. The existing therapy is inadequate and has many adverse effects. It needs a better therapeutic molecule preferably of natural origin, which has negligible or no adverse effects. In view of this, we evaluated Glycyrrhizin (GRZ), a major constituent of a plant *Glycyrrhiza glabra*, for its efficacy on asthmatic features in a mouse model of asthma. BALB/c mice were sensitized and challenged with ovalbumin (OVA) to develop the asthmatic features such as airway hyperresponsiveness: allergen induced airway constriction and airway hyperreactivity (AHR) to methacholine (MCh), and pulmonary inflammation. The mice were orally treated with GRZ (2.5, 5, 10 and 20 mg/kg) during or after OVA-sensitization and OVA-challenge to evaluate its protective or reversal effect, respectively on the above asthmatic features. The status of airway hyperresponsiveness was measured by monitoring specific airway conductance (SGaw) using a non-invasive method and the pulmonary inflammation was assessed by haematoxylin and eosin staining of lung sections. Several other parameters associated with asthma such as interleukin (IL)-4, IL-5, interferon- γ (IFN- γ), OVA-specific IgE, total IgG_{2a} and cortisol were measured by ELISA. GRZ (5 mg/kg) markedly inhibited OVA-induced immediate airway constriction, AHR to MCh ($p < 0.01$), lung inflammation, and infiltration of eosinophils in the peribronchial and perivascular areas. It prevented the reduction of IFN- γ ($p < 0.02$), and decreased IL-4 ($p < 0.05$), IL-5 ($p < 0.05$) and eosinophils ($p < 0.0002$) in the BAL fluid. Also, it reduced OVA-specific IgE levels ($p < 0.01$) and prevented the reduction of total IgG_{2a} ($p < 0.01$) in serum. We have also showed that it has no effect on serum cortisol levels. Our results demonstrate that GRZ alleviates asthmatic features in mice and it could be useful towards developing a better therapeutic molecule in the future.

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1. Introduction

Glycyrrhizin (GRZ) is a triterpene glycoside which consists of one molecule of 18 β -glycyrrhetic acid and

two molecules of glucuronic acid having structure, 18-beta-glycyrrhetic acid-3-O-beta-D-glucuronopyranosyl-(1 $\rightarrow$ 2)-beta-D-glucuronide [1,2]. It is a major active constituent of licorice root (*Glycyrrhiza glabra*) and has been attributed numerous pharmacological effects like anti-inflammatory, anti-viral, anti-tumor and hepatoprotective activities [3–6]. It has been shown that GRZ and its biologically transformed metabolite called 18 β -glycyrrhetic acid, an aglycon component of GRZ, inhibit

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the passive cutaneous anaphylaxis and skin contact inflammation in mice model of contact hypersensitivity [2]. It is unclear about the mechanism of action of GRZ and its metabolite against inflammation and other activities. But researchers believe that it could be due to steroid like structure of 18 β -glycyrrhetic acid which can produce a cortisol mimicking effect that may inhibit inflammation [7]. This effect is due to the inhibition of 11 β -hydroxysteroid dehydrogenase (11-HSD) which catalyses the conversion of cortisol to its inactive metabolite cortisone [8,9].

Asthma is a chronic respiratory disease characterized by reversible airway obstruction, increased mucus production, infiltration of eosinophils and nonspecific airway hyperresponsiveness [10]. The development of asthma is mediated by proinflammatory cytokines, such as IL-4, IL-5 and IL-13 secreted primarily by CD4⁺ Th2 cells. On the other hand, IFN- γ secreted by CD4⁺ Th1 cells inhibits the Th2 cell differentiation [11]. The asthmatic response is divided into early and late phase responses in human. The early phase begins immediately after exposure of allergen, which is often associated with mast cell degranulation [12]. The late phase response, associated with inflammation of the airways and airway hyperresponsiveness, occurs 8 to 24 h after allergen exposure [13]. This response is associated with the infiltration of eosinophils in the airways [14]. Similar early and late airway responses were also observed in mouse model after an allergen provocation [15].

The incidence of allergic and asthmatic disorders is increasing rapidly all over the world [16]. The current therapy is inadequate and also the majority of the prescribed drugs have side effects [17]. Consequently, a major thrust has been given to identify new molecules preferably from natural origin for mitigating these disorders. In view of this, we selected GRZ and evaluated its effect on allergen-induced immediate airway constriction, airway hyperreactivity to MCh, lung inflammation, eosinophil levels, Th1/Th2 cytokines, and also serum cortisol levels.

2. Methods

2.1. Development of experimental mouse asthma

Experimental protocols were approved by the Institutional Animals Ethics Committee. Male BALB/c mice (8–10 weeks old, 18–22 g) were obtained from National Institute of Nutrition, Hyderabad (India). Mice were acclimatized for one week under the laboratory conditions (25 $\pm$ 2 °C, 60% humidity) before starting the experiments. Airway hyperreactivity and airway inflammation were induced as described previously [18] with some minor modification. Briefly, mice were sensitized with 0.2 ml saline suspension containing 10 μ g oval-

bumin (OVA) (Sigma, USA) and 2 mg aluminum hydroxide in saline intraperitoneally (i.p.) on days 0, 7 and 14. From days 19 to 26, mice were subjected to aerosol of OVA (2%) inhalation 30 min daily (Fig. 1) in a Plexiglas chamber (20 $\times$ 20 $\times$ 10 cm³). The aerosol was generated by a nebulizer (DeVilbiss, model 645, USA) with an airflow rate of 7 l/min. Developed airway hyperresponsiveness (OVA-induced immediate airway constriction and airway reactivity to methacholine (MCh)) and airway inflammation were examined as described below. Control (sham-sensitized) mice received i.p. injections of 0.2 ml saline containing 2 mg alum and were challenged with saline alone. They were also checked for the airway hyperresponsiveness and airway inflammation as mentioned.

2.2. Mice groups and administration of GRZ

Mice were divided into eleven groups ($n=6$ each group) in two sets: Set I and Set II. Set I (groups 1–7) mice were used to find the protective effect of GRZ (Sigma, USA; dissolved in alkaline water) and Set II (groups 8–11) for the reversal effect as shown in Fig. 1. All groups were named according to the status of sensitization/challenge/treatment. For example, OVA/OVA/GRZ 5 mg indicates OVA-sensitized, OVA-challenged and treated orally with 5 mg/kg of glycyrrhizin. Set I mice were given vehicle (alkaline water, 20 μ l) or various concentrations of GRZ (2.5, 5, 10, 20 mg/kg, in 20 μ l alkaline water) orally twice daily from day 0 to 23. Set II mice except group 8 were examined for OVA-induced SGaw fall of at least 40% of the basal levels before the treatment. Vehicle/5 mg GRZ (orally twice per day) or dexamethasone (orally once per day) were given from 26 to 32. The selection of 5 mg dose was based on our pilot study (three independent experiments).

2.3. Measurement of airway function: specific airway conductance (SGaw)

Airway function of the mice was measured as specific airway conductance (SGaw) by using a dual-chamber whole body plethysmograph as described previously [18,19]. On day zero, all the mice were checked for basal SGaw and initial MChPD₃₅ without any sensitization and allergen challenge.

2.4. Measurement of OVA-induced immediate airway constriction

OVA-induced airway constriction of mouse was determined as a fall of SGaw after OVA-aerosol challenge as compared to its basal SGaw values. This measurement was performed on day 24 for the protective experiment and on days 24 and 33 for the reversal experiment. Each mouse was challenged with 3% OVA-aerosol inhalation for 10 min in a Plexiglas chamber (20 $\times$ 10 $\times$ 10 cm³) and then rechallenged with 3% OVA for 60 s inside the body plethysmograph. The fall of SGaw within 10 min was recorded. This decreased SGaw from its basal value was described as airway constriction. The sham control mice were challenged with saline as an aerosol inhalation instead of OVA-aerosol inhalation.

2.5. Measurement of airway reactivity to methacholine (MCh)

The airway reactivity to MCh was measured as described earlier [18,19]. Briefly, each mouse was acclimatized for 5 min in the plethysmograph, and an aerosol of PBS followed by aerosol of increasing doses of methacholine (3.1, 6.25, 12.5, 25, 50, 100 and 200 mg/ml in PBS) were given by nebulization till SGaw levels decreased below or equal to 60% of the basal level. A MCh dose–SGaw response curve was drawn for each mouse and the dose, which produced a 35% decrease in SGaw level (MChPD₃₅), was derived by intrapolation of the curve and was used as an index of airway reactivity as described previously [18,19].

2.6. Collection of bronchoalveolar lavage (BAL) fluid and blood

24 h after last challenge, the mice were sacrificed using an overdose of sodium pentothal (100 mg/kg, i.p.). Blood samples were collected by heart puncture and sera were separated by centrifugation at 1500 ×g for 10 min and kept at –70 °C till the measurements of IgE, IgG_{2a}, ALT and cortisol levels. BAL was performed by tracheal cannulation with total 2 ml of PBS and around 1.5 ml of BAL fluid was recovered after 4 lavages. The BAL fluid was centrifuged (400 ×g, 4 °C, 6 min) and the supernatant was kept at –70 °C until cytokines measurement were done. The BAL cells were washed three

times with PBS and the pellet was resuspended in 100 µl cold PBS. Total cell count was done by Neubauer haemocytometer. For differential counts, cytospin preparations were stained with Leishman's stain. The cells were identified by standard morphological criteria and at least 300 cells (all types) per cytospin preparation were counted and the percentage of eosinophils was calculated. Absolute eosinophil count was calculated by multiplying eosinophil percentage with total cell count.

2.7. Measurement of OVA-specific IgE, total IgG_{2a} and cytokines

OVA-specific IgE levels in sera were measured by enzyme linked immunosorbent assay (ELISA) as described previously [18]. Total IgG_{2a} in sera and IL-4, IL-5 and IFN-γ in BAL fluid supernatant were measured by ELISA method as per manufacturer's instructions (BD Pharmingen, USA).

2.8. Measurement of cortisol and alanine aminotransferase (ALT)

The serum cortisol levels were measured by competitive solid phase ELISA as per manufacturer's instructions (United Biotech Inc, CA). The levels of serum ALT were measured by UV kinetic method of TECO Diagnostics (Anaheim, CA). The result was expressed in IU/l at 37 °C.

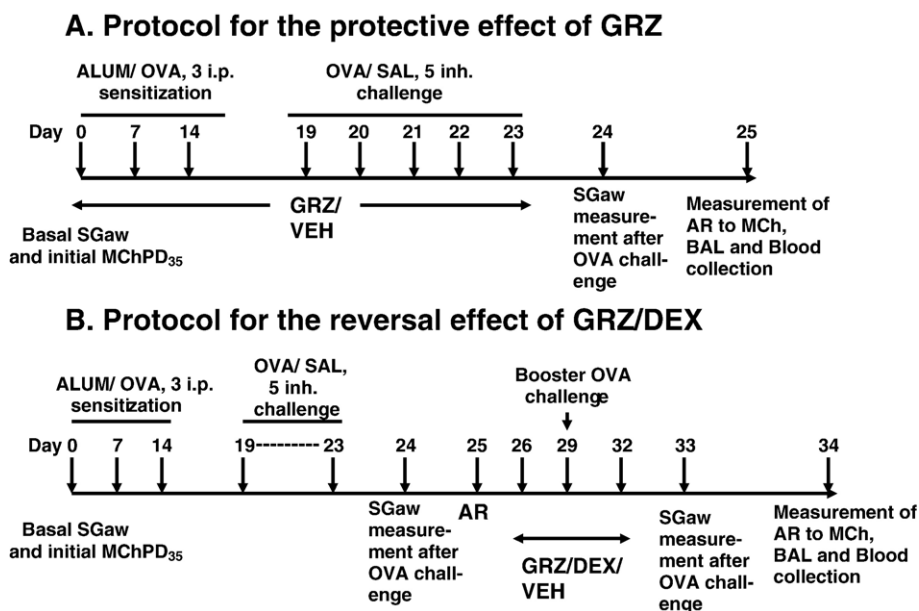


Fig. 1. Experimental protocols. Mice were sensitized and challenged as described in Methods. A. To evaluate the protective effect of glycyrrhizin (GRZ), mice were sensitized with ALUM or OVA and challenged with saline or OVA, and were given vehicle or GRZ orally twice per day (20 µl per dose). OVA induced SGaw and airway reactivity (AR) to MCh were measured on days 24 and 25 respectively. B. To evaluate the reversal effect, mice were sensitized with ALUM or OVA and challenged with saline or OVA to develop asthma and then treated with vehicle or GRZ twice or DEX once per day as described in Methods. One booster OVA challenge was given on day 29. OVA induced SGaw and AR to MCh were measured on days 33 and 34 respectively. i.p. = intraperitoneal, inh. = inhalation, SAL = saline.

2.9. Histological analysis of lung inflammation

The excised lung portion was fixed in 10% buffered formalin. The fixed, paraffin embedded tissue were sectioned into 4 μm sections and stained with haematoxylin and eosin (H&E) to assess inflammation. The inflammatory cell distribution in lung sections was determined by counting at least 300 inflammatory cells. This counting was done by light microscopic method in serial transverse section of lung. We were not able to count the inflammatory cells in lung sections of SHAM/SAL/VEH or SHAM/SAL/GRZ 5 mg mice, as no inflammation was found in those sections. It required two adjacent bronchovascular regions of single transverse section for OVA/OVA/VEH or GRZ 2.5 mg mice to count 300 inflammatory cells as dense inflammation was found in those

sections. But it required 3–8 adjacent peribronchial and/or perivascular regions of single transverse section to count 300 cells of OVA/OVA/GRZ 5, 10 mg mice. After counting 300 inflammatory cells, the percentage of eosinophils was calculated. Histopathological analysis was done in four mice from each group.

2.10. Statistics

All data were presented as means $\pm$ SEMs. Mann–Whitney *U*-test was applied for the difference of SGaw and MChPD₃₅ between two groups of mice. Student's *t*-test was performed for AEC in BAL fluid, cortisol levels, ALT levels, cytokines, OVA-specific IgE and total IgG_{2a} levels. A *p* value less than 0.05 was considered to be significant.

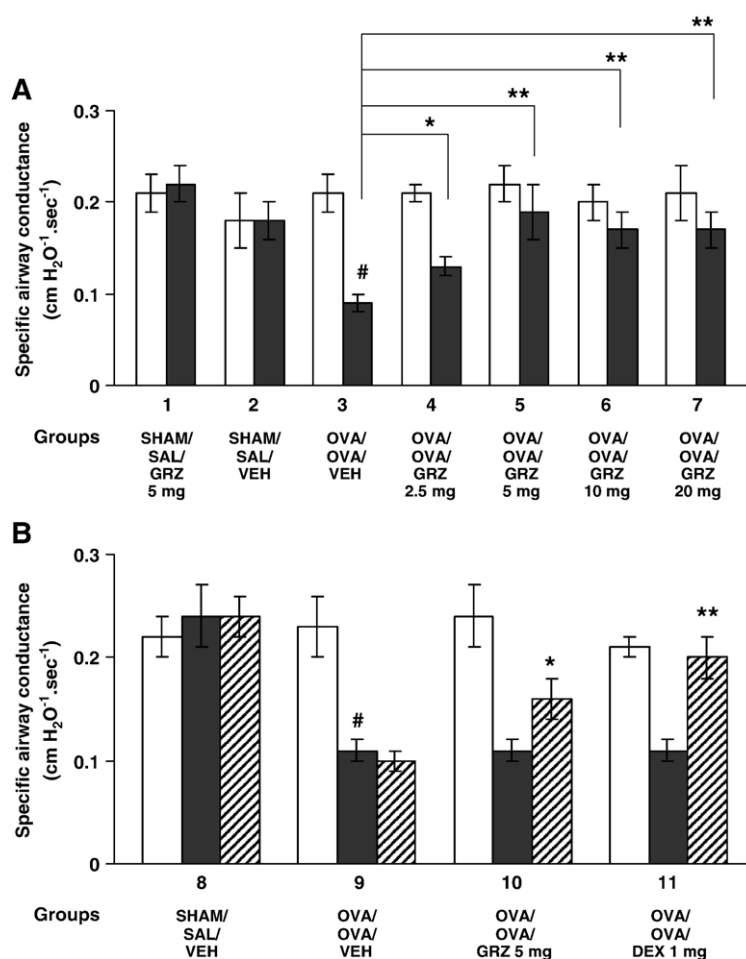


Fig. 2. GRZ inhibits immediate airway constriction. A. Experiments were performed with seven groups (1–7, $n=6$ in each) to examine the protective effects of GRZ as described in Methods. White bar indicates the SGaw values on day 0 (before starting sensitization) and black bar indicates the OVA-induced SGaw values on day 24 after vehicle or GRZ treatment. [#] $p<0.001$ as compared to before sensitized SGaw level; ^{*} $p<0.01$ and ^{**} $p<0.001$ as compared to OVA/OVA/VEH. B. To examine the reversal effect of GRZ, experiments were done on another set of four groups (8–11, $n=6$ in each) of mice as mentioned in the Methods. White bar indicates the SGaw values before starting the sensitization, black bar indicates the SGaw at the OVA-sensitization and OVA-challenge (on day 24), and hatched bar indicates the SGaw values after one week vehicle or GRZ treatment (on day 33). [#] $p<0.001$ as compared to before sensitized level of SGaw; ^{*} $p<0.01$ and ^{**} $p<0.001$ as compared to vehicle treated group 9.

3. Results

3.1. Effects of GRZ/DEX on general health of mice

To assess the general health and liver function of mice treated with GRZ, we estimated the weight of mice and serum ALT levels. OVA/OVA/GRZ 5 mg mice showed no significant change in weight when compared to OVA/OVA/VEH mice (22.16 ± 0.16 g versus 21.73 ± 0.62 g; $P=0.53$). In contrast, OVA/OVA/DEX 1 mg mice showed a significant decrease in weight compared to OVA/OVA/GRZ 5 mg mice (20.3 ± 0.16 g

versus 22.16 ± 0.16 g; $P=0.001$). Hair loss in abdomen was also observed in OVA/OVA/DEX 1 mg mice whereas this effect was not observed in GRZ treated mice. Also, there was no significant difference in the levels of ALT between OVA/OVA/VEH mice and OVA/OVA/GRZ 5 mg (25.09 ± 6.21 IU/l versus 20.47 ± 6.59 IU/l; $P=0.628$).

3.2. GRZ attenuates OVA-induced airway constriction

To test the effect of GRZ in OVA-sensitized and OVA-challenged mice, mice were treated with or without various

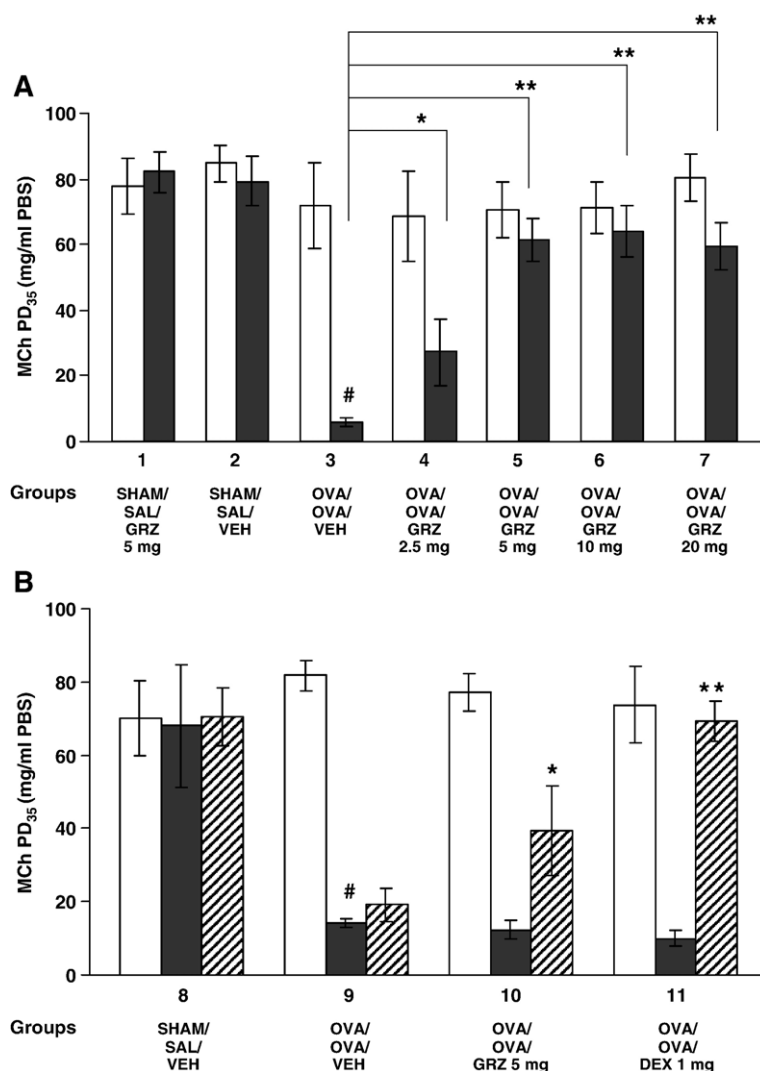


Fig. 3. GRZ inhibits airway hyperreactivity (AHR) to methacholine (MCh). A. Experiments were performed as described in Fig. 1A and MChPD₃₅ values were measured on day 25 to examine the protective effect of GRZ as described in Methods. White bar indicates MChPD₃₅ before sensitization and black bar indicates MChPD₃₅ values on the 25th day after vehicle or GRZ treatment. # $p<0.001$ as compared to before sensitized MChPD₃₅; * $p<0.01$ and ** $p<0.001$ as compared to OVA/OVA/VEH group. B. To evaluate the reversal effect on airway reactivity, experiments were performed as described in Methods and MChPD₃₅ values were measured on day 34. White bar indicates MChPD₃₅ before sensitization, black bar indicates MChPD₃₅ at sensitized and challenged condition (on day 25) and hatched bar indicated after one week treatment of GRZ (on day 34). # $p<0.001$ as compared to before sensitization level of MChPD₃₅; * $p<0.01$ and ** $p<0.001$ as compared to OVA/OVA/VEH group.

concentrations of GRZ as described in Methods. In the protective study (Fig. 2A), SHAM/SAL/VEH or GRZ 5 mg mice did not show any fall of SGaw from their basal values (groups 1 and 2). On the other hand, OVA/OVA/VEH mice showed 57% fall of SGaw (black bars) ($p<0.001$) as compared to their basal values (white bars). GRZ treatment markedly reduced the OVA induced fall of SGaw (groups 4–7 of Fig. 2A). The dose of 5 mg/kg of GRZ significantly protected from the fall of SGaw (up to 86% of the basal level, $p<0.001$, Fig. 2A). Interestingly, in the reversal study also, OVA/OVA/GRZ 5 mg mice significantly reversed the SGaw (up to 74% of basal level, $p<0.01$, Fig. 2B). This reversal effect was comparable to dexamethasone treatment ($p<0.001$ as compared to OVA/OVA/VEH mice).

3.3. GRZ reduces airway hyperreactivity to MCh

In the protective study (Fig. 3A), significant decrease in MChPD₃₅ values was found in OVA/OVA/VEH mice com-

pared to SHAM/SAL/VEH mice ($p<0.001$). This indicates that OVA-treated mice had developed airway hyperreactivity to MCh. Interestingly, OVA/OVA/GRZ 2.5, 5, 10 and 20 mg mice showed markedly increased MChPD₃₅ values, thereby demonstrating the attenuation of AHR to MCh. GRZ at a dose of 5 mg/kg increased MChPD₃₅ values up to 87% ($p<0.001$) of its basal level (Fig. 3A). In the reversal study, OVA/OVA/GRZ 5 mg mice showed significant increase in MChPD₃₅ values ($p<0.01$) (Fig. 3B). OVA/OVA/DEX 1 mg mice also showed significant increase in MChPD₃₅ values ($p<0.001$ as compared to OVA/OVA/VEH mice).

3.4. GRZ reduces OVA specific IgE levels

To test the effect of GRZ in OVA specific IgE levels, sera samples were collected from mice treated with or without different concentrations of GRZ and OVA-specific IgE levels were measured as described in Methods. OVA-specific IgE levels increased markedly in the OVA/OVA/VEH mice

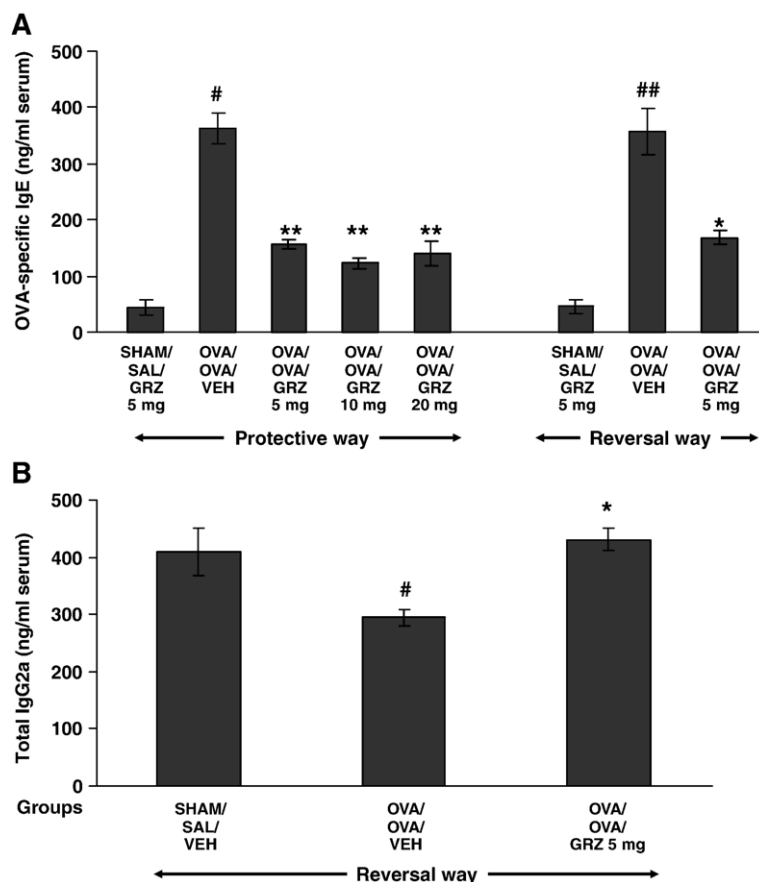


Fig. 4. Effects of GRZ on OVA-specific IgE and total IgG_{2a} in serum. Experiments were performed as described in Methods. Data are expressed as means $\pm$ SEMs of two independent experiments. A. GRZ reduces OVA-specific IgE in serum. Results are expressed as nanograms per ml serum. # $p<0.0002$ compared with SHAM/SAL/VEH (protective); ## $p<0.0002$ compared with SHAM/SAL/VEH (reversal); ** $p<0.002$ compared with OVA/OVA/VEH (protective) and * $p<0.005$ compared with OVA/OVA/VEH (reversal). B. GRZ restores total IgG_{2a} in serum. Results are expressed as nanograms per ml serum. # $p<0.002$ compared with SHAM/SAL/VEH and * $p<0.015$ compared with OVA/OVA/VEH.

compared to SHAM/SAL/VEH mice (362.5 ± 27.1 ng/ml versus 43.2 ± 13.3 ng/ml; $p < 0.0002$) (Fig. 4A). The OVA/OVA/GRZ 5, 10 and 20 mg mice showed reduced IgE levels significantly ($p < 0.002$ for all groups as compared to OVA/OVA/VEH mice). In the protective study, maximum reduction was found in OVA/OVA/10 mg GRZ mice as compared to OVA/OVA/VEH mice (122.5 ± 8.7 ng/ml versus 362.5 ± 27.1 ng/ml). Also, in the reversal study, a significant reduction in the IgE levels was observed in OVA/OVA/GRZ 5 mg mice as compared to OVA/OVA/VEH mice (Fig. 4A, $p < 0.005$).

3.5. GRZ restores total serum IgG_{2a} levels

To assess the effect of GRZ on general humoral immunity, total IgG_{2a} was measured as described in Methods. OVA/OVA/VEH mice showed decreased levels of IgG_{2a} in mice when compared to SHAM/SAL/VEH mice (294.9 ± 14.25 ng/ml versus 408.45 ± 41.53 ng/ml) (Fig. 4B). GRZ treatment, however, significantly restored IgG_{2a} levels back to baseline ($p = 0.014$ compared to OVA/OVA/VEH mice) indicating that GRZ had no adverse effect on general antibody response.

3.6. GRZ modulates cytokines and reduces eosinophilia

As shown in Table 1, the levels of Th2 cytokines, IL-4 and IL-5 were found to be increased ($p < 0.01$) in OVA/OVA/VEH mice but Th1 cytokine, IFN- γ was significantly decreased ($p < 0.01$) as compared to SHAM/SAL/VEH mice. These results indicate that OVA induced mice developed Th1/Th2 imbalance. However OVA/OVA/GRZ 5, 10, 20 mg corrected this imbalance by preventing the reduction of IFN- γ levels and restoring IL-4 and IL-5 levels back towards the baseline in

Table 1
Effect of Glycyrrhizin treatment on cytokine levels in the BAL fluid

	IFN- γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
Protective effect			
SHAM/SAL/VEH	1402 ± 162.7	64.2 ± 6.5	121.7 ± 11
OVA/OVA/VEH	$402.6 \pm 146.1^{\#}$	$316.8 \pm 51.7^{\#}$	$250 \pm 37.4^{\#}$
OVA/OVA/GRZ	$1373.6 \pm 261^{**}$	$125 \pm 16.6^{**}$	$139.4 \pm 37^{*}$
5 mg			
OVA/OVA/GRZ	$1043.7 \pm 375.4^{*}$	$123.3 \pm 17.6^{**}$	$105.6 \pm 24.6^{**}$
10 mg			
OVA/OVA/GRZ	$864.4 \pm 80.2^{*}$	$92.2 \pm 15.1^{**}$	$120.8 \pm 19.3^{*}$
20 mg			
Reversal effect			
SHAM/SAL/VEH	1253 ± 123	69.3 ± 7.4	101.8 ± 21.7
OVA/OVA/VEH	$391.1 \pm 21.2^{\diamond}$	$300.5 \pm 44.4^{\diamond}$	$275.3 \pm 39.2^{\diamond}$
OVA/OVA/GRZ	$755.3 \pm 82.3^{\heartsuit}$	$134.4 \pm 22.4^{\heartsuit}$	$160 \pm 31.1^{\heartsuit}$
5 mg			

Experiments were performed and the levels of IFN- γ , IL-4 and IL-5 were measured as described in Methods. Results are expressed as means $\pm$ SEMs ($n = 6$ in each group) of two independent experiments. $^{\#}p < 0.01$ compared with SHAM/SAL/VEH group (protective); $^{\diamond}p < 0.01$ compared with SHAM/SAL/VEH group (reversal). $^{**}p < 0.02$ and $^{*}p < 0.05$ compared with OVA/OVA/VEH group (protective); $^{\heartsuit}p < 0.05$ compared with OVA/OVA/VEH group (reversal).

Table 2

Effect of Glycyrrhizin treatment on absolute eosinophil count (AEC) in the BAL fluid

Treatment	Absolute eosinophil count ($\times 10^4$ /ml BAL fluid)
Protective effect	
SHAM/SAL/VEH	0.08 ± 0.03
OVA/OVA/VEH	$145.46 \pm 14.55^{\#}$
OVA/OVA/GRZ	$9.61 \pm 0.9^{*}$
5 mg	
OVA/OVA/GRZ	$6.15 \pm 0.78^{**}$
10 mg	
OVA/OVA/GRZ	$3.31 \pm 0.29^{**}$
20 mg	
Reversal effect	
SHAM/SAL/VEH	0.10 ± 0.03
OVA/OVA/VEH	$155.16 \pm 16.2^{\diamond}$
OVA/OVA/GRZ	$11.69 \pm 1.76^{\heartsuit}$
5 mg	

Total cell count (TCC) was quantified by Neubauer haemocytometer. For differential counts, cytospin preparations were stained with Leishman's stain. At least 300 cells (all types) per cytospin were counted and the percentage of eosinophils was calculated. AEC was calculated by multiplying eosinophil percentage with TCC. Results are expressed as means $\pm$ SEMs ($n = 6$ in each group) of two independent experiments. $^{\#}$ and $^{\diamond}p < 0.00001$ versus SHAM/SAL/VEH of protective and reversal respectively; $^{*}p < 0.005$ and $^{**}p < 0.0002$ versus OVA/OVA/VEH group (protective). $^{\heartsuit}p < 0.0002$ versus OVA/OVA/VEH (reversal).

protective study. The maximal effect was found with 5 mg/kg dose for IFN- γ compared to OVA/OVA/VEH mice (1373.6 ± 261 pg/ml versus 402.6 ± 146.1 pg/ml). The similar results, albeit less, were also found in OVA/OVA/GRZ 5 mg mice in the reversal experiments.

As shown in Table 2, in the protective experiments, OVA/OVA/VEH mice showed a significant increase in eosinophil numbers in BAL fluid as compared to SHAM/SAL/VEH ($p < 0.00001$). On the other hand OVA/OVA/GRZ 5, 10, 20 mg showed a significant reduction of eosinophil numbers as compared to OVA/OVA/VEH mice ($p < 0.005$ for 5 and 10 mg doses and $p < 0.0002$ for 20 mg dose). However this reduction was not significant with 2.5 mg dosage (data not shown). Similarly, in the reversal experiments, OVA/OVA/GRZ 5 mg mice showed a significant decreased number of eosinophils ($p < 0.0002$ as compared to OVA/OVA/VEH mice).

3.7. GRZ reduces lung inflammation

The extent of the inflammation and the eosinophil infiltration in the mice lungs were assessed by H&E staining of the paraffin embedded section. Representative micrographs are shown in Fig. 5. The lungs of SHAM/SAL/VEH mice from both protective and reversal protocol showed normal lung architecture with no sign of inflammation (Fig. 5A). The lungs of OVA/OVA/VEH mice showed dense perivascular and peribronchial distribution of inflammatory cells with destruction of mucosal epithelium (Fig. 5B). Among the inflammatory

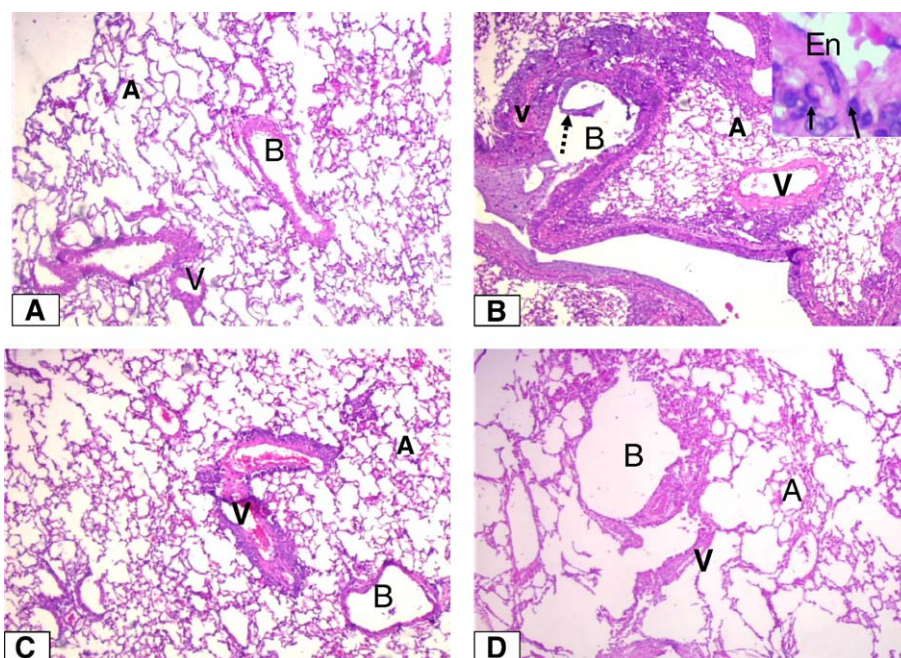


Fig. 5. Lung histopathology. (A) Photomicrograph shows the normal lung parenchyma of sham-sensitized and saline-challenged mice with bronchi (B), vessel (V) and alveoli (A) without inflammatory cells; (B) OVA-sensitized and OVA-challenged mice show dense inflammatory cell infiltration both in peribronchial and perivascular areas with 28% of eosinophils, and the destruction of bronchial mucosal epithelium (broken arrow). Inset shows eosinophils adjacent to the vascular endothelium (En) both inside and outside indicated the infiltration process from the vessel to the lung tissue. Arrow inside the inset indicates eosinophils; (C) 5 mg/kg GRZ treatment shows no significant inflammatory cell infiltration in the peribronchial area and perivascular area. (D) 10 mg/kg GRZ treatment shows no significant inflammatory cell infiltration in the bronchovascular area. Sections shown are from a representative of three independent experiments. Magnification (a–d) $\times 250$. B = bronchus, V = vessel, A = alveolus, En = endothelium.

cells, the eosinophils were 26.5 ± 1.7 (mean $\pm$ SEM, $n=4$) in bronchovascular region compared to near absence in the SHAM/SAL/VEH. In the GRZ treated mice, although OVA/OVA/GRZ 2.5 mg mice did not show any significant reduction of inflammation (data not shown), OVA/OVA/GRZ 5, 10 mg mice showed significant reduction of both peribronchial and perivascular inflammation (Fig. 5C and D). The percentages of lung tissue eosinophils were 14.75 ± 1.8 ($p=0.003$) and 9 ± 1.9 ($p=0.0004$) (mean $\pm$ SEM, $n=4$ each group) for OVA/OVA/GRZ 5 and 10 mg mice, respectively.

Table 3
Effect of GRZ/DEX on cortisol levels in mice sera

Groups	SHAM/ SAL/VEH	OVA/ OVA/VEH	OVA/OVA/ GRZ 5 mg	OVA/OVA/ DEX 1 mg
Serum cortisol ($\mu\text{g/dl}$)	8.225 ± 0.32	7.743 ± 0.48	7.518 ± 0.257	1.233 $\pm 0.488^*$

Experiments were performed as described in Methods. Sera were separated and cortisol levels were measured as described in Methods. Results are expressed as means $\pm$ SEMs ($n=4$ in each group).

* $p < 0.0001$ versus OVA/OVA/GRZ 5 mg group.

3.8. GRZ does not affect serum cortisol levels

To test whether or not GRZ exerts its anti-asthmatic effect through increasing systemic steroid like cortisol, serum cortisol levels were measured in GRZ-treated mice and compared with DEX-treated mice as described in Methods. As shown in Table 3, OVA/OVA/VEH mice showed mild reduction in serum cortisol levels when compared to SHAM/SAL/VEH mice 7.743 ± 0.48 $\mu\text{g/dl}$ versus 8.225 ± 0.32 $\mu\text{g/dl}$. Importantly, OVA/OVA/GRZ 5 mg mice showed almost similar levels of serum cortisol levels as OVA/OVA/VEH mice. In contrast, OVA/OVA/DEX 1 mg mice showed very less cortisol levels compared to other groups of mice (Table 3).

4. Discussion

Asthma is a chronic inflammatory airway disease in which multiple complex pathways are involved. Because of its chronic nature, long term medications are required for therapy. Currently available therapeutic agents have major adverse effects. So it is imperative to find out drugs preferably non-steroidal in nature, which can be taken for a long time without any adverse effects. With this basis, we

selected a natural compound, Glycyrrhizin, a major anti-inflammatory compound in licorice, to test its effects on OVA-induced asthma in mice. The animal models of asthma as well as human asthmatics show allergen induced immediate airway constriction and late airway hyper-reactivity [13,15]. In this report, we demonstrated that GRZ significantly reduces OVA-induced airway constriction, airway hyperreactivity to methacholine and decreases lung inflammation including marked eosinophil infiltration in the mouse model of asthma. In addition, it reduces OVA-specific IgE levels in serum and Th2 cytokines, IL-4 and IL-5 in BAL fluid. On the other hand, it prevents the decrease in total IgG_{2a} levels in serum and Th1 cytokine, IFN- γ in BAL fluid (Table 2, Fig. 4A and B). These results indicate that GRZ modulates Th1/Th2 paradigm without affecting the general immunity. Also, GRZ does not affect the general health of mice as determined by alanine aminotransferase (ALT) levels in serum, body weight and normal behavior in comparison to GRZ untreated mice. Indeed, it has been found that GRZ has hepato-protective activity in rats even at high concentration (200 mg/kg) [20]. It is, however, to be noted that GRZ used in our experiments is only 75% pure. Therefore, the effective doses are actually 75% of the doses mentioned in the results. In any event, the impurity present in the commercially available GRZ does not affect the general health of mice.

The early airway response in asthma is IgE dependent and caused by the activation and degranulation of pulmonary mast cells by allergen mediated cross-linking of IgE antibodies bound to the high affinity IgE receptor (Fc ϵ RI). As a result, numerous mediators are released which ultimately cause immediate airway constriction [21]. The higher production of IgE, therefore, is more favorable for degranulation of mast cells and thereby causes more airway constriction after allergen exposure. In the present study, we have observed an increased level of OVA-specific IgE and increased airway constriction in OVA/OVA/VEH mice as compared to SHAM/SAL/VEH group. The decreased airway constriction by the treatment of GRZ could be therefore by inhibiting the IgE levels. Further, it increases IFN- γ which probably inhibits IL-4 production and in turn reduces the IgE production. It is, therefore, possible that GRZ may have inhibitory effects on mast cell degranulation by reducing the production of IgE which ultimately reduce the OVA-induced immediate airway constriction.

The late phase airway response in asthma is associated with the infiltration of the inflammatory cells especially eosinophils to the site of inflammation. The recruitment of eosinophils into the airway and its long-term survival are mediated by IL-5 [22]. It is known that inflammation in allergic asthma is mediated by the increased expression of

multiple pro-inflammatory genes, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors which are mostly regulated by increased gene transcription controlled by transcription factors such as nuclear factor-kB (NF-kB) and activating protein-1 (AP-1) [23]. NF-kB was found to be markedly activated which in turn regulates many of the inflammatory genes that are abnormally expressed in asthma [24,25]. Although GRZ has not been demonstrated to inhibit NF-kB binding in the airway or lung inflammation, however, it has been shown that it inhibited NF-kB binding activity in the rat liver cirrhosis model [26]. The recruitment of inflammatory cells from blood vessel to the airway needs various selectins and the blocking of these selectins has been demonstrated to inhibit antigen induced airway inflammation and late airway responsiveness in animal models [27,28]. Interestingly sodium salt of GRZ has been demonstrated to inhibit E-selectin, L-selectin and P-selectin-mediated human eosinophils and neutrophils adhesion to the umbilical vein endothelial cells [29]. It is interesting to note that GRZ inhibits eotaxin-1 level via STAT6 in human lung fibroblasts [30]. It is quite possible that GRZ may block eotaxin levels in vivo as well and block infiltration of eosinophils in the lungs. Also, in our study we demonstrated that GRZ treatment could reduce pathophysiological as well as immunological components of late phase airway response like reduction of airway hyper-reactivity to methacholine (Fig. 3), reduction of absolute number of eosinophils in the BAL fluid (Table 2), reduction of eosinophil percentage in the lung and reduction of IL-5 cytokine in the BAL fluid (Table 1). Hence, it might be possible that the reduced infiltration of eosinophils into the airway lumen could be due to the effect of GRZ in blocking the activation of NF-kB in the airways. However, further investigations are required to elucidate its effect on NF-kB and other transcription factors, including AP1, CEBP (CCAAT enhancer-binding protein) and CREB (cyclic adenosine monophosphate response element-binding protein)-binding protein (CBP) in airways [23].

To find the mode of action of GRZ against asthma, we also measured the serum cortisol levels. Interestingly, our experimental results indicate that it had no effect on serum cortisol levels (Table 3). These results are similar to initial observations in which 11-HSD inhibition was not found at low concentrations of GRZ metabolite. But inhibition was observed when glycyrrhithenic acid was given in high concentrations [8,9]. This indicates that the mechanism of action of GRZ in our study is cortisol independent. In contrast, dexamethasone treatment to OVA induced mice caused significant reduction of serum cortisol levels. This kind of suppression was reported not only for oral corticosteroids but also for inhaled steroids [31–33].

In conclusion, GRZ significantly alleviates the asthmatic features such as OVA induced immediate airway constriction, airway hyperreactivity to methacholine, eosinophilia and lung inflammation. GRZ, therefore, could be useful towards developing better therapeutic molecule in the future.

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