Effects of A Polyherbal Agent on Structural Changes and Biochemical Markers During Airway Remodelling in Experimental Model of Bronchial Asthma

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Abstract
UNIM-352, a polyherbal preparation used in the traditional Unani system of medicine for bronchial asthma. The present study evaluated the possible effects of UNIM-352 on biochemical markers and structural changes in allergen induced airway-remodelling in rats. Wistar rats were immunized on day 1 with ovalbumin and Al(OH)3 and challenged with aerosolized ovalbumin from day 15 to 21. They were then divided into four groups and treated orally with vehicle, UNIM-352 (200 or 400 mg/kg) or prednisolone (10 mg/kg). After 24 hours of last challenge, blood, bronchoalveolar lavage (BAL) fluid and lung tissue were collected and assayed for (a) cytokine levels (TGF-β and IL-13); (b) hydroxyproline content, and (c) histopathology, and the effects of various drug treatments were compared with vehicle controls. The results showed that UNIM-352 markedly reduced the levels of TGF-β, IL-13 in blood and BAL fluid and hydroxyproline content in lung homogenates. Histopathological examination of lung tissue showed that the polyherbal agent had an attenuating effect on inflammatory cells infiltration, goblet cell hyperplasia and sub epithelial fibrosis. The results suggest that UNIM-352 prevents the development and progress of the structural and biochemical changes seen during airway remodelling and thus could be beneficial in cases of chronic refractory bronchial asthma.

Keywords: Airway remodelling; Bronchial asthma; Herbal; Hydroxyproline; IL-13; TGF-β; UNIM-352

Introduction
Bronchial asthma is a chronic inflammatory disorder of the airways that leads to airway inflammation, tissue injury and subsequent abnormal structural changes collectively referred to as airway remodeling [1]. These changes include airway wall thickness, epithelial alterations, inflammatory cell infiltration, sub epithelial fibrosis, goblet cell hyperplasia, hypertrophy and hyperplasia of airway smooth muscle and increased vascularity [2-7]. The airway smooth muscle cells may involve in promoting inflammation and remodeling by the release of several inflammatory mediators such as transforming growth factor-β (TGF-β), Th2 cytokines (IL-13, IL-5, IL-9 etc.) and extracellular matrix (ECM) proteins [8]. Corticosteroids and bronchodilators are the standard treatment for bronchial asthma that markedly inhibits the lung inflammation and bronchial constriction. But, the significant incidence of side effects and refractoriness to these drugs is a major problem [9,10]. Therefore, there has been a search for novel compounds and has been extended to drugs used in the traditional system of medicine that can provide a better treatment for bronchial asthma.

In Indian traditional systems of medicine, the herbal drugs play a significant role and being used in the treatment of various diseases. Most of the drugs used in Unani and Ayurveda are natural products mainly based on plant sources [11,12]. UNIM-352 is a traditional Unani formulation, which has been clinically used in India for the treatment of bronchial asthma [13,14]. UNIM-352 contain six different herbal ingredients and all are well known traditional medicines, viz. Linum usitatissimum [15], Trigonella foenum-graecum [16], Allium sativum [17], Strychnos potatorum [18], Caesalpinia bonducella [19], and Pongamia glabra [20]. Previous studies showed that UNIM-352 has anti-inflammatory, immunomodulatory, mast cell stabilizing and Broncho relaxant effects in the experimental model of bronchial
Asthma [21,22]. Experimental studies also showed that UNIM-352 prevents the infiltration of neutrophil and eosinophil cells as well as inhibits the pro-inflammatory and Th-2 cytokines such as TNF-α, IL-4, GM-CSF. UNIM-352 also blocks the inflammatory gene transcription by inhibiting NF-κB and activating histone deacetylase [23]. Based on these observations, the present study was designed to evaluate the effects of UNIM-352 on biochemical markers and structural changes during airway remodelling in rats.

**Materials and Methods**

**Experimental animals**

Wistar rats, weighing 180-220g were used in the present study. They were housed in polyacrylic cages and kept in standard controlled room with natural light and dark cycle. Rats were fed with standard food pellets and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC/7/2011) and the guidelines of Indian National Science Academy, New Delhi were followed for care and safety of animals in scientific research.

**Drugs and chemicals**

UNIM-352 (Batch No. 01/11-12) used in the present study, is a standardized formulation prepared and supplied by Central Research Institute of Unani Medicine (CRIUM), Hyderabad, under the auspices of Central Council for Research in Unani Medicine (CCRUM), Ministry of AYUSH, Government of India, New Delhi. It contains a mixture of following ingredients: *Linum usitatissimum* L. (1g), *Trigonella foenum-graecum* L. (2g), *Allium sativum* L. (3.5g), *Strychnos potatorum* L. (7g), *Caesalpinia bonducella* Fleming (1g), *Pongamia glabra* Vent (1g), and Honey (q.s.) in 20g of preparation. This dark brown colored semi-solid preparation was dissolved in distilled water before administration to rats. Prednisolone, Albumin from chicken egg white (OVA), Chloramine-T-hydrate, Trans-4-hydroxy-L-proline, 4-(dimethylamino) benzaldehyde were purchased from M/s. Sigma-Aldrich, St Louis, MO, USA. Rat TGF-β and IL-13 ELISA kits were procured from USCN Life Science Inc. (Wuhan, China) and Cusabio Biotech Co., Ltd. (Wuhan, China) respectively.

**Experimental groups and treatment**

Wistar rats were divided randomly into four groups each containing six rats and subjected to treatment protocol for three weeks: (i) Control group: rats were immunized and challenged with OVA and treated orally with distilled water (vehicle); (ii) and (iii) UNIM-352 (200) and (400) groups: rats were immunized and challenged with OVA and treated orally with UNIM-352 at the dose of 200 or 400 mg/kg from day 1 to 21; (iv) Prednisolone group: rats were immunized and challenged with OVA and treated orally with prednisolone (10 mg/kg) from day 1 to 21.

**Immunization and challenge protocol**

All animals in each group were immunized and challenged with OVA according to the procedure described by Dong, *et al.* (2008) with some modifications [24]. Briefly, rats were immunized by intraperitoneal injection of OVA (50 mg per rat) emulsified with 100 mg of aluminium hydroxide in 1 ml of normal saline on the first day of the study. From day 15 to 21, rats were daily exposed to aerosolized OVA (2% in normal saline) for 30 minutes. The challenge was carried out in a Plexiglas box (38cm x 30cm x 22cm) by using ultrasonic nebulizer (Aerneb Lab Nebulizer System, Ireland).

**Blood and BAL fluid collection**

After 24 hours of last OVA challenge, all animals were anesthetized. Blood was collected by cardiac puncture and centrifuged at 3000 rpm for 10 minutes at 4°C and the serum was separated. After sacrificing the animals, the trachea was cannulated and normal saline was slowly injected into the lung and withdrawn. After the collection of BAL fluid, the samples were maintained at 4°C and centrifuged at 1500 rpm for 10 minutes and the supernatant was collected for biochemical analysis. The serum and BAL fluid supernatant were stored at -80°C for assay of TGF-β and IL-13.
Histopathological study of lung tissue

Immediately after the BAL fluid collection, the left lung of each rat was removed, fixed in 10% formalin solution and processed. The sections of the lungs were stained with hematoxylin-eosin and histopathological examination was carried out and comparison done among various drug treatment groups.

Hydroxyproline assay

The right lung was removed and the total collagen content, an indicator of lung fibrosis was evaluated by determining hydroxyproline content according to the procedure described by Stegemann & Stalder (1967) [25]. Briefly, lung tissue was weighed and homogenized in normal saline and then digested in 3 ml of 6N HCl for 8 hours at 130⁰C. Each sample was neutralized and then freshly prepared chloramine-T reagent was added. The samples were left at room temperature for 20 minutes and then dimethylamino benzaldehyde-perchloric acid reagent was added to each sample. The samples were incubated at 60⁰C for 15 minutes and then cooled under tap water. Finally, the absorbance of each sample was read at 550 nm on a spectrophotometer. The hydroxyproline content is expressed as μg/g tissue.

Assay of cytokines

TGF-β and IL-13 in serum and BAL fluid were analysed by using commercially available ELISA test kits as per manufacturer’s instructions. Briefly, the microtiter plate was pre-coated with an antibody specific to TGF-β and IL-13. Antigen and biotin-conjugated polyclonal antibody preparation specific for TGF-β and IL-13 were simultaneously incubated for specified periods. Then, streptavidin horseradish peroxidase and TMB substrate was added to produce a coloured reaction product. The absorbance was read at a wavelength of 450 nm using ELISA plate reader and results were expressed as pg/ml.

Statistical analysis

GraphPad Prism software was used for statistical analysis. All values were represented as means ± S.E.M. and analysed by using one-way ANOVA followed by post hoc Dunnett’s multiple comparison test. p < 0.05 was considered as statistically significant.

Results

Effect of UNIM-352 on cytokine levels

The results showed that sensitization and challenge with ovalbumin resulted in significant increase in the level of TGF-β to 302.3 ± 58.74 pg/ml in blood and 551.0 ± 62.15 pg/ml in BAL fluid, as compared to normal group, where the levels were lower than the detectable limit.

Analysis of the data after different treatments (UNIM-352 and prednisolone) showed that the values of TGF-β levels were significantly different across all groups [F (3, 23) = 5.9, for blood; and F (3, 23) = 5.6, for BAL fluid; p < 0.05 for both blood and BAL fluid]. Intergroup analysis showed that 21 days’ treatment with UNIM-352 at both the doses (200 and 400 mg/kg) and prednisolone (10 mg/kg) in ovalbumin immunized and challenged rats reduced the levels of TGF-β in both blood and BAL fluid, as compared to vehicle treated control group. The reduction in TGF-β levels were significantly marked with the higher dose (400 mg/kg) of UNIM-352 and prednisolone (p < 0.05). These results are summarized in Figure 1.

Similar to that observed for TGF-β, the levels of IL-13 were significantly enhanced in ovalbumin sensitized and challenged rats as compared to normal group, i.e. the levels were 7.94 ± 0.47 pg/ml (blood), 10.47 ± 0.78 pg/ml (BAL fluid) in normal and 80.95 ± 3.10 pg/ml (blood), 141.40 ± 5.01 pg/ml (BAL fluid) in sensitized rats and the levels were increased by 919% in blood and 1250% in BAL fluid.

Analysis of the data of IL-13 levels after different treatments showed the values were significantly different across all groups [F (3, 23) = 3.2, for blood; and F (3, 23) = 4.9, for BAL fluid; p < 0.05 in each case]. Intergroup analysis showed that 21 days’ treatment with UNIM-352 at both the doses (200 and 400 mg/kg) and prednisolone in ovalbumin immunized and challenged rats reduced the levels of IL-13 in both blood and BAL fluid, as compared to vehicle treated control group. The reduction in IL-13 levels were significantly marked with the higher dose of UNIM-352 and prednisolone (p < 0.05). These results are summarized in Figure 2.
Effects of A Polyherbal Agent on Structural Changes and Biochemical Markers During Airway Remodelling in Experimental Model of Bronchial Asthma

Figure 1: Effects of UNIM-352 on TGF-β levels in blood and BAL fluid of OVA immunized and challenged rats. Data are expressed as Mean ± SEM. *p < 0.05, as compared to control group. Control: OVA immunized and challenged rats treated with vehicle. UNIM-352 (200) & UNIM-352 (400): OVA immunized and challenged rats treated with UNIM-352 (200 mg/kg) and UNIM-352 (400 mg/kg) respectively. Prednisolone: OVA immunized and challenged rats treated with Prednisolone (10 mg/kg).

Figure 2: Effects of UNIM-352 on IL-13 levels in blood and BAL fluid in OVA immunized and challenged rats. Data are expressed as Mean ± SEM. *p < 0.05, as compared to control group. Control: OVA immunized and challenged rats treated with vehicle. UNIM-352 (200) & UNIM-352 (400): OVA immunized and challenged rats treated with UNIM-352 (200 mg/kg) and UNIM-352 (400 mg/kg) respectively. Prednisolone: OVA immunized and challenged rats treated with Prednisolone (10 mg/kg).

Effect of UNIM-352 on hydroxyproline content

The results showed that sensitization and challenge with ovalbumin resulted in significant increase in the level of lung hydroxyproline content as compared to normal group, i.e. the levels were 111.6 ± 4.4 μg/g tissue and 250.4 ± 8.1 μg/g tissue in normal and sensitized rats respectively and the levels were increased by 124%.

Analysis of the data showed that there were significant changes in hydroxyproline content of lung homogenate after various treatments across all groups [F (3, 23) = 5.3; p<0.05]. Intergroup analysis revealed that, in ovalbumin immunized and challenged rats, UNIM-352 at both the dose levels (200 and 400 mg/kg) significantly reduced the lung hydroxyproline content in a dose dependent manner; when compared with the vehicle treated control group rats (p<0.05). The reductions were comparable with the standard drug, prednisolone (p<0.05). UNIM-352 (200 and 400 mg/kg) decreased the hydroxyproline content of lung homogenate to 199.1 ± 17.3 μg/g tissue and 191.7 ± 15.63 μg/g tissue respectively, whereas prednisolone induced suppression was 174.2 ± 14.07 μg/g tissue, versus vehicle treated control group (250.4 ± 8.1 μg/g tissue). These results are summarized in Figure 3.

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**Effects of A Polyherbal Agent on Structural Changes and Biochemical Markers During Airway Remodelling in Experimental Model of Bronchial Asthma**

Figure 3: Effects of UNIM-352 on hydroxyproline content in lung tissue of OVA immunized and challenged rats. Data are expressed as Mean ± SEM. *p < 0.05, as compared to control group. Control: OVA immunized and challenged rats treated with vehicle. UNIM-352 (200) & UNIM-352 (400): OVA immunized and challenged rats treated with UNIM-352 (200 mg/kg) and UNIM-352 (400 mg/kg) respectively. Prednisolone: OVA immunized and challenged rats treated with Prednisolone (10 mg/kg).

**Histopathological findings**

Lung tissue sections from the vehicle treated ovalbumin sensitized and challenged control group rats showed marked histopathological changes, such as larger numbers of inflammatory cells infiltration, increased goblet cell hyperplasia and sub epithelial fibrosis as compared to that of normal rats. The extent of the above histopathological changes was much reduced in the UNIM-352 (200 and 400 mg/kg) treated group, which was comparable with the prednisolone treated groups. These results are shown in Figure 4.

Figure 4: Effects of UNIM-352 on histopathological changes in lung tissue of OVA immunized and challenged rats stained with hematoxylin-eosin, 10x. Control group showed more eosinophils, sub epithelial fibrosis and goblet cell hyperplasia as shown with arrows. Control: OVA immunized and challenged rats treated with vehicle. UNIM-352 (200) & UNIM-352 (400): OVA immunized and challenged rats treated with UNIM-352 (200 mg/kg) and UNIM-352 (400 mg/kg) respectively. Prednisolone: OVA immunized and challenged rats treated with Prednisolone (10 mg/kg).

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Discussion

Airway remodelling is a crucial feature in the pathophysiology of severe bronchial asthma. It plays a key role in the gradual irreversible loss of lung function in asthmatic patients [26]. Airway remodelling results from dysregulated repair processes in response to the tissue injury caused by inflammation, thus leading to permanent structural changes in the airways of asthmatic patients [27]. The histopathologic features of airway remodelling involve epithelial detachment, thickening of the basement membrane or subepithelial fibrosis, inflammatory cell infiltration, bronchial smooth muscle cell hypertrophy and hyperplasia along with increased mucus production. One of the most important features of airway remodelling is fibrosis caused by the deposition of collagen which is also a potential marker for disease severity [28]. Inflammatory cells and structural cells secrete several important mediators including TGF-β and IL-13, which regulate the process of airway remodelling. TGF-β was found to be of particular importance, because it has a broad spectrum of activities in pulmonary inflammation and fibrosis and is particularly increased in asthmatic patients, with severe form of disease [29]. Such remodelling changes can also be mediated through Th-2 pathway. IL-13, which is primarily a Th-2 cytokine, is reported to play an important role in subepithelial fibrosis, goblet cell hyperplasia and infiltration of inflammatory cells [30]. The standard therapy of bronchial asthma which include corticosteroids and β2 agonists do not always revert all these features of asthma. Furthermore, no therapeutic agent is known to have a satisfactory effect against airway remodelling. Therefore, safer and effective alternative forms of therapy are required for the management of bronchial asthma and associated airway remodelling. UNIM-352 is one such polyherbal formulation with well documented clinical use in acute and chronic asthmatic patients in Unani system of traditional medicine [13,14]. Therefore, in our present study, we experimentally evaluate the possible effects of UNIM-352 in the rat model of airway remodelling.

TGF-β is one of the most important and extensively studied mediators of tissue remodelling in asthmatic lung. TGF-β, a potent profibrotic cytokine has a role in enhancing proliferation of goblet cells and secretion of mucus [31]. TGF-β plays a significant role in subepithelial fibrosis by promoting proliferation of fibroblast and myofibroblast cells that secrete interstitial collagen and increases the apoptosis of airway epithelial cells [32]. TGF-β mRNA and protein expression is elevated in the airways and BAL fluid of patients with bronchial asthma and correlate with the severity of disease and degree of airway remodelling [33, 34]. In animal studies, administration of TGF-β in mice for four weeks has shown evidence of increased deposition of collagen, subepithelial fibrosis and airway hyper responsiveness [35]. In the present study, our results showed that both the doses of UNIM-352 (200 and 400 mg/kg, p.o.) reduced the level of TGF-β in both blood and BAL fluid in ovalbumin immunized and challenged rats. This observation indicates the reduction in TGF-β could be a mechanism of beneficial effect of the polyherbal agent in airway remodelling. McMillan et al. also showed that treatment with anti-TGF-β antibody reduced number of mucus producing goblet cells in an allergen induced airway remodelling in mouse model of asthma [36].

Bronchial asthma is a Th-2 lymphocyte mediated inflammatory response and IL-13, a Th-2 cytokine is considered as a key mediator of allergic asthma and airway remodelling [37,38]. Moreover, IL-13 stimulate various cells that are important in bronchial asthma, such as eosinophils, mast cells, B-cell, fibroblasts, epithelial cells and airway smooth muscle cells [39]. In humans, basal levels of IL-13 are elevated in the lungs during asthmatic attacks [40]. The role of IL-13 is evident from the study of Yang et al. who demonstrated that the administration of an anti-IL-13 monoclonal antibody effectively inhibited airway hyper responsiveness, inflammatory cells infiltration as well as airway remodelling [41]. Murine studies have shown that, IL-13 plays a key role in the differentiation of goblet cells [42] and mucus overproduction [30]. IL-13 is also a key fibrogenic factor associated with and induces subepithelial fibrosis that is mediated through upregulation and activation pathway of TGF-β [43]. In chronic asthma model, sub epithelial fibrosis and deposition of collagen were attenuated in IL-13 deficient mice or anti-IL-13 antibody treated mice [38]. Taken together, these factors strongly supported that IL-13 plays a broad role in the pathogenesis of asthma. The results of the present study showed that treatment with the different doses of polyherbal agent, UNIM-352 (200 and 400 mg/kg, p.o.) attenuated the levels of IL-13 in both blood and BAL fluid of ovalbumin immunized and challenged rats. Since IL-13 is a Th-2 dependent cytokine and plays a key role in various aspects of airway remodelling, UNIM-352 could provide protection against development of permanent structural changes in the bronchial airways.

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The deposition of extracellular collagen in the lung is an indicator of pulmonary fibrosis. Excessive accumulation of collagen has been reported in various diseases such as lung fibrosis, liver cirrhosis, tumour growth etc. Collagen is one of the important proteins which have the amino acid hydroxyproline, and measurement of hydroxyproline content of lung is widely accepted as an efficient marker of collagen production during lung fibrosis [44]. The results of our present study showed significantly reduced levels of hydroxyproline after UNIM-352 (200 and 400 mg/kg, orally) treatment in lung homogenate of ovalbumin immunized and challenged rats. Since collagen deposition is one of the important features of lung fibrosis and airway remodelling, UNIM-352 may be beneficial in the treatment and prevention of lung fibrosis and airway remodelling.

Asthmatic airway remodelling is characterized by abnormal epithelium formation, excessive deposition of extracellular collagen, infiltration of inflammatory cells, sub epithelial fibrosis, mucous gland hyperplasia and oedema. The increase in the number and size of goblet cells and submucosal glands results in excessive mucus production. This mucus hypersecretion may eventually contribute to airway hyper responsiveness and airway obstruction [3,45]. The histopathologic reports of the present study showed that treatment with the polyherbal agent, UNIM-352 (200 and 400 mg/kg, p.o.) attenuated the inflammatory cells infiltration, goblet cell hyperplasia and sub epithelial fibrosis, when compared with the control group rats. These results strongly suggest that UNIM-352 could be beneficial in various aspects of airway remodelling during asthma.

Conclusion

In conclusion, it can be inferred that both the doses of UNIM-352 (200 and 400 mg/kg) reduces the level of a) TGF-β, b) IL-13 and c) hydroxyproline content in a dose dependent manner. In addition, the lung histopathology report showed that it also attenuated inflammatory cells infiltration, goblet cell hyperplasia and sub epithelial fibrosis. These results suggest the anti-remodelling mechanism of UNIM-352, and propose it as an effective agent in the prevention of airway remodelling during bronchial asthma.

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Effects of A Polyherbal Agent on Structural Changes and Biochemical Markers During Airway Remodelling in Experimental Model of Bronchial Asthma


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