

5-Aminosalicylic Acid attenuates allergen-induced airway inflammation and oxidative stress in asthma



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ABSTRACT

Pro-inflammatory cytokines regulate the magnitude of allergic reactions during asthma. Tumor necrosis factor – alpha (TNF- α), interleukin-6 (IL-6) and interleukin-13 (IL-13) play a crucial role in aggravating the inflammatory conditions during allergic asthma. In addition, oxidative stress contributes to the pathogenesis of asthma by altering the physiological condition resulting in the development of *status asthmaticus*. Anti-inflammatory corticosteroids are being widely used for treating allergic asthma. In the present study 5-aminosalicylic acid (5-ASA), a salicylic acid derivative, was evaluated, *in vivo* for its potential to suppress TNF- α , IL-6 and IL-13 using ovalbumin (OVA) induced allergic asthma in Balb/C mice. Oral administration of 65, 130 and 195 mg/kg 5-ASA significantly reduced the OVA induced total and differential leucocyte count, TNF- α , IL-6, IL-13, nitrite, nitrate, MDA, MPO and TPL levels in the lung lavage samples. Collectively, these findings suggest that 5-ASA is a potent immunomodulator and suppresses key Th₂ cytokines production and oxidative stress in OVA-induced asthma.

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1. Introduction

According to the 'Global Burden of Asthma' report, the numbers of asthmatics in central and southern Asia have reached over 50 million. Worldwide, currently, about 300 million people are reported to be suffering from asthma. More alarmingly, by 2025, the number of patients suffering from asthma is expected to increase by an additional 100 million cases [1]. In addition, asthma has been declared as the most common chronic disease among children [2]. In India alone, about 57,000 deaths were reported in 2004 making this disease as one of the major health concerns requiring an immediate development of potent therapies [3,4].

Asthma is often characterized by episodic bronchoconstriction due to airway inflammation, remodeling and hyper-responsiveness [5]. The pathophysiological features of asthma include infiltration of eosinophils [6], neutrophils [7], lymphocytes and monocytes [8], activation of which results in excessive release of cytokines and chemokines in the lung parenchyma. In addition, mast cells play a predominant role in inflammation and immediate allergic reactions [9]. The cytokines further aggravate the inflammation inducing structural and functional changes in resident airway cells. Inflammatory mediators like histamine, [10] cytokines [11], leukotrienes, prostaglandins, thromboxanes [12], and free radicals like reactive oxidative species (ROS) [13] and reactive nitrogen species (RNS) [14] derived from infiltrated inflammatory cells and airway structural cells (e.g. epithelial cells, mucous cells) have been implicated in the pathophysiological changes in allergic asthma. Inflammatory cytokines such as TNF- α , IL-6 and IL-13 play critical role in the development and progression of airway inflammation.

Tumor necrosis factor-alpha (TNF- α) acts by binding to its receptors (p55), on cell membrane and induce a cascade of intracellular signaling events ultimately resulting in the activation of

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transcription factor nucleus-factor-kappa B (NF κ B) [15]. The activated NF κ B is then trans-located into the nucleus, initiating the coding of mRNA responsible for the synthesis of several key mediators including pro-inflammatory cytokines (TNF- α , IL-1 β , GM-CSF), adhesion molecules (ICAM-1, VCAM-1), chemokines (MIP-1 α , RANTES, eotaxin) and inflammatory enzymes (iNOS, cyclooxygenase-2) [16]. Interleukin-6 (IL-6) released by multitude of triggered cells including macrophages and epithelial cells inflates the magnitude of allergic responses during asthma by regulating the fate of effector CD4T cells, inhibiting Th₁ differentiation thereby promoting Th₂ differentiation leading to the production of IL-4. IL-13 released from T-helper 2 lymphocytes induces Stat-6 dependent signaling and transcription of several genes.

Oxidative stress contributes significantly to asthma pathogenesis. Activated inflammatory cells like eosinophils and neutrophils produce reactive oxygen species which when combined with nitric oxide form a potent peroxy nitryl radical that eventually results in nitrosylation of proteins and lipid peroxidation. S-nitrosylation (nitrosylation) involves the covalent incorporation of a nitric oxide moiety into thiol groups to form S-nitrosothiol (SNO). S-nitrosylation is a form of post translational protein modification with similarities to phosphorylation. They also activate T cells by increasing the production of Th2 cytokines [17,18].

Anti-inflammatory corticosteroids are used as mainstay anti-asthma medications. However, development of drug resistance followed by non-responsiveness of patients to corticosteroids such as dexamethasone on long term use pose a serious threat to clinical management of asthma [19]. In this regard, it is necessary to explore possible chemical moieties for their potential in suppressing aggravated immunological responses in asthmatic airways [20]. 5-ASA (mesalazine or mesalamine) is a derivative of salicylic acid and is being used in the treatment of inflammatory bowel disease (IBD) for over 30 years, primarily due to its immunomodulatory effect. 5-ASA inhibits I κ B kinase α -mediated phosphorylation of I κ B α in mouse intestinal epithelial cells where it was hypothesized to inhibit the production of TNF- α and other Th₂ cytokines [44]. Apart from this, 5-ASA is also a potent peroxy radical scavenger [45].

In this present study we evaluated the potential of 5-ASA in suppressing the production of TNF- α , Th₂ cytokines and oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin, Sodium potassium tartrate, Folin–Ciocateau's reagent and WBC diluting fluid were obtained from Loba Chemie, India. Giemsa stain was obtained from Rankem, India. Tumor necrosis factor – α (Cat#: K0331186) and interleukin – 6 (Cat#: K0331230) kits were obtained from Koma Biotech, South Korea. Interleukin – 13 kit (Cat#: ELM-IL13-001) was obtained from Ray Bio, USA. FAD was obtained from Hi-media, India. Greiss Reagent and Hexa-decyl-trimethyl ammonium bromide were obtained from Fluka, Germany. Thiobarbituric acid, trichloroacetic acid, NADPH, tetraethoxypropane, May-Grunwald stain, O-dianisidine and eosin were obtained from Sigma Aldrich, Germany. Hematoxylin was obtained from SDFCL, India. Dexamethasone was obtained as a gift sample from Ranbaxy, India. 5-aminosalicylic acid was procured from Enzo Life Sciences, USA.

2.2. Animals and grouping

Male Balb/C mice (20–28 g) were obtained from the central animal house facility of JSS College of Pharmacy, Udhagamandalam and maintained under standard environmental conditions

(22–28 °C, 60–70% relative humidity, and 12/12 light and dark cycle). Animals had access to standard laboratory feed (M/S Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The experimental protocol was approved by the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA) recognized Institutional Animal Ethics Committee (IAEC). The mice were divided randomly into six groups ($n = 6$). Group A served as control administered with normal saline. Group B served as OVA control administered with ovalbumin as an aerosol (2.5% w/v) using nebulizer (Smart care™ Ultrasonic Nebulizer, WH 2000). Group C served as positive control administered with ovalbumin followed by dexamethasone (30 mg/kg) from days 14 to 21. Groups D, E and F served as treatment groups administered with 5-ASA at 65, 130, and 195 mg/kg respectively by oral gavage one hour prior to airway challenge.

2.3. Ovalbumin (OVA) induced allergic asthma

All animals except vehicle control (Group A) were injected intraperitoneally (i.p.) with a mixture of OVA (50 μ g) and alum (1 mg) in 0.2 ml of normal saline, on days 0 and 7. On days 14 and 21, the mice were challenged with 2.5% w/v OVA aerosol through a nebulizer (Smart care™ Ultrasonic Nebulizer, WH 2000) delivering particles of 0.5–5 μ m size at a pressure range of 30–36 psi for 20 min. The saline control mice were exposed to saline aerosol for 20 min on day 14 and 21 (Fig. 1).

2.3.1. Treatment

5 ASA suspended in 1% carboxy methyl cellulose (CMC) was administered by oral gavage from days 15 to 20. The dose of 5-ASA was selected by converting human dose to a dose of 130 mg/kg (a median of minimal and maximal dose levels of 65 and 195 mg/kg were derived). The drug was prepared by suspending 0.65 g, 1.30 g and 1.95 g of 5-ASA in 10 ml of 1% CMC suspension respectively. The drug suspensions were prepared fresh daily prior to dosing and were administered according to individual body-weights. Dexamethasone was also prepared in a similar manner by suspending 100 mg in 10 ml of 1% CMC.

2.3.2. Collection of bronchoalveolar lavage fluid (BAL fluid)

After 24 h of final challenge, the mice were euthanized by administering thiopentone (80 mg/kg) intraperitoneally. A tracheal cannula was inserted via mid cervical incision and lavaged twice with 1 ml of ice-cold phosphate buffered saline (PBS, pH = 7.4). After collecting the BAL fluid, the lungs of the mice were extracted and a part of it was stored at –20 °C for the estimation of malonyldialdehyde, myeloperoxidase and total protein [20], while the remaining portion was stored in 10% formalin for histology studies.

2.3.3. Estimation of total and differential leucocyte count, enzyme-linked immunosorbent assay (ELISA) of IL-6, IL-13 and TNF- α , nitrate and nitrite levels in BALF

Estimation of total and differential leucocyte count was performed according to Thompson et al., 1996 [22]. IL-6, IL-13 and TNF- α were determined in the BAL fluid using respective ELISA kits according to the manufacturer's instructions. The levels of nitrate and nitrite were estimated according to the method reported by Toward et al., 2000 [23].

2.3.4. Estimation of malonyldialdehyde (MDA), myeloperoxidase (MPO) and total protein in lung samples

Malonyldialdehyde activity was determined using the method specified by Aruoma et al., 1989 [24]. Measurement of myeloperoxidase activity in lungs was performed by the method specified by

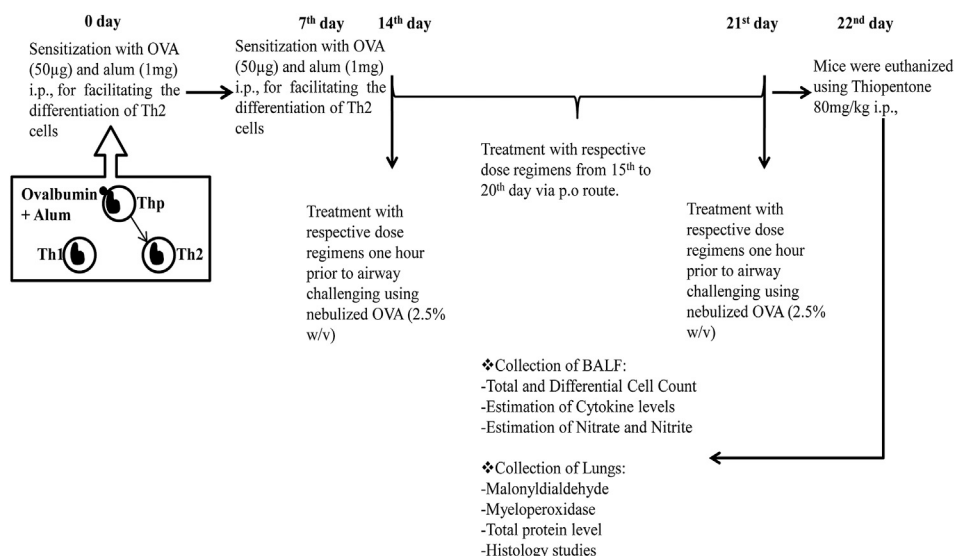


Fig. 1. Schematic depiction of the phase wise procedure of ovalbumin (OVA) induced allergic asthma model in Balb/C mice.

Bradley et al., 1982 [25]. The total protein level in the lungs was determined by using the method of Lowry et al., 1951 [26].

2.3.5. Histology study of lungs

The isolated lung tissue was immediately stored in 10% formalin. The paraffin embedded tissues were cut into 6 µm sections using tissue microtome (Leica, UK; Model No. RM2135). The sections were stained with hematoxylin and eosin stain for histological observations and with periodic acid Schiff stain (PAS) for morphological study of bronchi and goblet cells. The stained tissues were then mounted onto a microscope (Motic, Japan) and examined [27].

2.4. Statistical analysis

The statistical analysis was performed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparison tests. p value <0.05 was considered statistically significant. The analysis was carried out using Graph pad Prism software V.5.01 in Microsoft Windows 7 workstation.

3. Results

3.1. Effect of 5-ASA on total and differential leucocyte count in the BALF

To determine the effect of 5-ASA on total and differential leucocyte count in the BALF, mice were immunized with OVA and challenged with two OVA aerosol sprays. Compared to vehicle control, in OVA-treated mice a significant ($p < 0.001$) increase in

total cell count (from 8.17 to 26.17), eosinophils (from 0.73 to 20.17), monocytes (1.77–10.17) and neutrophils (26.21–39.5) was observed in the BAL fluid collected at 24th h after the last OVA challenge, confirming the induction of inflammation in mice (Table 1). Administration of 5-ASA through oral gavage reduced the OVA-induced total cell count, eosinophils, monocytes and neutrophils level in a dose dependent manner. At a dose of 195 mg/kg 5-ASA, the total leucocyte count and neutrophil number was found to be similar to those of vehicle control treated mice. In addition, oral administration of 195 mg/kg 5-ASA significantly ($p < 0.05$) improved the number of lymphocytes from 30.17 ± 2.91 (in OVA treated animals) to 63.17 ± 2.24 (which is very close to vehicle control animals i.e., 71.29 ± 2.19). The efficacy of 195 mg/kg 5-ASA was similar to 30 mg/kg dexamethasone, a known anti-inflammatory agent and a potent drug for treating asthma.

3.2. Effect of 5-ASA on $TNF-\alpha$, IL-6 and IL-13 in BAL fluid

$TNF-\alpha$ is a key cytokine which activates pro-inflammatory transcription factors NF κ B and AP-1 which then activate many inflammatory genes in asthmatic airway. Interleukin-6 (IL-6) is another pro-inflammatory marker secreted by T-cells and macrophages in response to an antigenic challenge and Interleukin-13 (IL-13) is a crucial cytokine which is responsible for the activation of B-cells to produce IgE and is also necessary for the survival of mast cells. We evaluated whether administration of 5-ASA reduces the OVA induced production of $TNF-\alpha$, IL-6 and IL-13 in BAL fluid. The BAL fluid was collected from vehicle control treated mice, mice challenged with OVA, and mice treated with 5-ASA. Analysis of the levels of $TNF-\alpha$, IL-6 and IL-13 in BAL fluid showed a significant

Table 1
Effect of 5-ASA on total and differential leucocyte counts in BALF of OVA sensitized Balb/C mice.

Group no.	Treatment	Total cell count (10^3 /ml)	Percentage of			
			Eosinophils	Monocytes	Lymphocytes	Neutrophils
1	Vehicle control	8.17 ± 1.42	0.73 ± 0.19	1.77 ± 1.27	71.29 ± 2.19	26.21 ± 1.25
2	OVA control	$26.17 \pm 2.64###$	$20.17 \pm 1.54###$	$10.17 \pm 1.08###$	$30.17 \pm 2.91###$	$39.50 \pm 1.41###$
3	(Dexamethasone 30 mg/kg)	$9.33 \pm 1.33***$	$6.50 \pm 1.12***$	$3.50 \pm 1.18**$	$61.83 \pm 1.64***$	$28.17 \pm 1.38***$
4	5 ASA 65 mg/kg	$17.83 \pm 1.05**$	$13.50 \pm 1.94**$	9.17 ± 1.45	$45.50 \pm 2.86**$	$32.17 \pm 1.70**$
5	5 ASA 130 mg/kg	$11.33 \pm 1.28***$	$8.83 \pm 1.08***$	$4.67 \pm 1.56*$	$56.33 \pm 2.26***$	$29.83 \pm 1.30***$
6	5 ASA 195 mg/kg	$8.83 \pm 1.19***$	$5.67 \pm 1.20***$	$3.17 \pm 1.14**$	$63.17 \pm 2.24***$	$28.00 \pm 1.59***$

Values expressed as mean $\pm$ SEM ($n = 6$) Statistical significance: $###p < 0.001$ vs. vehicle control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. OVA control.

increase in OVA group compared to vehicle control administered mice. Treating OVA administered mice with 5-ASA reduced the TNF- α levels in BAL fluid in a dose dependent manner (Fig. 2a). At a dose of 195 mg/kg 5-ASA, the TNF- α level was similar to that of 30 mg/kg dexamethasone treated mice. The data in case of IL-6 (Fig. 2b) showed a dose dependent reduction with increasing concentration of 5-ASA. At 195 mg/kg dose, an approximate 4-fold decrease in IL-6 compared to OVA control was observed. Similarly, treating OVA administered mice with 5-ASA reduced the IL-13 levels in BAL fluid in a dose dependent manner (Fig. 2c). At a dose of 195 mg/kg 5-ASA, the IL-13 level depicted a two-fold decrease compared to OVA control. Moreover, at this dose, the

level of IL-13 was significantly decreased compared to dexamethasone 30 mg/kg treated group.

3.3. Effect of 5-ASA on nitrates and nitrites in BAL fluid

The levels of nitrate and nitrite in BAL fluid determine the intensity of nitrogen radicals. Nitric Oxide Synthase (NOS) is an inflammatory enzyme released in response to allergens which amplifies the inflammation by synthesizing Nitric Oxide (NO). BAL fluid was collected from vehicle control treated mice and mice exposed to OVA and treated with 65 mg/kg, 130 mg/kg and 195 mg/kg 5-ASA. The data showed a dose dependent reduction in both nitrate and nitrite levels with increasing concentration of 5-ASA. At 195 mg/kg dose, an approximate four-fold decrease in nitrate level and a near two-fold decrease in nitrite level compared to OVA control were observed (Fig. 3a and b). Moreover at this dose, the nitrate and nitrite levels were similar to those of untreated control mice as well as the mice exposed to positive control dexamethasone 30 mg/kg.

3.4. Effect of 5-ASA on levels of malonyldialdehyde (MDA), myeloperoxidase (MPO) and total protein (TLP)

Malonyldialdehyde is a biomarker for oxidative stress estimated to quantify the degree of lipid peroxidation in lungs. MPO is a

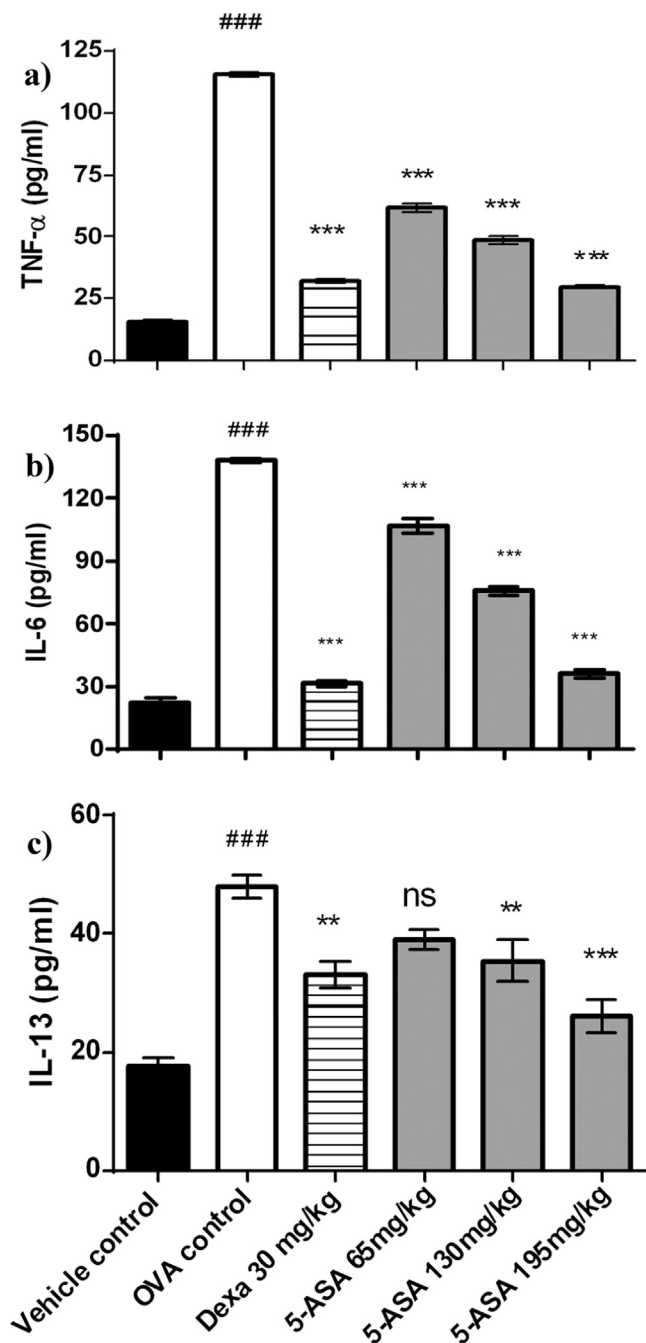


Fig. 2. Effect of 5-ASA on (a) TNF- α , (b) IL-6 and (c) IL-13 in BAL fluid of OVA sensitized Balb/C mice respectively. Graph represents mean $\pm$ SEM ($n = 6$). #### $p < 0.001$ vs. vehicle control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. OVA control.

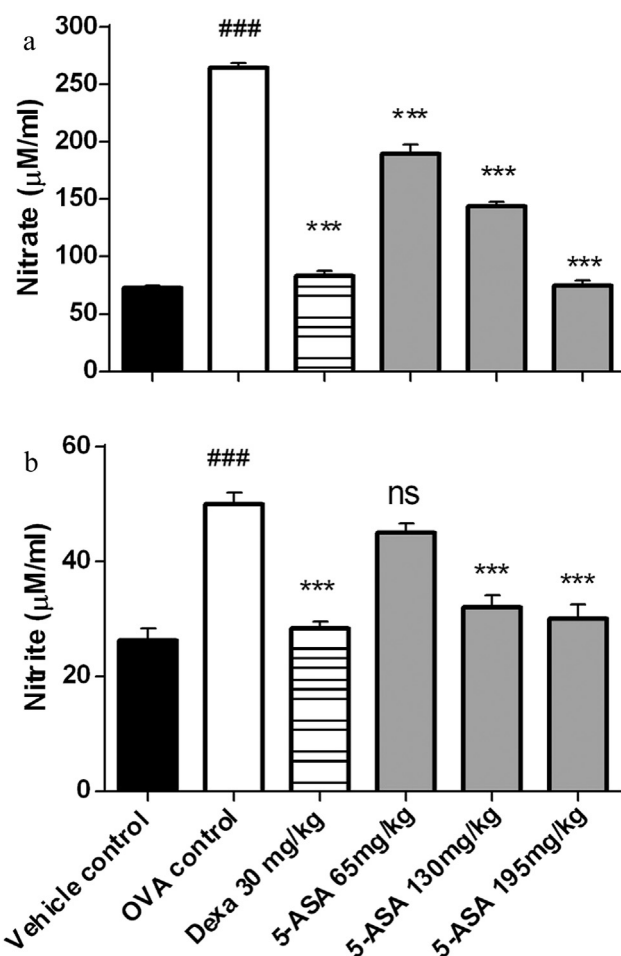


Fig. 3. Effect of 5-ASA on (a) nitrate and (b) nitrite level in BAL fluid of OVA sensitized Balb/C mice. Graph represents mean $\pm$ SEM ($n = 6$). #### $p < 0.001$ vs. vehicle control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. OVA control.

biomarker which reveals the extent of cell injury while total protein level in lungs sheds light on the degree of protein degradation. To evaluate the effect of 5-ASA on lipid peroxidation, myeloperoxidase and total protein, the lungs were collected from vehicle control treated mice, mice challenged with OVA and those treated with dexamethasone and 5-ASA at test doses. Analysis of the levels of MDA, MPO and TLP in lung homogenate showed a significant increase ($p < 0.05$) compared to vehicle control mice. Treating OVA administered mice with 5-ASA reduced the levels of MDA, MPO and TLP in lung homogenate in a dose dependent manner (Figs. 4a–c respectively). At a dose of 195 mg/kg 5-ASA, the MDA levels were similar to dexamethasone 30 mg/kg treated group; a threefold decrease was observed in case of MPO levels compared to OVA treated group and a twofold decrease in comparison to dexamethasone treated group. Similarly, at a dose of 195 mg/kg 5-ASA, the total protein content was significantly decreased by a near twofold and this was similar to that exhibited by dexamethasone treated group compared to OVA control.

3.5. Histology of bronchi

The histology of bronchi stained with hematoxylin and eosin (H&E) revealed normal architecture of sub-epithelial cells and sub-mucosal mucus glands with a mild degree of peribronchial inflammation and active goblet cells in mice treated with vehicle control. The mice treated with OVA showed a severe increase in peribronchial inflammation and active metaplastic goblet cells which were confirmed in periodic acid Schiff (PAS) stained sections (Figs. 5a and b). Among all tested doses, maximum structural restoration in bronchi was observed in 5-ASA at 130 mg/kg, which showed occasional areas of focal inflammation with functional goblet cells in bronchi compared with OVA control. Similar findings were observed in mice treated with dexamethasone. Whereas, 5-ASA at 195 mg/kg showed mild peribronchial inflammation with diffused pneumonia, severe congestion and highly active goblet cells with a degree of sub epithelial fibrosis.

4. Discussion

Allergic asthma, one of the most prevalent pulmonary diseases, has affected humans with diverse manifestations and is responsible for significant morbidity and economic impact [28]. Some of the epochal causes are increase in environmental pollution and predisposition of individuals producing excessive IgE because of a major change in the gene pool. A variety of drugs have been synthesized and tested individually as well as in combination for the treatment of asthma [20]. Even though initial success has been reported with combination therapies, recent studies have reported the development of resistance to long-term use of corticosteroids. Therefore, studies of late are exploring the possibility of replacing corticosteroids [20].

In this report, we present a preclinical evaluation of 5-ASA as a potential candidate to suppress inflammatory responses in allergic asthma. We carried out a detailed pharmacological assessment of 5 ASA against OVA induced allergic asthma in Balb/C mice. OVA model is best known for its significance in depicting the mechanism of allergic responses and immunological responsiveness creating a platform for scientists to understand the etiology of allergic asthma [30]. The cytokine secretion by activated T-lymphocytes, mast cells and injured airway epithelial cells confer to the pathogenesis of airway hyper reactivity and other features of asthma [31]. The regulatory role of CD4⁺ T-lymphocytes in asthmatic inflammation and the importance of Th2 cytokines have been highlighted by numerous studies in mice. The adjuvant (alum) used

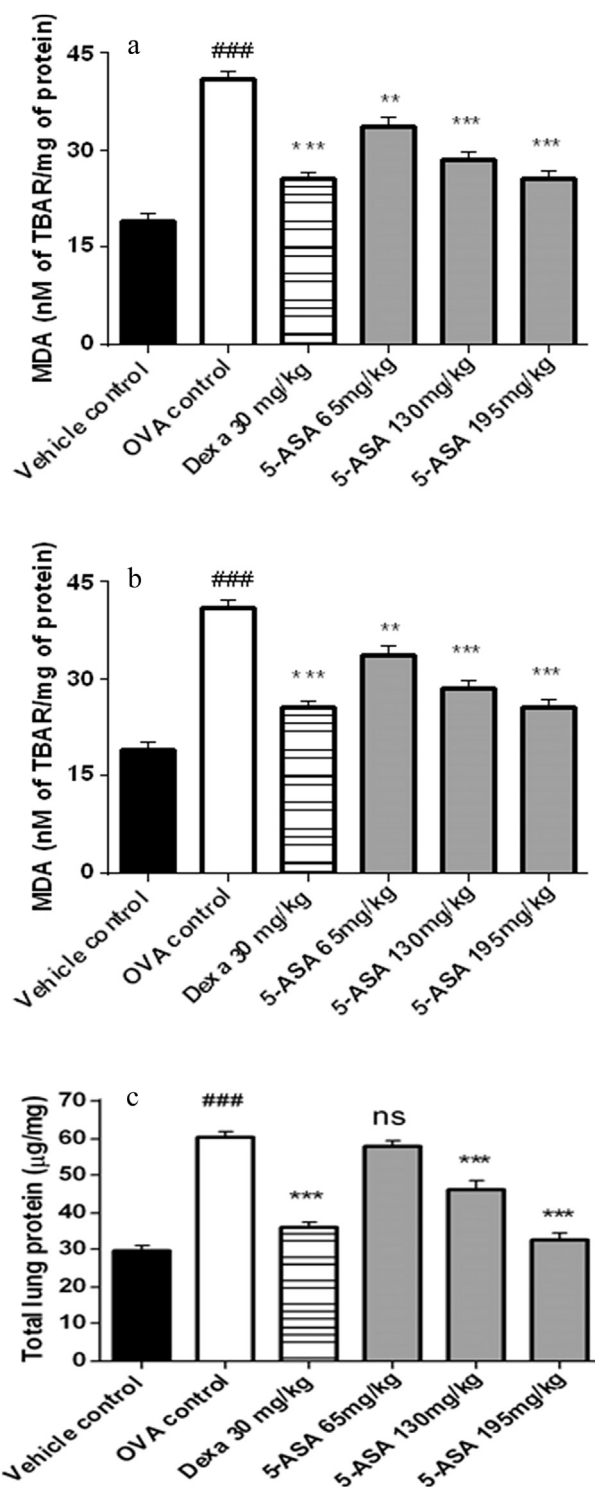


Fig. 4. Effect of 5-ASA on levels of (a) malonyldialdehyde, (b) myeloperoxidase and (c) total protein in lungs of OVA sensitized Balb/C mice. Graph represents mean $\pm$ SEM ($n = 6$). ^{###} $p < 0.001$ vs. vehicle control; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. OVA control.

in sensitization phase is known to promote Th₂ cells on coming in contact with the allergen [32].

One of the characteristic features of allergic inflammation is eosinophil infiltration, which is associated with the development of airway hyper responsiveness (AHR) by releasing basic proteins and oxygen derived free radicals [33,34]. Experimentally activated

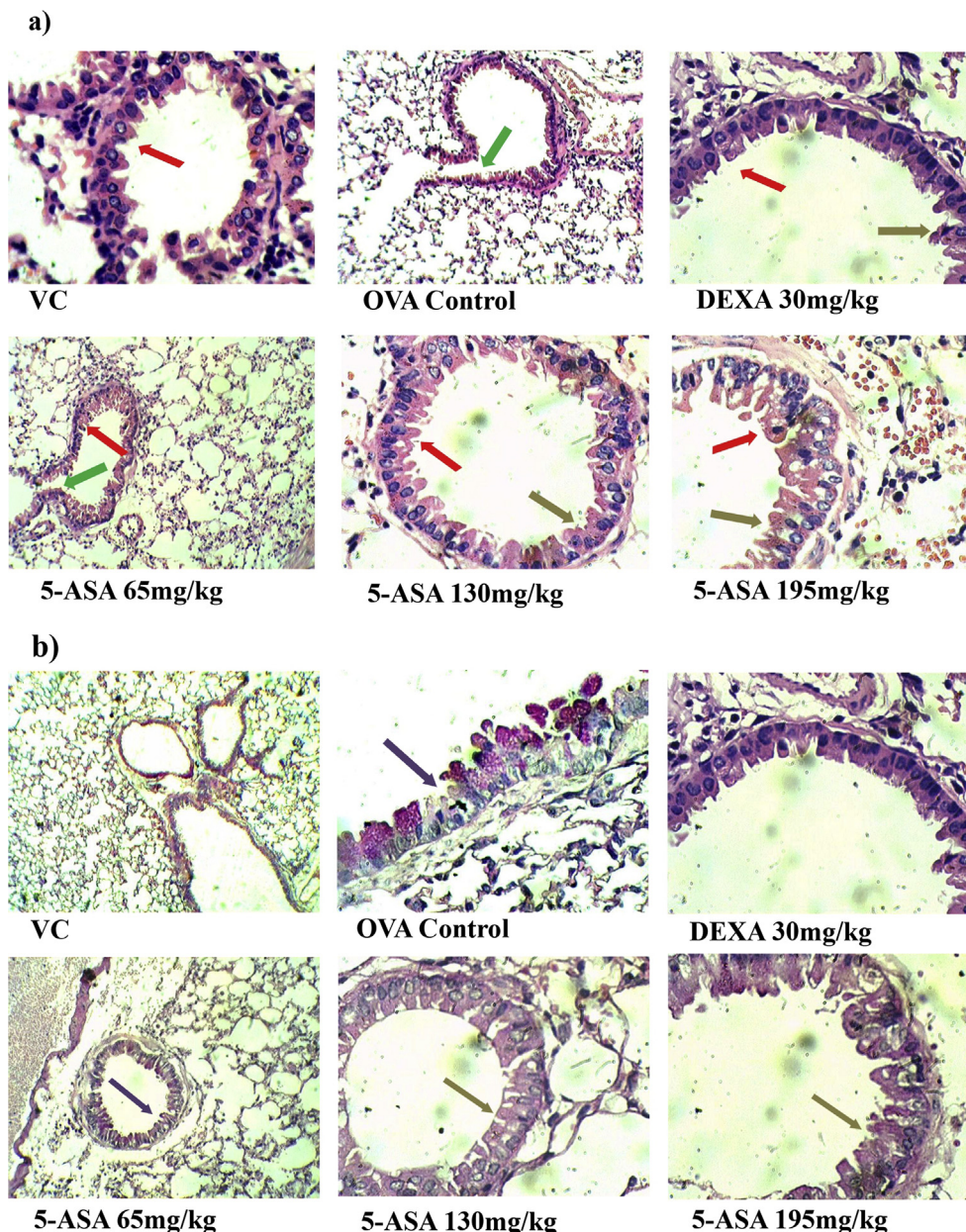


Fig. 5. Histological examination of lung tissues was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μm thicknesses, and stained with (A) hematoxylin and eosin (H&E) solution and (B) periodic acid Schiff solution (PAS), scale bars equal 50 μm . Vehicle control (VC), OVA control, DEXA 30 mg/kg, 5-ASA 65 mg/kg, 5-ASA 130 mg/kg and 5-ASA 195 mg/kg. Red, green, gray, violet and blue arrows denote active goblet cell, rupture of bronchi, epithelial regeneration, metaplastic goblet cells and subepithelial fibrosis respectively. Treatment of DEXA or 5-ASA was performed 1 h before the airway challenge. (Br) Bronchi. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eosinophils have been shown to induce airway epithelial damage, which is a characteristic of patients with asthma [35]. Neutrophils are not a predominant cell type observed in the airways of patients with mild-to-moderate chronic asthma. They appear to be a more prominent cell type in airways and induced sputum of patients with more severe asthma [12,36,37]. Lymphocytes play a crucial role in coordinating inflammatory response in asthma. T-lymphocytes are coded to express a distinctive pattern of cytokines, which are similar to that described in the murine Th_2 type of T-lymphocytes, which characteristically express IL-4, IL-5, IL-9 and IL-13 [38]. While, in allergic diseases B-lymphocytes secrete IgE and the factors regulating IgE secretion are now much better understood [39].

In this study, we found a significant increase in total and differential leucocyte count in BAL fluid of mice treated with OVA. There was a progressive decrease in total leucocytes count in mice treated with 5 ASA with a remarkable decrease at dose level of 195 mg/kg. Similar findings were observed in cases of neutrophil, eosinophil and monocyte count but vice versa in case of lymphocyte count when compared to OVA control.

The levels of $\text{TNF-}\alpha$ reveal the extent of sensitization of inflammatory cells to further amplify immune responses [40]. $\text{TNF-}\alpha$ acts as an amplifying mediator in asthma produced in increased amounts in the airways of asthmatics. It activates the pro-inflammatory transcription factors, nuclear factor-kappa B (NF κ B)

and activator protein-1 (AP-1) which then switch on many inflammatory genes [41]. The findings show that heightened levels of TNF- α and IL-6 were significantly reduced by 5-ASA at test doses, suggesting that 5-ASA could potentially be used in clinical practice. The elevated levels of IL-13 were significantly reduced in mice treated with 5-ASA at test doses. IL-13 mainly helps in determining the isotype switching in B-cells that result in IgE formation [42]. Reports have suggested that IL-13 was found in elevated levels in established disease rather than in the initial phase of the disease [43]. The maximum inhibition was observed at 195 mg/kg dose level for all three cytokines. This effect of the drug on cytokine levels might be due to its inhibitory property of I κ B degradation, which leads to NF κ B activation. NF κ B is a transcription factor present in the cytoplasm bound to I κ B in inactive state. Various cytokines and allergens cause ubiquitination of I κ B leading to activation of NF κ B, which is then displaced into nucleus and helps in production of mRNA. This mRNA then binds to ribosomes and various cytokines and inflammatory enzymes are synthesized [44].

In case of nitrate and nitrite levels, there was a significant decrease in the animals treated with 5-ASA at tested doses when compared to OVA control. This suggests a hypothesis that 5-ASA might possess inhibitory activity towards nitric oxide synthase (NOS). Increase in levels of nitrate and nitrite leads to development of oxidative stress contributing to the formation of peroxy nitrite radical which causes nitrosylation of proteins [45]. The levels of MDA and MPO were significantly reduced in mice treated with 5-ASA at tested doses. However, maximum reduction was observed in 195 mg/kg treated mice. Similar observations were recorded in case of total protein content. 5-ASA was effective in inhibiting the lipid peroxidation on the outer surface of membrane vesicles. It also inhibits oxidation of lipid membranes from both outside and within the cell which may be an important mechanism of cellular protection from oxidative stress [45]. 5-ASA acts as a reactive oxygen scavenger and also prevents xanthine oxidase – induced loss of DNA [46]. It was also found to be a strong peroxy radical scavenger in rabbit sarcoplasmic reticulum (SR) membrane [47]. 5-ASA was oxidized by the products of oxidative burst of the white blood cells, and protects CHO cells from damage caused by free radicals [48].

The above findings were further strengthened by histology studies. The tissues were stained with two types of stains, H&E and PAS. H&E staining was done for studying the morphological changes in the tissues, whereas PAS staining was done to study the detailed morphological changes of goblet cells. The airways of mice treated with OVA demonstrated structural changes which included goblet cell hyperplasia, goblet cell metaplasia, enlarged submucosal mucus glands, increased matrix deposition in the airway wall, increased airway smooth muscle mass, wall thickening and abnormalities in elastin. These changes were significantly reduced in the mice treated with 5-ASA at all three tested doses. However, there was a mild degree of sub-epithelial fibrosis observed in 195 mg/kg group which might be due to the acidic nature of the drug.

5. Conclusion

The findings from the present study demonstrate the anti-asthmatic effect of 5-ASA at a molecular level in ovalbumin induced allergic asthma in murine model. The results clearly indicate the regulatory effect of 5-ASA on inflammatory cells, cytokines (TNF- α , IL-6 and IL-13), nitrate and nitrite levels in the BAL fluid of mice. It also suggests the effect of 5-ASA on oxidative markers such as MDA, MPO and TLP by reducing their levels in the lung tissue of treated mice. Overall, the results suggest 5-ASA as a potent lead for regulating airway inflammation and can be a promising component of therapy for asthma patients.

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References

- [1] World asthma days. Global burden of asthma report. Embargoed; May 4, 2004.
- [2] <http://www.who.org/>.
- [3] South Asia Network for chronic Disease, Asthma in India. Available from: www.sancd.org/uploads/pdf/Asthma_factsheet.pdf [last accessed on 12.10.12].
- [4] Ganesh Kumar S, Premarajan KC, Sarkar S, Sahu K Swaroop, Sahana Ambika, Abhishek Antony. Prevalence and factors associated with asthma among schoolchildren in rural Puducherry, India. *Curr Pediatr Res* 2012;16(2): 159–63.
- [5] Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE. A new look at the pathogenesis of asthma. *Clin Sci* 2010;118:439–50.
- [6] Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma: relationship to bronchial hyperactivity. *Am Rev Respir Dis* 1988;137:62–9.
- [7] Sur S, Crotty TB, Kephart GM. Sudden onset fatal asthma: a distinct clinical entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev Respir Dis* 1993;148:713–9.
- [8] Romagnani S. The role of lymphocytes in allergic diseases. *J Allergy Clin Immunol* 2000;105:399–408.
- [9] Peachell P. Targeting the mast cell in asthma. *Curr Opin Pharmacol* 2005;5: 251–6.
- [10] Liu MC, Hubbard WC, Proud D, Stealey BA, Galli SJ, Kagey-Sobotka A. Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics: cellular, mediator and permeability changes. *Am Rev Respir Dis* 1991;144:51–8.
- [11] Chung KF, Barnes PJ. Cytokines in asthma. *Thorax* 1999;54:825–57.
- [12] Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997;156:737–43.
- [13] Kirkham P, Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol Ther* 2006;111:476–94.
- [14] Ricciardolo FL, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. *Eur J Pharmacol* 2006;533:240–52.
- [15] Barnes PJ, Karin M. Nuclear factor- κ B: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997;336:1066–71.
- [16] Demoly P, Basset-Seguín N, Chanez P. c-Fos proto-oncogene expression in bronchial biopsies of asthmatics. *Am J Respir Cell Mol Biol* 1992;7:128–33.
- [17] Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;111:291–7.
- [18] Lukacs NW. Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol* 2001;1:108–16.
- [19] Barnes PJ. Corticosteroid resistance in airway disease. *Proc Am Thorac Soc* 2004;1(3):264–8.
- [20] Hakim A, Adcock IM, Usmani OS. Corticosteroid resistance and novel anti-inflammatory therapies in chronic obstructive pulmonary disease: current evidence and future direction. *Drugs* 2012 Jul 9;72(10):1299–312. <http://dx.doi.org/10.2165/11634350-000000000-00000>.
- [22] Thompson AB, Teschler H, Wang YM, Konietzko N, Costabel U. Preparation of bronchoalveolar lavage fluid with microscope slide smears. *Eur Respir J* 1996;9:603–8.
- [23] Toward TJ, Broadley KJ. Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *Br J Pharmacol* 2000;131:271–81.
- [24] Aruoma OI, Halliwell B, Laughton MJ, Quinlan GJ, Gutteridge JM. The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron (II)–iron (III) complex. *Biochem J* 1989;258:617–20.

- [25] Bradley PP, Christensen RD, Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 1982;60:618–22.
- [26] Lowry OH, Rosenburgh NJ, Farr A, Randall RS. Protein measurement with folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [27] Aich Jyotirmoi, Mabalirajan Ulaganathan, Ahmad Tanveer, Agrawal Anurag, Ghosh Balam. Loss-of-function of inositol polyphosphate-4-phosphatase reversibly increases the severity of allergic airway inflammation. *Nature* 2012;3:877.
- [28] Mitchell EA. Was current treatment increasing asthma mortality and morbidity? *Thorax* 1989;44:81–4.
- [30] Kumar RK, Herbert Cristan, Foster PS. The “classical” ovalbumin challenge model of asthma in mice. *Curr Drug Targets* 2008;9:485–94.
- [31] Kumar RK, Temelkovski J, Mcneil HP, Hunter N. Airway inflammation in a murine model of chronic asthma: evidence for a local humoral immune response. *Clin Exp Allergy* 2000;30:1486–92.
- [32] Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 2008;1:213–20.
- [33] Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000;105:651–63.
- [34] Robinson DS, Kay AB, Wardlaw AJ. Eosinophils. *Clin Allergy Immunol* 2002;16:43–75.
- [35] Yukawa T, Read RC, Kroegel C. The effects of activated eosinophils and neutrophils on guinea pig airway epithelium in vitro. *Am J Respir Cell Mol Biol* 1990;2:341–54.
- [36] Jatakanon A, Uasaf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532–9.
- [37] Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;119:1329–36.
- [38] Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17:138–46.
- [39] Gould HJ, Beavil RL, Vercelli D. IgE isotype determination: epsilon-germline gene transcription, DNA recombination and B-cell differentiation. *Br Med Bull* 2000;56:908–24.
- [40] Thomas PS, Yates DH, Barnes PJ. Tumor necrosis factor- α increases airway responsiveness and sputum neutrophils in normal human subjects. *Am J Respir Crit Care Med* 1995;152:76–80.
- [41] Kips JC, Tavernier JH, Joos GF, Peleman RA, Pauwels RA. The potential role of tumor necrosis factor α in asthma. *Clin Exp Allergy* 1993;23:247–50.
- [42] Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J Allergy Clin Immunol* 2001;107:9–18.
- [43] Borish LC, Nelson HS, Corren J. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J Allergy Clin Immunol* 2001;107:963–70.
- [44] Yan Fang, Polk D Brent. Aminosalicic acid inhibits I κ B kinase α phosphorylation of I κ B α in mouse intestinal epithelial cells. *J Biol Chem* 1999;274:36631–6.
- [45] Pearson David C, Jourd'Heuil David, Meddings Jonathan B. The anti-oxidant properties of 5-aminosalicylic acid. *Free Radic Biol Med* 1996;21:367–73.
- [46] Craven PA, Pfanstiel J, Saito R, DeRubertis FR. Actions of sulfasalazine and 5-aminosalicylic acid as reactive oxygen scavengers in the suppression of bile acid-induced increases in colonic epithelial cell loss and proliferative activity. *Gastroenterology* 1987 Jun;92(6):1998–2008.
- [47] Dinis TC, Maderia VM, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem* 1994;315:161–9.
- [48] Dull Bob Jay, Salata Kalman, Van Langenhove Agnes, Goldman Peter. 5-Aminosalicylate: oxidation by activated leukocytes and protection of cultured cells from oxidative damage. *Biochem Pharmacol* 1987;36:2467–72.