

Intranasal curcumin and sodium butyrate modulates airway inflammation and fibrosis via HDAC inhibition in allergic asthma

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ABSTRACT

Asthma being an inflammatory disease of the airways lead to structural alterations in lungs which often results in the severity of the disease. Curcumin, diferuloylmethane, is well known for its medicinal properties but its anti-inflammatory potential via Histone deacetylase inhibition (HDACi) has not been revealed yet. Therefore, we have explored here, anti-inflammatory and anti-fibrotic potential of intranasal curcumin via HDAC inhibition and compared its potential with Sodium butyrate (SoB), a known histone deacetylase inhibitor of Class I and II series. Anti-inflammatory potential of SoB, has been investigated in cancer but not been studied in asthma before.

Materials and methods: In present study, ovalbumin (OVA) was used to sensitize Balb/c mice and later exposed to (1%) OVA aerosol. Curcumin (5 mg/kg) and Sodium butyrate (50 mg/kg) was administered through intranasal route an hour before OVA aerosol challenge. Efficacies of SoB and Curcumin as HDAC inhibitors were evaluated in terms of different inflammatory parameters like, total inflammatory cell count, reactive oxygen species (ROS), histamine release, nitric oxide and serum IgE levels. Inflammatory cell recruitment was analyzed by H&E staining and structural alterations were revealed by Masson's Trichrome staining of lung sections.

Results: Enhanced Matrix Metalloproteinase-2 and 9 (MMP-2 and MMP-9) activities were observed in bronchoalveolar lavage fluid (BALF) of asthmatic mice by gelatin zymography which was inhibited in both treatment groups. Protein expressions of MMP-9, HDAC 1, H3ack9 and NF-kB p65 were modulated in intranasal curcumin and SoB pretreatment groups.

Conclusion: This is the first report where intranasal curcumin inhibited asthma severity via affecting HDAC 1 (H3ack9) leading to NF-kB suppression in mouse model of allergic asthma.

1. Introduction

Airway inflammation, remodeling and hyperresponsiveness are characteristic features of allergic Asthma [1]. Structural changes within the airways include loss of epithelial membrane, subepithelial fibrosis, thickening of basement membrane, increased smooth muscle mass, neovascularity, myofibroblast and goblet cell metaplasia called as airway remodeling [2–6]. Due to these changes, airway wall becomes narrow leading to airway hyperresponsiveness (AHR) and irreversible loss of lung function [1].

Airway inflammation reflects specific pattern like intermittent wheezing, dyspnea, cough, and chest tightness [7]. As reported earlier, specific pattern in asthmatic patients is characterized by activation and degranulation of mast cells, increased levels of IL-4, IL-5 and IL-13 generated from Th2 cells, infiltration of eosinophils, and mucous

hypersecretion [8]. Upon activation and cross-linking of high affinity IgE receptors present on mast cells, preformed inflammatory mediators such as histamine, serotonin, TNF- α , leukotriens, prostaglandins, various other cytokines and chemokines are released [9]. Reactive Oxygen Species (ROS) generated during normal metabolic processes and from activated phagocytic cells like neutrophils, eosinophils, monocytes and macrophages where large amount of ROS is produced, can damage lipid, proteins, DNA and carbohydrates leading to abnormal cellular functions, tissue injury and enhanced inflammatory response [10,11].

Eosinophils, upon recruitment to the lungs, perform multiple functions including antigen presentation, degranulate to secrete various mediators like chemokines (CCL-11, CCL-22, MMPs), Cytokines (IL-13, IL-5, TGF- β , etc.) and various mediators like major basic protein (MBP) and erythropoietin [12]. Current anti-inflammatory agents mainly glucocorticoids are the cornerstone in the treatment of asthma, however,

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this is not effective in all patients. Severe asthmatics display glucocorticoid resistance [13] or effective only at higher doses which results in many side effects [14]. This may provide a rationale to develop new therapeutic strategies for asthma that can address airway remodeling and hyperresponsiveness in addition to inflammation.

Extracellular matrix is formed by dynamic assemblage of its components which include elastin, fibronectin, glycoproteins, vitronectin and members of the MMP family (MMP-1, MMP-2, MMP-9, and MMP-12) in addition to TIMPs (tissue inhibitors of MMPs) [15]. To maintain architect and integrity of airways, equilibrium is required between degradation and synthesis of ECM components [16]. Inflammatory and structural cell derived MMPs has been found associated with the progression of AHR, inflammatory cell trafficking and remodeling in asthmatic airways which also affects contraction, fibroblast invasion and accumulation of collagen in airways [17].

Histone acetylation and deacetylation, i.e., epigenetic modifications could directly affect the chromatin structure thus subsequently affect gene transcription and expression. Opposing activities of HATs and HDACs helps in maintaining steady state of acetylation in core histones [18]. Acetylation of lysine residues on H3 has been suggested to involve in transcription of inflammatory genes [19]. Methylation and acetylation of lysine 9 on histone 3 (H3K9) has got particular importance in context of inflammatory gene expression [20]. Recently, increased expression of H3acK9 was found in association with inhibition of inflammation and it has also proposed that hyperacetylation of H3K9 at A20 promoter region leads to subsequent increase in A20 expression [21]. A20, also known as TNFAIP3, is well-studied for its inhibitory action against NF- κ B. It encodes a dual ubiquitin editing protein and act as a negative feedback regulator in NF- κ B signaling [22,23].

A large array of genes involved in inflammation and immunity is regulated by a family of inducible transcription factors, NF- κ B. The family is composed of 5 members including p50 (NF- κ B 1), p52 (NF- κ B 2), p65 (Rel A), Rel B and Rel C [24]. Among these, p65 and p50 heterodimers are the common NF- κ B isoforms playing key role in immunoregulation, inflammation, cell survival and proliferation [25]. These proteins are normally associated with a family of inhibitory proteins, like I κ B family present in cytoplasm [26]. In addition to regulating inflammatory responses, NF- κ B also mediates activation, differentiation and function of T cells [27]. Besides these, NF- κ B also influences cell motility by activation of adhesion or matrix remodeling related genes, such as MMP-9 and vimentin [28].

Altered acetylation level of histones caused by overexpression of histone deacetylases result in abnormal transcriptional repression of several molecular pathways, as reported in oncological studies [29,30]. It is reported that, HDACs are involved in regulation of inflammatory responses and onset of immune diseases [31,32]. The HDAC family consist of 18 members that have important role in development and tissue homeostasis and their expression levels are related with glucocorticosteroid resistance [33,34]. Highly elevated expression of HDAC 1 was reported in case of another inflammatory disease such as arthritis [35]. HDAC1 and 2 have been reported to be involved in G1-to-S progression and B cell proliferation, either during development or activation of mature cells [36].

Nowadays, histone deacetylase inhibitors, a new class of anti-cancer drugs are gaining attention for its application in other diseases. It has been reported that HDACi can notably alleviate airway hyperresponsiveness and reduce the number of bronchial eosinophils, lymphocytes, and mucosal infiltration of inflammatory cells [37,38]. Nausea, vomiting, anorexia, dehydration, hematological adverse events are some common side effects associated with synthetic HDACi [39]. Discovering naturally occurring HDACi and using them alone, or in combination may reduce these adverse effects.

Curcumin (diferuloylmethane), a phytochemical derived from *Curcuma longa* (turmeric) is commonly used as spice and food coloring agent and is endowed with numerous biological properties related to human health. It has been shown to possess anti-inflammatory [40], anti-

allergic [41], anti-tumor and anti-oxidant properties [42] since long. Due to therapeutic efficacy, curcumin is being used for the treatment of wide variety of disorders including osteoarthritis [43], solid tumors [44], immune related diseases [45,46], inflammation and oxidative stress, etc. [47,48]. It is considered as a natural pan-HDAC inhibitor. A study in B cell non-Hodgkin Lymphoma (B-NHL) cell line (Raji cells) reported that curcumin has blocked Raji cell proliferation and down-regulated the expression of HDACs 1, 3 and 8 in dose-dependent manner [49]. Another study in medulloblastoma xenografts and transgenic mouse model of medulloblastoma revealed down regulation of HDAC 4 [50].

Butyrate, the simplest HDAC inhibitor, is metabolic product of intestinal bacteria [51]. Butyrate has been reported to play important role in regulation of epithelial cells in the colon region [52] and also suppresses interleukin (IL-8) production and enhances differentiation of epithelial cells in colon [53]. Low concentrations (IC50 in the mM range) of butyrate has been found effective in inhibiting class I and II HDACs except HDAC6 and 10, although it is not a specific HDAC inhibitor [54,55].

We hypothesized that sodium butyrate (SoB), an HDAC inhibitor, can be effective in attenuating structural changes in airways. As mentioned earlier, both CUR and SoB inhibit HDAC 1 expression which might be associated with the disease progression in OVA induced asthmatic model.

So, anti-asthmatic potential of curcumin and SoB were explored along with their respective efficacies and underlying pathways in modulating pathological features of asthma.

2. Material and methods

2.1. Experimental groups

Balb/c mice (6–8 weeks old; 20–24 g) were procured from central animal facility of Central Drug Research Institute, Lucknow, India and were maintained in animal house at 25 °C temperature and 12 h light: dark cycle. Procedures for experimental animal handling and sacrifice were approved by the Institutional Animal Ethical Committee Banaras Hindu University, Varanasi, India.

2.2. Reagents

Ovalbumin (OVA, grade V), Sodium butyrate and DCFDA (Dichlorofluorodiacetate) were purchased from Sigma- Aldrich (St Louis, MO, USA). Antibodies for HDAC 1, H3acK9, NF- κ B, β -actin and HRP-conjugated secondary antibody were purchased from cell signaling technology, whereas MMP-9 antibody was purchased from Thermo Scientific (US). Polyvinylidenedifluoride (PVDF) membranes were purchased from Genetix Biotech Asia Pvt. Ltd. whereas Immobilon western chemiluminescent HRP substrate kit was purchased from Merck (Darmstadt, Germany).

2.3. Development of OVA induced Asthma model

For developing acute phase asthmatic model, Balb/c mice (6–8 weeks; 20–25 g) were used. Mice were randomly divided into four groups (5mice/group) and named according to sensitization/challenge/treatment protocol. Group I- **Control** (Con), Group II- **(OVA)** (OVA + alum sensitized/OVA challenge); Group III- **OVA + DMSO** (OVA + alum sensitized/OVA challenge/ DMSO treated as vehicle, i.n.); Group IV- **OVA + CUR**, (OVA + alum sensitized/OVA challenge/ CUR treated (5 mg/kg, i.n.). Group V-**OVA + SOB**, (OVA + alum sensitized/OVA challenge/ treated with SoB 50 mg/kg, i.n.). Control mice received saline containing 4 mg alum intraperitoneally and challenged with saline alone. Rest of three groups were immunized with 0.2 ml saline suspension containing ovalbumin (50 μ g) emulsified in 4 mg of aluminium hydroxide on days 0, 7, and 14. From days 19 to 22, mice were subjected

Table 1

Grouping of animals. (OVA ovalbumin, CUR curcumin, SoB sodium butyrate, DMSO dimethylsulphoxide, i.n intranasal, i.p intraperitoneal).

Sr. No.	Group	Sensitization (i.p)	Challenge (aerosol)	Treatment (i.n)
1.	Control	saline	saline	–
2.	OVA	OVA-alum	1% OVA	–
3.	OVA + DMSO	OVA-alum	1% OVA	DMSO
4.	OVA + CUR	OVA-alum	1% OVA	CUR (5 mg/kg)
5.	OVA + SOB	OVA-alum	1% OVA	SoB (50 mg/kg)

to 1% OVA aerosol inhalation 30 min daily [56].

2.4. Treatment schedule and experimental plan

To evaluate the therapeutic effects of sodium butyrate and curcumin, each administered separately, an hour prior to OVA aerosol challenge. After the last OVA aerosol challenge, mice were sacrificed. BALF, serum and lungs were collected and stored for biochemical and histopathological analysis (Table 1 and Fig. 1).

2.5. Collection of Bronchoalveolar Lavage Fluid (BALF), serum and lungs

Mice were sacrificed after 24 h of last OVA aerosol challenge, BALF was collected and washed. Briefly, lung washings were centrifuged at 3000 rpm for 10 min at 4 °C. After centrifugation, total and differential cell counts were analyzed in BALF cell pellet. Lung lobes were fixed in 10% neutral buffer formalin and were stored at –80 °C along with BALF supernatants for further analysis. Serum was collected and stored at –20 °C for further analysis.

2.6. Total cell count

Total BALF cells were enumerated by using trypan blue dye exclusion test. For differential count, cells were cytospun onto glass slides, fixed in methanol and stained with giemsa. Inflammatory cells were counted by selecting five random fields on the basis of nuclear morphology.

2.7. Reactive oxygen species (ROS) in BALF

ROS was measured in BALF [57]. Briefly, 1×10^6 cells were separated from BALF pellet of each group and washed with PBS, plated in a 96 well black plate. Freshly prepared DCFDA (10 μ M) in DMSO was incubated at 37 °C in dark for 30 min. Microplate fluorescence reader (Bio-Tek instruments Inc., 9 Winooski, VT, USA) at excitation (485 nm) and emission (530 nm) was used to measure fluorescence and ROS levels

were represented as fluorescence intensity in arbitrary units.

2.8. Eosinophil peroxidase (EPO) activity

EPO activity was measured in both BALF and lungs [58]. Homogenized lungs (10% PBS (w/v) were centrifuged (6000 rpm at 4 °C for 15 min) and cell pellet was resuspended in PBS containing 0.5% HTAC and homogenized again followed by three freeze thaw cycles. Reaction mixture containing 1.5 mM OPD and 6.6 mM hydrogen peroxide H_2O_2 in 75 mM Tris–HCl (pH-8.0) mixed with samples followed by 30 min incubation at 20 °C in dark, H_2SO_4 (4 M) added to stop the reaction and results were shown in absorbance units.

2.9. Myeloperoxidase (MPO) activity determination

To investigate neutrophil infiltration and activation, MPO activity was calculated in lung tissue homogenate [59]. Briefly, lungs were homogenized in potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% CTAB (cetyl trimethyl ammonium bromide) and subjected to three freeze/thaw cycles (at –80 °C). The reaction mixture containing 0.002% hydrogen peroxide (H_2O_2) and 0.167 mg/ml O-dianisidine dihydrochloride was mixed with homogenate (300 μ l volume). Absorbance was calculated at 460 nm over 20 min.

2.10. Nitrite level estimation

Nitrite level in serum was detected as a measure of nitric oxide (NO release) [60]. Serum diluted in PBS (1:3) and mixed with 100 μ l VCl_3 (0.8 g/ml in 1 N HCl). Griess reagent (100 μ l) was prepared by mixing 1:1 solution of 1% sulphanilic acid in 5% orthophosphoric acid and 0.1 % N-(1-naphthyl) ethylenediamine and added to the above sample. The reaction mixture was incubated at 37 °C for 30 min and absorbance was taken in microplate reader (at 540 nm). Nitrite concentration was calculated against standard curves and expressed as (l M/ml) of serum.

2.11. Histamine assay in serum

Histamine release was estimated using flat bottomed (black well) plates in dark [61]. In brief, diluted serum (180 μ l) in 0.1 M HCl was mixed with NaOH (1 M, 36 μ l) and 1% w/v (9 μ l) OPT dissolved in acetone-free methanol. The reaction was stopped using 3 M HCl (18 μ l), after 20 min of incubation. Fluorescence intensities were measured using 360 nm excitation and 450 nm emission filters.

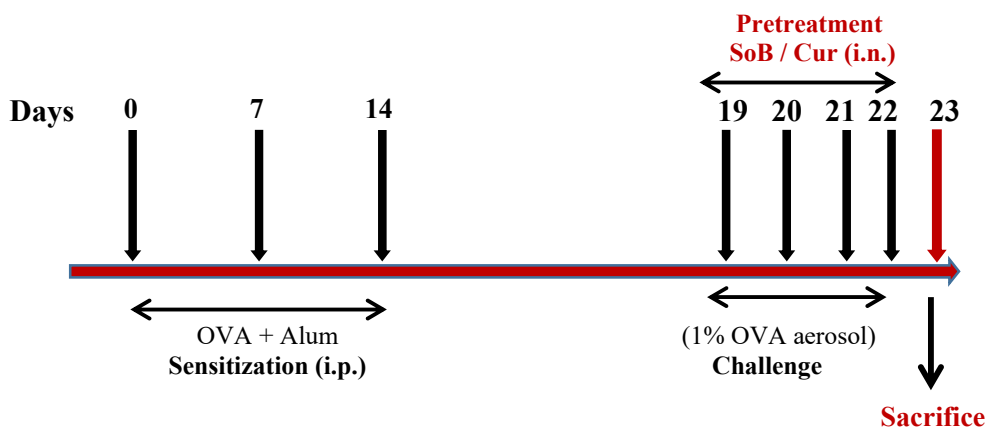


Fig. 1. Sensitization, challenge and treatment schedule of animals. Mice were sensitized and challenged with OVA+Alum and OVA aerosol respectively and treated with SOB or CUR accordingly.

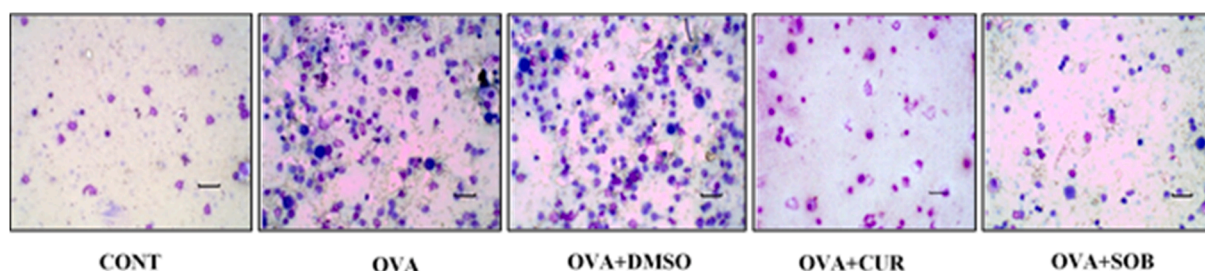


Fig. 2. Cytopsin preparations of BALF cells after giemsa staining (40 $\times$). Inflammatory cell accumulation was very high in OVA group as compared to control group whereas the number of cells was reduced in CUR and SOB treated groups.

2.12. Quantification of serum IgE level

OVA specific-IgE was measured in serum as reported earlier [61]. OVA (20 μ g) was used to coat microtitre plates with coating buffer (100 μ l, sodium carbonate and sodium bicarbonate, pH 9.6) at 4 $^{\circ}$ C overnight. Plate was washed 5 times on day 2 with PBST (0.05% Tween-20) and blocked with 1% BSA at 37 $^{\circ}$ C for 2 h, followed by washing. Samples (1:9) were incubated for 2 h at 37 $^{\circ}$ C, followed by three subsequent washings followed by incubation with HRP labelled anti-mouse IgE

(1:1000) for 2 h at 37 $^{\circ}$ C. OVA specific serum IgE was detected using TMB and reaction was stopped by using 100 μ l of 2.4 M sulphuric acid. Absorbance was measured at 450 nm.

2.13. Hydroxyproline content in lungs

Hydroxyproline content was estimated in lungs to study collagen deposition [62]. Lung homogenate (10%) was prepared in 12 N HCL and hydrolyzed (at 110 $^{\circ}$ C; 16–18 h) then suspended in citrate-acetate buffer

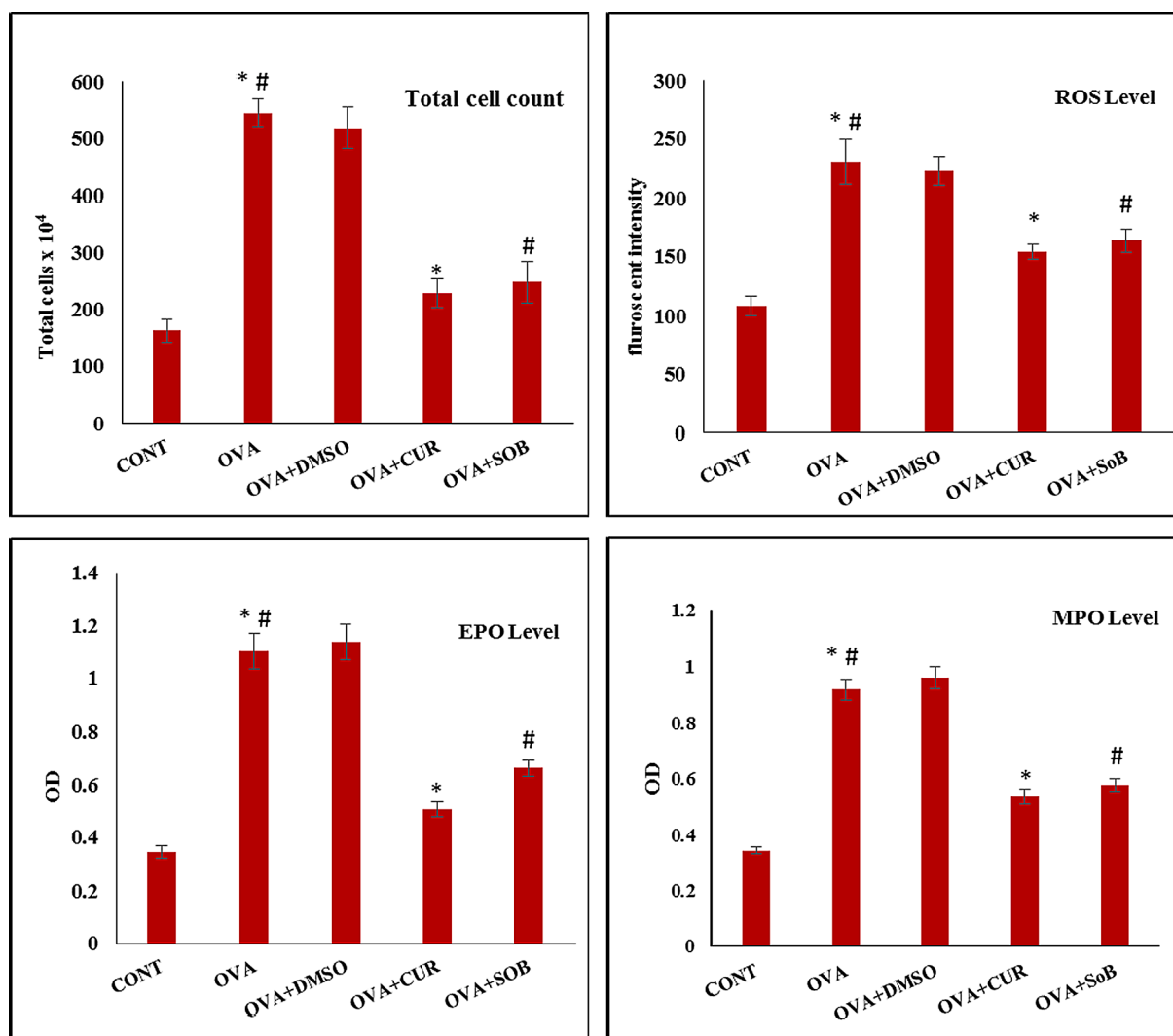


Fig. 3. Measurement of inflammatory cell recruitment and oxidative stress. Total cell count, ROS level, EPO and MPO activities in BALF pellet and lungs respectively, where marked increase in these parameters was observed in OVA induced group which was significantly reduced in CUR and SOB treated groups. Results are shown as means $\pm$ SEM ($p < 0.05$) * OVA vs. CUR group; # OVA vs. SOB group.

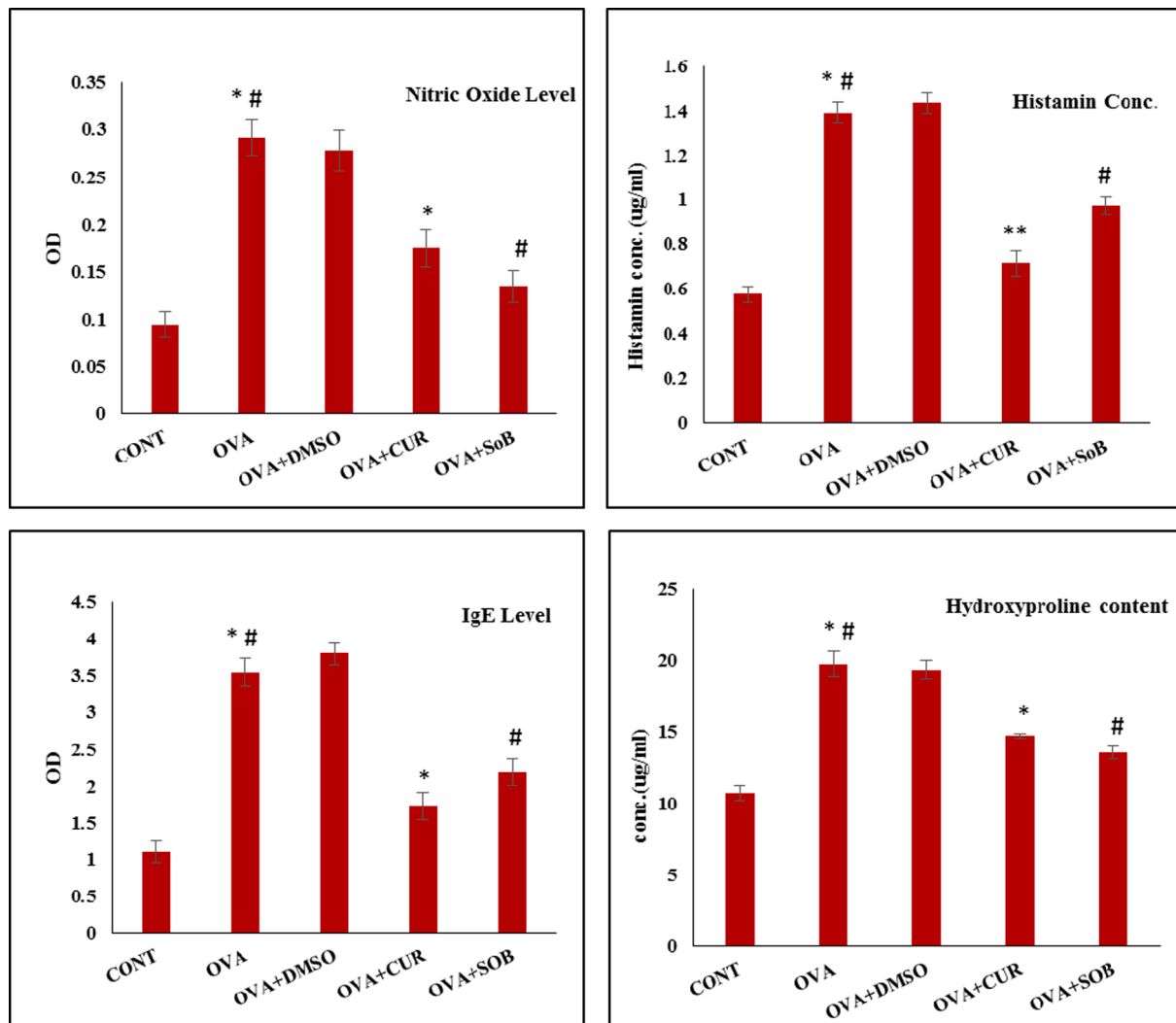


Fig. 4. Measurement of histamine, nitric oxide, IgE level in serum and hydroxyproline content in lungs. The level was significantly increased in OVA induced group as compared to control group which was significantly decreased in CUR and SoB treated groups. Results are shown as means $\pm$ SEM ($p < 0.05$) * OVA vs. CUR group; # OVA vs. SOB group; ** OVA+SoB vs. OVA+CUR group.

(5% citric acid, 7.24% sodium acetate, 1.2% glacial acetic acid, and 3.4% NaOH, pH 6.0), freshly prepared chloramine-T solution (1.4% chloramine-T, 10% N-propanol; 150 μ l) and 80% citrate-acetate buffer was added and incubated at room temperature for 20 min. Freshly prepared Ehrlich's solution (150 μ l; 4.5 g 4-dimethyl amino benzaldehyde dissolved in 18.6 ml n-propanol, 7.8 ml of 70% perchloric acid) was added at 65 $^{\circ}$ C for 15 min. Absorbance was taken at 550 nm and results were expressed in micrograms (hydroxyproline content in total lung tissue).

2.14. Gelatin zymography to determine collagenase activity

Gelatin zymography was performed in BALF to assess collagenase activity of MMP-2 and 9 [63]. Briefly, SDS-PAGE gel was prepared using gelatin (10%, 20 mg/ml). Protein concentrations of BALF supernatant were estimated using Lowry's method [64] and samples (50 μ g) from each group were electrophoresed at 4 $^{\circ}$ C and gel was washed thrice for 15 min in renaturing buffer containing 2.5% Triton X-100 on rocking platform. Gel was incubated in incubation buffer (50 mM Tris-HCl, 50 mM Tris base, 0.2 M NaCl, 5 mM CaCl₂ and 0.02% NaN₃; pH 7.5) at 37 $^{\circ}$ C for 48 h, then stained with Coomassie Brilliant Blue R 250 stain for 30 min and destained (5% methanol, 7% acetic acid in distilled water) and white bands were observed due to gelatinolytic activity of MMPs.

Bands were analyzed using image J software.

2.15. Lung histopathology

Lungs were fixed (10% formalin), embedded in paraffin and thin sections (5 μ m) were made followed by staining with hematoxylin and eosin (H&E) to assess inflammation and structural alterations. To detect collagen deposition as measure of airway remodeling, lung sections were subjected to Masson's trichrome staining and observed under light microscope (40 $\times$).

2.16. Immunoblotting

Lung homogenate (10%) was prepared (in homogenizing buffer containing 1 mM sodium orthovanadate, phenylmethane sulfonyl fluoride, 2.0 μ g/ml lipoprotein, 1 mM dithiothreitol and 2.0 μ g/ml aprotinin) and centrifuged at 12,000 rpm for 20 min. After protein quantification using Folin's Ciocalteu reagent samples were electrophoresed on 12% SDS-PAGE and transferred on polyvinylidene difluoride (PVDF) membrane in semidry transfer system (Bio-Rad trans-Blot SD Semi-dry electrophoretic transfer cell) and blocked in 4% BSA. Blot was probed with mouse primary antibodies HDAC 1, and β -actin. Proteins were detected using HRP linked mouse anti-IgG secondary

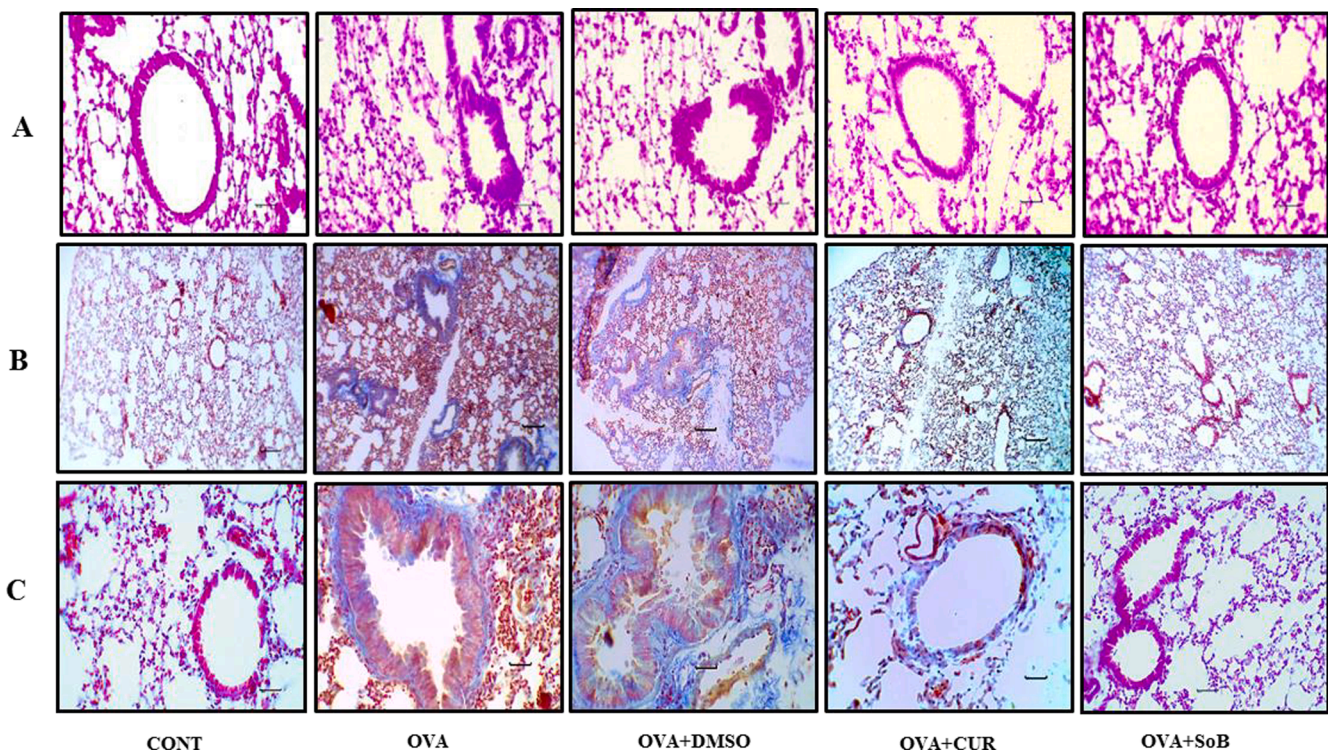


Fig. 5. (A-C) Effect of CUR and SOB on airway inflammation and collagen deposition. Lung sections from experimental groups were stained with H&E (A) and Masson's trichrome stain (B and C) then examined under light microscope. OVA and DMSO group showing increased cell recruitment around bronchioles and alveolar spaces, constricted and thickened airway lumen in contrast to control group. Masson's trichrome staining (B and C) revealed collagen deposition in lungs (10 $\times$ and 40 $\times$). Thick and intense blue bands seen in OVA and DMSO group confirming high collagen deposition which was found significantly reduced in curcumin and sodium butyrate group.

antibody and chemiluminescence kit. Gene expression was analyzed after normalizing β -actin expression using image J software.

2.17. Statistical analysis

Values were expressed as mean $\pm$ SEM. Values were compared between the groups and analyzed statistically by applying student's *t* test and one-way ANOVA using SPSS 16 followed by Tukey's test. Statistical significance was considered at $p < 0.05$. The experiments were repeated thrice and one set of data has been presented here.

3. Results

3.1. Effect of Curcumin and SoB on inflammation and oxidative stress in the lungs

Inflammatory cell infiltration to asthmatic lungs were observed in OVA sensitized and challenged mice and total inflammatory cells were enhanced in the BALF of OVA and DMSO groups as compared to control and treatment groups. Inflammatory cells were significantly reduced in CUR and SoB treated groups. Analysis of cytospun and giemsa stained slides also confirmed enhanced inflammatory cell infiltration in OVA and DMSO groups. Very few inflammatory cells were observed in the alveolar spaces of control mice lungs. Recruitment of inflammatory cells was significantly reduced in both CUR and SoB groups (Figs. 2 and 3) but CUR was better in inhibiting inflammatory cell infiltration than SoB.

The production of intracellular reactive oxygen species (ROS) and nitrite levels were assessed to detect oxidative stress. ROS and nitrite levels were found higher in OVA and DMSO groups as compared to control and treated groups. The level was significantly reduced in CUR and SoB treated groups. ROS level was effectively reduced by CUR whereas serum nitrite level was better modulated by SoB (Figs. 3 and 4).

3.2. Effect of CUR and SoB on eosinophil and neutrophil recruitments to the lungs

Eosinophils are the major cells involved in allergic inflammation but neutrophils also contribute to the inflammatory cascade. Activated neutrophils and eosinophils promote inflammation by secretion of inflammatory mediators like MMPs, chemokines, cytokines and other molecules stored in their granules. EPO and MPO activity was measured as an indicator of eosinophil and neutrophil recruitment. EPO and MPO activities were suppressed in both the treatment groups which indicate that eosinophil recruitment and neutrophil infiltration in lungs was significantly reduced by curcumin and sodium butyrate administration where curcumin was found better than SoB (Fig. 3).

3.3. CUR and SoB ameliorates IgE and histamine levels

Challenge and treatment with OVA resulted in increased IgE level in serum and histamine level in BALF. Histamine release in BALF and IgE level in serum were measured which were significantly increased in asthmatic group but SoB and CUR treatment groups have shown significant decrease (Fig. 4).

3.4. Histopathological analysis: Inflammation and structural alterations in lungs

To observe inflammatory cell infiltration and structural changes, hematoxylin and eosin stained lung sections were examined under microscope where OVA induced group have shown bronchoconstriction and enhanced inflammatory cell infiltration in alveolar spaces (Fig. 5A).

Structural alterations characterized by sub epithelial fibrosis, loss of epithelial integrity, deposition of collagen leads to narrowing and thickening of airways. Hydroxyproline content was measured to detect

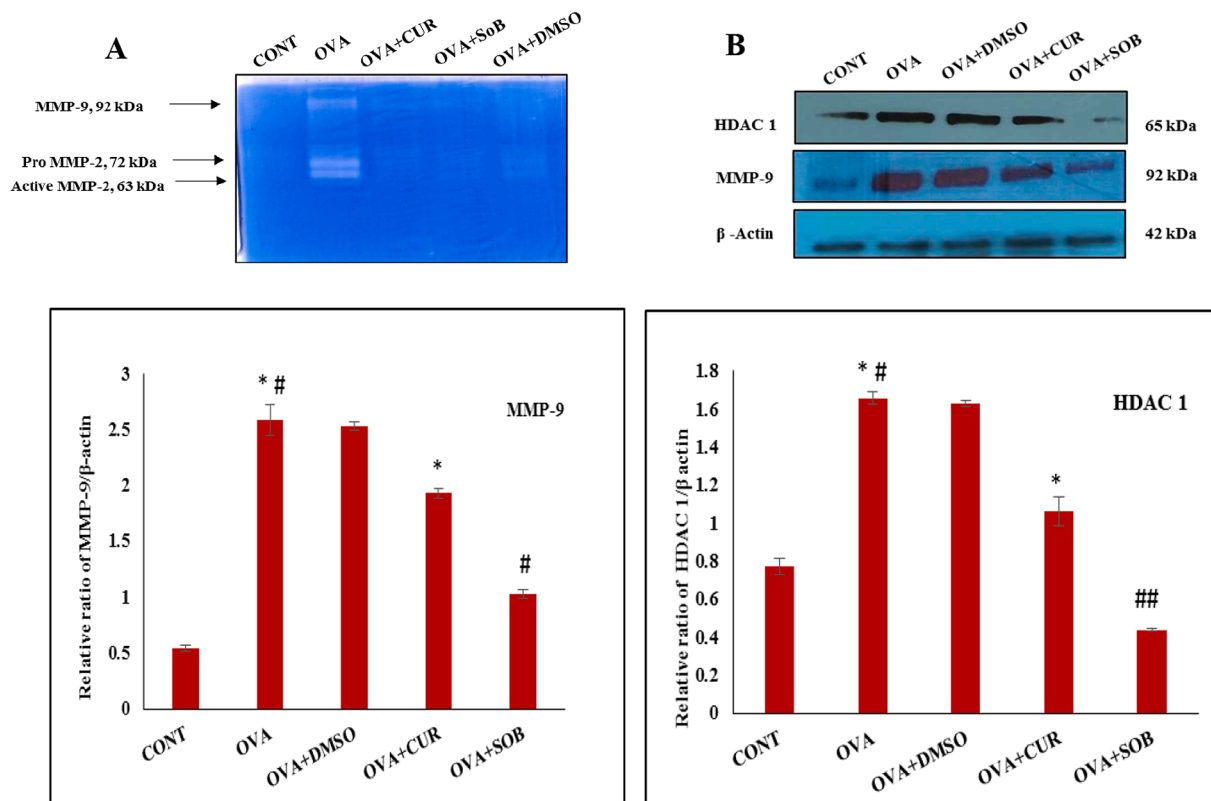


Fig. 6. Gelatin zymography (in BALF) and protein expressions of MMPs and HDAC 1 in lung tissue. (A) White bands in gelatin zymography revealed higher levels of active MMPs (MMP-9 and MMP-2) in OVA induced group as compared to control. Pretreatment of curcumin and sodium butyrate had significantly suppressed activities of MMP-2 and MMP-9 in BALF; (B) Significant elevation was observed in protein expression of HDAC 1 and MMP-9 in OVA induced group whereas significant reduction was observed in CUR and SOB treated groups. Results are shown as means $\pm$ SEM ($p < 0.05$) * OVA vs. CUR group; # OVA vs. SOB group; ## OVA+CUR vs. OVA+SoB group.

collagen deposition around bronchioles which was increased in OVA group but reduced in CUR and SoB treatment groups (Fig. 4). Further confirmed by Thickening in epithelial and smooth muscle layers in CUR and SOB treatment groups in Masson's trichrome stained lung sections due to reduced collagen deposition (Fig. 5B&C).

3.5. Curcumin and Sodium Butyrate inhibit matrix metalloproteinase-2 and 9 (MMP-2 & MMP-9) activity and expression

MMP-2 and MMP-9 are proteolytic enzymes that regulate extracellular matrix turnover and important for wound healing and normal wear and tear processes in lungs. MMPs play important role in inflammation and fibrosis. Collagenase (gelatinase; MMP-2 and MMP-9) activity in BALF was studied by gelatin zymography where SoB and curcumin treatment has significantly inhibited increased collagenase activities of MMP-2 and MMP-9 in OVA induced asthma (Fig. 6).

3.6. Effect of CUR and SoB on HDAC 1, NF- κ B, H3acK9 and MMP-9 expression

HDAC1, NF- κ B (p65), MMP-9 and H3acK9 expressions were analyzed in lung homogenates by western blotting. Significant elevation in HDAC1, NF- κ B (p65) and MMP-9 expressions were observed in OVA induced groups which was reduced in SoB treatment groups whereas curcumin was not found much effective. On the other hand, H3acK9 expression was enhanced in OVA and DMSO groups as compared to control and both the treated groups (Fig. 7).

4. Discussion

Allergen-induced immune responses and lung injury stimulate airway inflammation, hyper responsiveness and remodeling. Interdependent inflammatory and remodeling processes result into structural changes in the asthmatic airways [65]. These structural changes include increased vascularity in airways, basement membrane thickening, mucus gland hyperplasia, extracellular matrix deposition that ultimately results in impaired wound healing thereby increased fibroblast proliferation in lungs [66–68]. Invasion and transformation of airway fibroblasts to myofibroblasts lead to subepithelial collagen deposition [69–71]. These pathological changes are believed to contribute in AHR and irreversible airway obstruction responsible for morbidity and mortality in severe asthma [72,73].

In the current study, the effects of HDAC inhibitor, sodium butyrate (SoB) on airway inflammation and airway remodeling processes was investigated along with curcumin in OVA induced mouse model of asthma at given doses. Anti-asthmatic effects of SoB and curcumin on fibrosis, histopathological changes and protein expression of HDAC 1, H3acK9, MMP-9 and NF- κ B were investigated where significant reduction in inflammation and some aspects of airway remodeling (subepithelial fibrosis) was observed.

Several changes like cellular inflammation where numbers of eosinophils, mast cells and neutrophils were enhanced in OVA induced asthmatic group, where eosinophil infiltration has been reported to be responsible for airway remodeling. As reported earlier, eosinophils are responsible for generation of major fibrotic factors which stimulate differentiation of fibroblasts to myofibroblasts and also synthesis and secretion of collagen into the airway submucosal space which ultimately leads to airway fibrosis [74–77].

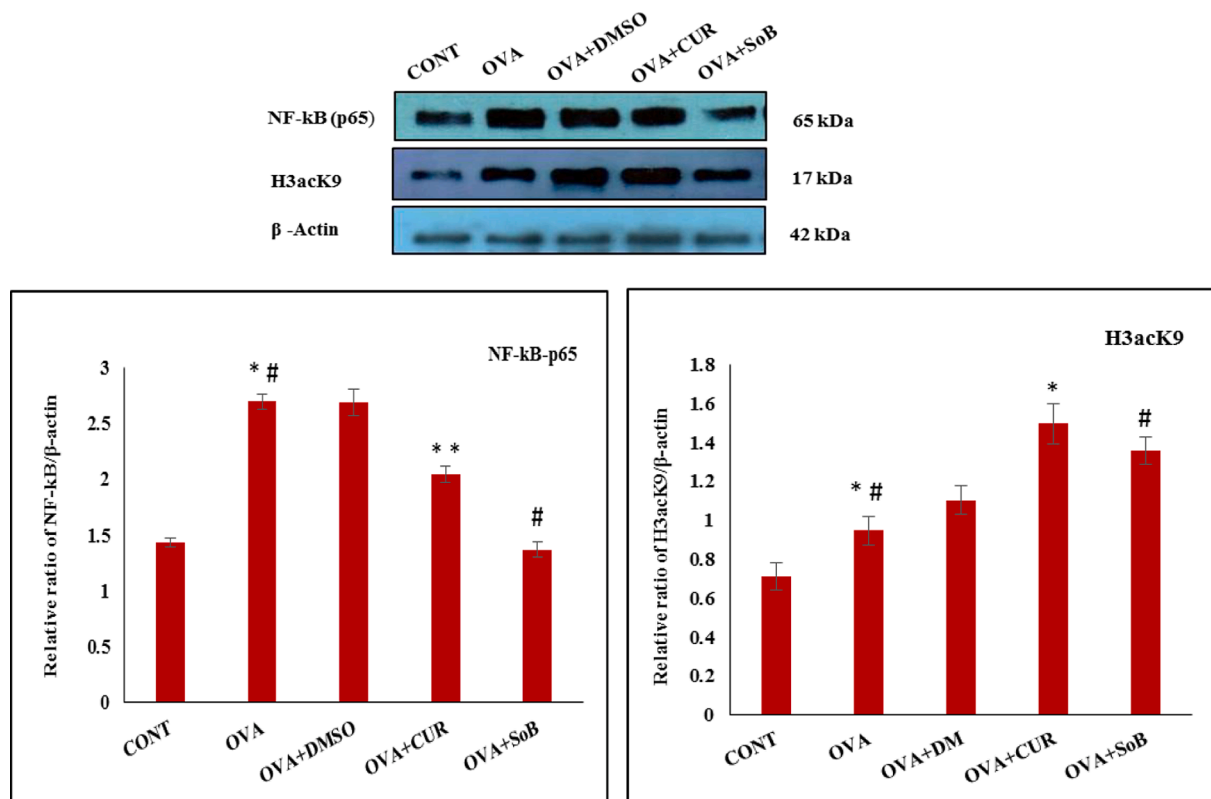


Fig. 7. Protein expressions of NF-kB (p65) and H3acK9 in lung tissue. Significant elevation was observed in p65 expression in OVA induced mice whereas there was significant reduction observed in CUR and SOB treated groups. Whereas level of H3acK9 was reduced in OVA induced group which was significantly restored by both the treatment groups. Results are shown as means $\pm$ SEM ($p < 0.05$) * OVA vs. CUR group; # OVA vs. SOB group; ** OVA+CUR vs. OVA+SoB group.

Accumulation of eosinophils, basophils, mast cells and neutrophils in asthmatic BALF was reduced in both curcumin and sodium butyrate treatment groups (Figs. 3 and 4). Further, elevated levels of EPO (eosinophil peroxidase activity), MPO (myeloperoxidase activity) and histamine in OVA induced group were reduced after curcumin and SoB treatment. Intranasal CUR and SoB has effectively suppressed infiltration and activation of these inflammatory cells, thus, reduced airway inflammation. Oxidative stress has also been known to contribute initiation and/or worsening of respiratory diseases. In case of asthma, ROS released by activated macrophages and granulocytes can increase mucus secretion and enhance permeability followed by augmented airway obstruction [78]. We also have observed reduced ROS levels in both treatment groups (Fig. 3). Lung histopathology has also revealed remarkable bronchoconstriction, thickening of airway wall and inflammatory cell recruitment in OVA induced asthmatic group. Treatment with CUR and SoB has significantly reduced the inflammation and bronchoconstriction (Fig. 5).

Epithelial alterations, subepithelial fibrosis in lamina reticularis and increased smooth muscle mass are key features of remodeling in asthma [79,3]. Increased deposition of extracellular matrix components including collagens I, III, and V, fibronectin, tenascin, and biglycan by fibroblasts lead to fibrosis [4,80]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes and are known for their ability to regulate ECM turnover by degrading ECM [81]. MMPs have important roles in both normal wound healing process [82] as well as pathogenic processes like inflammation and fibrosis [83,84]. In asthmatic condition, both inflammatory cells such as macrophages, eosinophils, leukocytes and structural cells such as epithelial cells and fibroblast cells secrete MMPs in airways [85–87]. Unlike inflammation for which several therapies like corticosteroids, salbutamol, theophylline etc. are available; but there is no effective therapy that targets remodeling.

In the present study, assessment of ECM deposition was verified by histological observation of Masson's trichrome stained lung sections and measurement of hydroxyproline content in lungs. Ovalbumin-induced mice have shown thick and intense collagen fibers around bronchioles and high collagen fibers in lungs. Both curcumin and sodium butyrate had significantly inhibited elevated hydroxyproline level as well as collagen deposition around bronchioles.

Under normal conditions, MMP-2 is present at basal levels and expressed only by endothelial cells or sometimes by macrophages [88] and do not contain MMP-9. In inflammatory conditions, MMP-9 transcription and expression increases by cells such as fibroblasts, endothelial and alveolar cells as well as due to the infiltration of neutrophils and eosinophils. We performed gelatin zymography in BALF samples, where enhanced expression and activities of MMP-2 and MMP-9 were suppressed in both the treatment groups. Similar trend was revealed after immunoblotting where SoB was much more effective in suppressing MMP-9 expression as compared to intranasal CUR (5 mg/kg) as compared to SoB (Fig. 6).

Since HDAC inhibitors play regulatory role in various cellular pathways, therefore, HDAC 1 expression in lungs were investigated to find out its correlation with inflammation and fibrotic factors. Sodium butyrate is well known inhibitor of class I and class II series [54,55] while curcumin is also considered as natural pan-HDAC inhibitor [49]. HDAC1 is the member of class I family and its localization is exclusively nuclear due to the lack of a nuclear export signal (NES) [29]. HDAC1 and HDAC3 have been suggested to be extensively involved in allergic inflammation-related diseases [35,89,90]. HDAC inhibition often lead to relative hyper acetylation of core histone proteins (H3 and H4), which probably regulates expression of some inflammation related genes [91].

OVA aerosol challenge led to increased HDAC1 and NF-kB expression in lungs whereas expression of H3acK9 was suppressed, suggesting, some kind of inter-relationship between these factors in allergic asthma

pathogenesis. Treatment with SoB resulted in significant suppression of NF- κ B and HDAC 1. Interestingly, SoB has proved to possess better potential in suppressing NF- κ B as compared to intranasal curcumin. In addition, some authors demonstrate inter-relation between short chain fatty acids and suppression of NF- κ B activation, which is a well-known inflammatory mediator [92].

It has been reported that HDAC 1 is involved in inflammatory response in different pathways like, IFN- γ mediated, B-cell proliferation and HIF-1 α mediated pathway [36,93–96]. Inhibition of HDAC 1 by CUR and SoB leads to affect inflammation via these pathways. As mentioned earlier, HDAC inhibition lead to hyperacetylation and that may be site specific i.e., increased expression of H3acK9 at A20 promoter region [21].

Our findings also suggest that HDAC1 inhibition may lead to increased expression of H3acK9. We observed reduced expression of NF- κ B (p65) unit in both the treatment groups, which may suggest that HDAC1inhibition lead to NF- κ B suppression probably by hyperacetylation of H3K9 at A20 promoter region leading to increased expression of A20 which in turn down regulates activation of nuclear factor kappa B (NF- κ B). We found restored H3acK9 expression in both, SoB and curcumin treatment groups (Fig. 7).

Anti-inflammatory and anti-allergic properties of curcumin has been already reported but poor bioavailability limits its therapeutic efficacy. Recently we have studied good absorption of curcumin after intranasal administration at lower doses in mouse model of asthma [56,59,97]. Lately, butyrate has been reported to be involved in eosinophil trafficking and survival and also induce apoptosis of eosinophils [98]. Analysis of observed parameters suggest that Sodium butyrate effectively ameliorates/restores structural changes in airways and HDAC 1 might have some important role in expression of these factors thereby regulate inflammation and airway remodeling.

This study demonstrates comparative therapeutic effects of Sodium butyrate and curcumin (a phytochemical) (at given doses) on inflammation and airway remodeling by inhibition of HDAC1 and NF- κ B. Fibrotic factors (epithelial damage, MMPs and collagen deposition) which lead to airway damage in asthma can be highly deleterious were suppressed by SoB and also activation of airway epithelial cells which secrete cytokines, matrix metalloproteases and growth factors [99]. Based on these investigations we can conclude that pretreatment of HDAC inhibitor, SoB and CUR has effectively inhibited inflammation and structural changes in airways.

Therefore, therapeutic potential of natural HDAC inhibitor may offer existing alternative to the treatment of wide variety of diseases due to minimal or no side effects. Available data are supporting enough to which may work on property of curcumin to act as HDAC inhibitor thereby affecting airway inflammation and remodeling. New targets and pathways related to HDACs can be discovered which might be helpful in better disease management.

CCRediT authorship contribution statement

Rashmi Singh: Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Rashmi Singh reports was provided by Banaras Hindu University. Dr. Rashmi Singh reports financial support was provided by Department of Science and Technology. Ramiya Islam reports a relationship with University Grants Commission that includes: funding grants.

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