



Original Contribution

Mutated glutathione S-transferase in combination with reduced glutathione shows a synergistic effect in ameliorating oxidative stress and airway inflammation

Prabhanshu Tripathi^{a,b}, Smitha Nair^a, B.P. Singh^a, Naveen Arora^{a,*}

^a Institute of Genomics and Integrative Biology (Council of Scientific and Industrial Research), Delhi 110 007, India

^b Department of Biotechnology, University of Pune, Ganeshkhind, Pune 411 007, India

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ABSTRACT

Oxidative stress is implicated in the pathogenesis of asthma, and antioxidant levels are reduced in asthma patients. Previously, glutathione S-transferase (GST) with reduced IgE binding suppressed oxidative stress and modulated airway inflammation to some extent in mice. GST catalyzes the quenching of reactive oxygen species by reduced glutathione (GSH) and the absence of any one of them may limit antioxidative behavior. This study evaluates the effects of mutated (m) GST with GSH in combination and individually in limiting oxidative stress and inflammatory responses in a mouse model. BALB/c mice were immunized and challenged with ovalbumin. The mice were treated with mGST, GSH, mGST + GSH, or α -lipoic acid by inhalation and sacrificed to evaluate inflammation and oxidative stress parameters. Treatment with the mGST + GSH combination showed significantly reduced total cell ($p < 0.01$) and eosinophil ($p < 0.01$) counts in BALF compared to other groups. The lung inflammation score was lowest for the mGST + GSH group, along with reduced IL-4 ($p < 0.01$) and OVA-specific IgE compared to the other treatment groups. Oxidative stress as per the lipid peroxidation and 8-isoprostane level in BALF of mGST + GSH mice was reduced significantly compared to the individual antioxidants. In conclusion, mGST in combination with GSH has a synergistic effect in reducing airway inflammation compared to the individual antioxidants and has potential for the treatment of asthma.

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Asthma is a chronic immune inflammatory disorder characterized by bronchial hyperresponsiveness (BHR) and increased vascular permeability as well as influx and activation of inflammatory cells in the airways [1,2]. Activation of macrophages, neutrophils, and eosinophils generates highly reactive oxidants such as superoxide, hydroxyl radicals, nitrogen dioxide, and peroxynitrite ions, collectively termed ROS and RNS [3]. The oxidants play a major role in exacerbating the symptoms of asthma by inducing mucus hypersecretion, bronchoconstriction, and lung tissue damage [4]. Excessive generation of oxidants results in depletion of inherent antioxidants thereby causing oxidative stress [5]. This imbalance favors a skewing of the T_H2 immune response while suppressing T_H1 differentiation [6]. Neutralization or quenching of free radicals in the body occurs through both nonenzymatic (vitamins C and E) and enzymatic (superoxide dismutase, catalase) mechanisms [7]. But dietary intake of antioxidants, namely α -lipoic acid, N-acetylcysteine, superoxide dismutase, and ambroxol, produced limited suppression of allergic airway inflammation and BHR in a mouse model of asthma [8,9].

In the lung epithelial cells and air spaces, enzymatic antioxidants, particularly glutathione peroxidase and glutathione S-transferase (GST), catalyze the conjugation of reactive intermediates with reduced

glutathione (GSH) to produce less reactive water-soluble compounds [10]. GSH in the lungs and surrounding tissue protects from oxidative stress. Oxidative stress alters the cellular redox buffer (GSH/GSSG) by depleting intracellular GSH [10]. Thus, the modulation of intracellular levels of glutathione and its enzymes can buffer the antioxidant potential of the cell. The oxidant-scavenging and anti-inflammatory properties of GST may be exploited to modulate the innate immune response to achieve a therapeutic benefit. In our previous study, the allergenicity of *Alternaria alternata* GST was reduced by mutations at positions 21 and 27 with no effect on enzymatic activity [11]. The inhalation of mutated GST (mGST) reduced oxidative stress and T_H2 response to a limited extent in an ovalbumin-induced mouse model [11]. Studies have shown that a combination of antioxidants may have a better potential to reduce oxidative stress while maintaining cellular redox homeostasis (high GSH/GSSG) [7,9]. Hence, this study aimed to investigate the effects of mGST in combination with reduced glutathione in a mouse model of airway inflammation. The antioxidants were also tested individually, taking α -lipoic acid as the standard.

Materials and methods

Animal study protocol

BALB/c mice were randomly divided into six groups of six mice each. Group 1 mice were sensitized, challenged, and treated with

* Corresponding author. Fax: +91 11 27667471.

E-mail address: naveen@igib.res.in (N. Arora).

phosphate-buffered saline (PBS) as control. Group 2–6 mice were given *ip* injection of 10 µg ovalbumin (OVA) with 1 mg alum in 100 µl of PBS on days 1 and 14. The mice were subjected to airway challenge with OVA 4 µg/mouse on days 28, 29, and 30. After 1 h of the challenge, the mice were given 2 µg of antioxidant or combination per mouse. The antioxidant dose was selected based on previous studies [11,12]. Group 2 mice were treated with PBS (positive control) and groups 3, 4, 5, and 6 were treated intranasally with 2 µg mGST, 2 µg GSH (Sigma), 1 µg mGST + 1 µg GSH, and 2 µg α-lipoic acid (SRL, India), respectively.

Collection of bronchoalveolar lavage fluid (BALF) and blood

After inhalation treatment, the mice were sacrificed with an overdose of sodium pentobarbital on day 31 and a tracheotomy was performed. PBS (0.5 ml) was instilled three times into the lungs and BALF was collected by aspiration (total 1.5 ml) via tracheal cannulation. BALF was centrifuged and the supernatant collected and stored at -70°C . The total inflammatory cells were counted using a hemocytometer after staining with trypan blue. One hundred microliters of BALF was spread on the slide, fixed, and stained with Leishman's stain for differential cell counts. Leishman's stain is based on a mixture of methylene blue and eosin [13]. It was used for staining blood smears to differentiate and identify leukocytes. Blood was collected, and sera were separated and used for measurement of serum immunoglobulins.

Determination of immunoglobulins by ELISA

OVA-specific IgE, IgG1, and IgG2a were measured in serum by ELISA as described elsewhere [14]. Briefly, microtiter plates (Nunc, Denmark) were coated with 5 µg/ml OVA. The plates were washed with PBS, blocked with 3% defatted milk, and incubated with mouse sera for IgE (1:10), IgG1 (1:50), and IgG2a (1:50), individually. After being washed with PBS + Tween 20 followed by PBS, the plates were incubated with IgG1–peroxidase or IgG2a–peroxidase (1:1000 in PBS; BD Pharmingen, San Diego, CA, USA). For IgE, biotinylated anti-mouse IgE (2 µg/ml; BD Pharmingen) was used, and the plate was washed and incubated with streptavidin–peroxidase (1:1000; BD Pharmingen). Color was developed using orthophenylene diamine and absorbance read at 492 nm.

Determination of cytokines by ELISA

IL-4, IL-10, and IFN-γ levels were determined in BALF by ELISA using paired antibodies according to the manufacturer's instructions (BD Pharmingen). Briefly, capture antibody (1:250 v/v) for each cytokine was coated separately on microtiter plates, incubated overnight, and blocked with 10% fetal calf serum. BALF samples (1:2 v/v) were added to wells and incubated for 2 h at 30°C . Biotinylated anti-IL-4 or anti-IL-10 or anti-IFN-γ was used to detect specific cytokine levels. The detection limit for IL-4 was 7.8 pg/ml and for IL-10 and IFN-γ was 31.3 pg/ml.

Histopathology

Lungs were fixed with 10% neutral-buffered formalin and embedded in paraffin. The 4-µm sections were cut and stained with hematoxylin and eosin for histological evaluation. The sections were scanned using a light microscope for OVA-induced peribronchial and perivascular inflammation. Semiquantification of inflammation score was performed on a scale of 0–4 for perivascular accumulation of inflammatory cells followed by statistical analysis. Lung inflammation score [15] was calculated in terms of eosinophil infiltration and mucus secretion: 0, no or occasional inflammatory cells; 1, with a few inflammatory cells; 2, with scattered aggregates of inflammatory

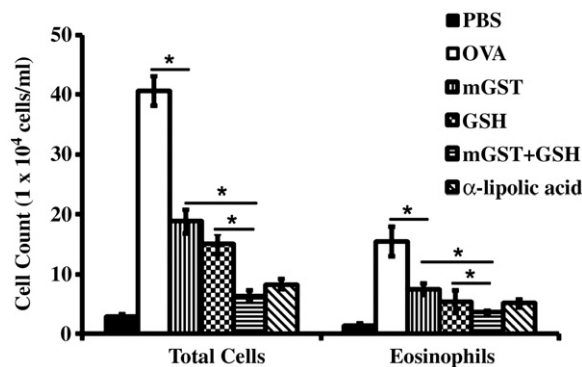


Fig. 1. Total cell and eosinophil counts in BALF posttreatment in OVA-sensitized and challenged mice and PBS control. Total cell count was determined using a hemocytometer. One hundred microliters of BALF was spread on the slide, fixed, and stained with Leishman for eosinophil count. Data are presented as mean $\pm$ SD; $N = 6$ mice per group; * $p < 0.01$.

cells; 3, with a thin layer of inflammatory cells surrounding the airways and vessels; and 4, with a thick layer of inflammatory cells surrounding the airways and vessels. Inflammation score was presented as the mean $\pm$ SD of the mice in each group.

Oxidative stress in BALF

Oxidative stress was determined by measuring thiobarbituric acid-reactive substance (TBARS) concentration spectrophotometrically [16]. Malondialdehyde and thiobarbituric acid (TBA) react to form a product with maximum absorption at 532 nm. BALF (200 µl) was mixed with 500 µl of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation at 10,000 g/ 4°C /15 min and the supernatant was treated with 0.67% TBA in boiling water for 15 min. OD was measured at 532 nm after cooling and compared with standard malondialdehyde.

The concentration of 8-isoprostanes was measured using an 8-isoprostane EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) as per the manufacturer's instructions. Briefly, plates were precoated with mouse monoclonal antibody and blocked with a formulation of proteins. After a wash, 50 µl of standard/BALF sample was added to the corresponding wells and incubated for 18 h at 4°C . The wells were washed five times with wash buffer. Ellman's reagent (200 µl) was added to each well and kept in the dark for 120 min for color development. The plates were read at 420 nm and the blank was subtracted. The concentration of 8-isoprostanes was calculated from a standard curve plotted with standard samples.

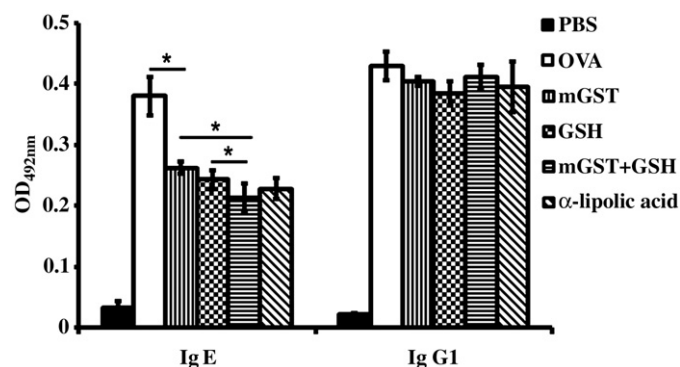


Fig. 2. Serum immunoglobulin levels after intranasal administration of antioxidants in OVA-sensitized and challenged mice. OVA-specific IgE, IgG1, and IgG2a were measured in serum by ELISA. Microtiter plates were coated with OVA, incubated with mouse sera for IgE (1:10) and IgG1 (1:50), and detected using secondary antibodies, individually. Data are presented as mean $\pm$ SD; $N = 6$ mice per group; * $p < 0.01$.

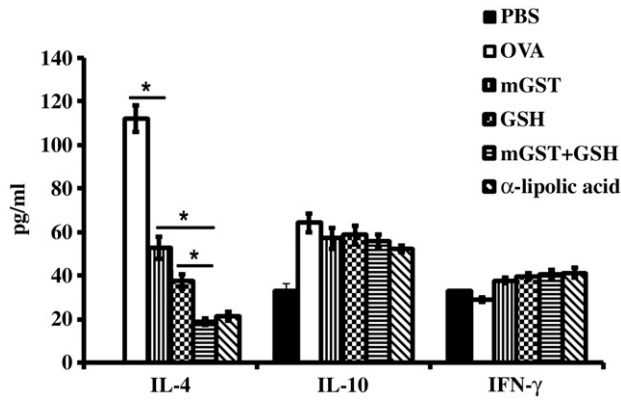


Fig. 3. Cytokine profiles in BALF of the antioxidant treatment groups. IL-4, IL-10, and IFN- γ were determined by ELISA. Capture antibody (1:250 v/v) for each cytokine was coated on microtiter plate, blocked, incubated with BALF samples (1:2 v/v), and detected by biotinylated anti-IL-4, anti-IL-10, or anti-IFN- γ . Data are presented as mean $\pm$ SD; $N = 6$ mice per group; * $p < 0.01$.

Nuclear extract preparation and NF- κ B (p65) estimation

Nuclear extract of lung tissues was prepared using the NuCLEAR Extraction Kit (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. PBS-rinsed tissue (100 mg) was suspended in

1 ml of lysis buffer containing dithiothreitol (DTT) and protease inhibitors. The tissue was homogenized and the disrupted cell suspension was centrifuged at 10,000 g for 20 min. The pellet was resuspended in 150 μ l of extraction buffer with DTT, protease inhibitor for 30 min and centrifuged for 5 min at 20,000 g. The supernatant was stored at -70°C as nuclear extract.

The NF- κ B (p65) transcription factor was determined in the above fraction using an immunoassay kit (Cayman Chemical Co.). Complete transcription factor binding buffer (90 μ l) was added to each well of a microtiter plate precoated with dsDNA consensus sequence containing the NF- κ B response sequence. Ten microliters of each sample was added per well and incubated overnight at 4°C without shaking. The wells were washed five times with wash buffer followed by addition of 100 μ l of diluted NF- κ B (p65) antibody and incubated for 1 h at 25°C . After five washes with wash buffer, 100 μ l of secondary antibody was added per well and incubated for 1 h at 25°C . One hundred microliters of developing solution was added to each well, the reaction was stopped after 30 min, and the absorbance was read at 450 nm. The blank and positive controls were taken as supplied with the kit.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical significance was determined by Student's two-tailed t test for independent means. $p < 0.01$ was considered statistically significant.

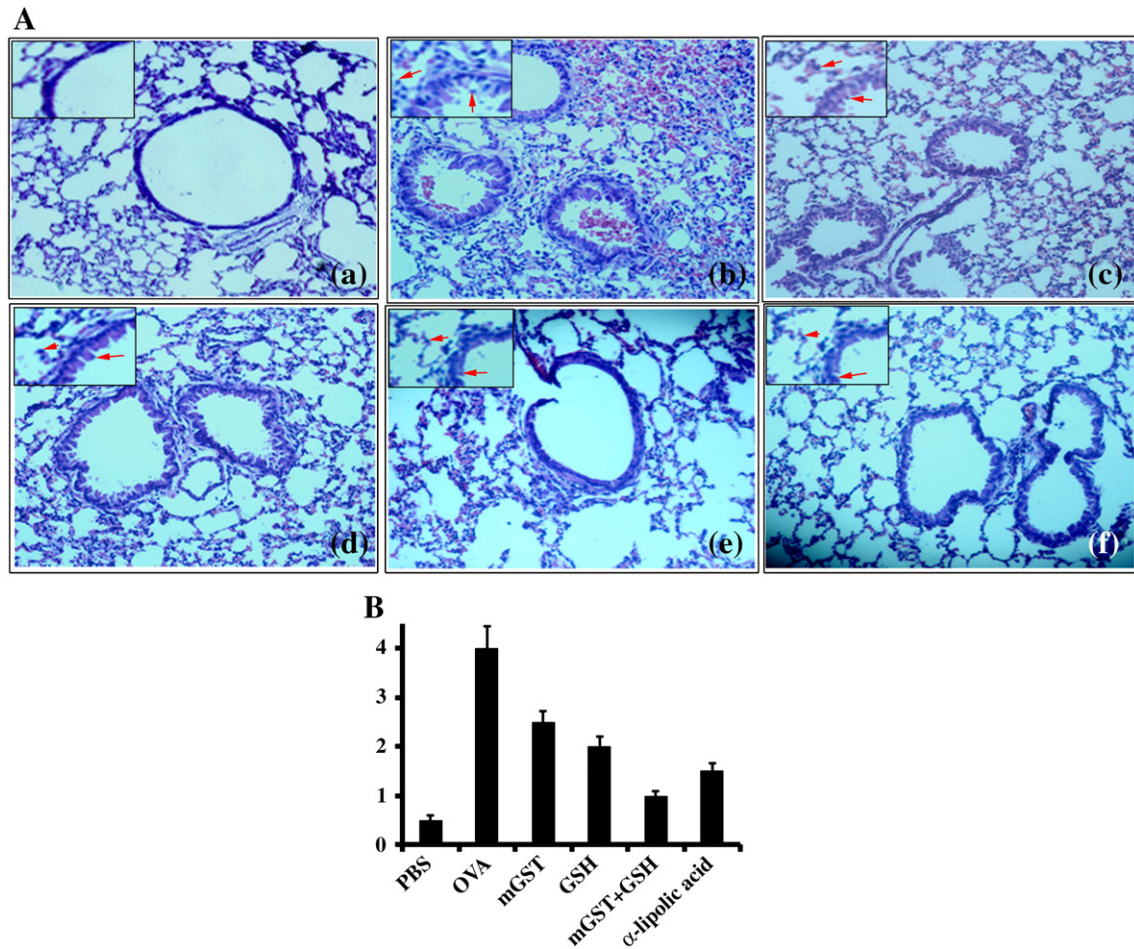


Fig. 4. (A) Hematoxylin and eosin-stained sections of lungs showing eosinophil infiltration in the treatment groups, OVA and PBS controls. Lungs were fixed with formalin, embedded in paraffin, cut into sections, and fixed on the slide for staining. (a) PBS control; (b) OVA challenged and PBS treated; (c) mGST treated; (d) GSH treated; (e) mGST + GSH treated; and (f) α -lipoic acid treated. (B) Lung inflammation score calculated in terms of eosinophil infiltration. The y axis shows the score: 0, no or occasional inflammatory cells; 1, with a few inflammatory cells; 2, with scattered aggregates of inflammatory cells; 3, with thin layer of inflammatory cells surrounding the airways and vessels; and 4, with a thick layer of inflammatory cells surrounding the airways and vessels. Data are presented as mean $\pm$ SD; $N = 6$ mice per group.

Results

mGST with GSH suppresses allergen-induced inflammatory cells

Analysis of BALF showed a large number of total cells $[(40.67 \pm 2.52) \times 10^4]$ and eosinophils $[(15.50 \pm 2.50) \times 10^4]$ in OVA-challenged mice compared to PBS control $[(2.87 \pm 0.31) \times 10^4]$ and $[(1.31 \pm 0.34) \times 10^4]$ (Fig. 1). Intranasal administration of antioxidants after 1 h of each challenge (days 28, 29, 30) significantly reduced the cellular infiltration ($p < 0.01$). The cellular infiltration depicting airway inflammation was higher in the mGST $[(18.84 \pm 2.05) \times 10^4]$ and $[(7.41 \pm 1.00) \times 10^4]$ and GSH $[(15.10 \pm 1.76) \times 10^4]$ and $[(5.41 \pm 1.90) \times 10^4]$ treatment groups compared to the combination mGST + GSH group $[(6.31 \pm 0.91) \times 10^4]$ and $[(3.58 \pm 0.30) \times 10^4]$. Treatment with α -lipoic acid taken as the standard antioxidant also showed a reduced number of inflammatory cells $[(8.28 \pm 0.86) \times 10^4]$ and $[(5.08 \pm 0.61) \times 10^4]$ comparable to the antioxidant combination treatment. The data demonstrate that treatment with a combination of antioxidants reduces inflammatory cells to an extent reaching toward the PBS control.

Individual and combined antioxidants reduce OVA-specific IgE

Specific IgE levels were elevated significantly in OVA-immunized and challenged mice (0.380 ± 0.032) compared to PBS control (0.033 ± 0.010) ; Fig. 2). IgE levels decreased substantially $(0.262 \pm 0.010$ to $0.213 \pm 0.023)$ in the various antioxidant treatment groups (mGST, GSH, mGST + GSH, or α -lipoic acid) compared to OVA-immunized PBS-treated mice. The reduction in IgE levels was significant (0.213 ± 0.023) , with mGST + GSH treatment reaching to nearly half the value of OVA-challenged PBS-treated mice. IgG1 levels, however, did not change on treatment with the antioxidants. Specific IgG2a levels were not detectable in any treatment group.

mGST with GSH treatment reduces TH2 cytokines in BALF

The IL-4 level in BALF of OVA-immunized and challenged mice was 112.00 ± 6.00 pg/ml, whereas it was below the detection limit (7.8 pg/ml) in the PBS-treatment group. Treatment with mGST and GSH reduced the IL-4 level to 52.77 ± 4.90 and 37.30 ± 3.34 pg/ml, respectively, whereas mGST + GSH decreased the IL-4 level to one-sixth $(18.67 \pm 1.52$ pg/ml) of the OVA-challenged PBS-treated mice. The reduction in IL-4 level with mGST + GSH treatment was similar to that of α -lipoic acid $(21.33 \pm 2.08$ pg/ml). IL-10 and IFN- γ levels did not change posttreatment in all the groups (Fig. 3).

Combination of mGST and GSH reduces cellular infiltration and airway inflammation

Lung sections stained with hematoxylin and eosin showed numerous eosinophils in lung interstitium around airways and blood vessels in OVA-challenged mice. The cellular infiltration was accompanied by narrowing of the airway lumen (Fig. 4A). Mice treated with mGST, GSH, or α -lipoic acid showed a substantial reduction in cellular infiltration in airways compared to OVA-challenged PBS-treated mice. Individual antioxidant treatment groups were assigned an inflammation score between 2 and 3 (Fig. 4B). The mGST + GSH and α -lipoic acid-treated mice showed a significant reduction in inflammation and were assigned a score of 1. The OVA-challenged PBS-treated mice showed a thick layer of inflammatory cells surrounding the airways and vessels (score 4). The combination of antioxidants reduced inflamed cells and improved the tissue damage caused by the ovalbumin.

mGST and GSH reduce allergen-induced oxidative stress

Oxidative stress in BALF was measured in terms of lipid peroxidation and 8-isoprostane level. OVA-challenged mice showed a TBARS level of 3.25 ± 0.34 pM/ μ l. Inhalation of mGST and GSH reduced this level to 2.07 ± 0.33 and 2.13 ± 0.23 pM/ μ l, respectively, whereas mGST + GSH treatment showed a significantly higher reduction in TBARS level $(1.33 \pm 0.27$ pM/ μ l) in BALF ($p < 0.01$). The reduction in TBARS level with the antioxidant combination was similar to that of α -lipoic acid $(1.39 \pm 0.07$ pM/ μ l) treatment (Fig. 5A).

The level of 8-isoprostane, a potent pulmonary vasoconstrictor, was significantly elevated in OVA-immunized and challenged mice $(82.79 \pm 4.58$ pg/ml) compared to PBS control $(22.55 \pm 2.40$ pg/ml; Fig. 5B). The level of 8-isoprostane was reduced significantly after treatment with mGST or GSH $(61.82 \pm 3.59$ and 56.65 ± 5.33 pg/ml, respectively). Its level was reduced to nearly half in the mGST + GSH treated group $(44.53 \pm 6.78$ pg/ml), similar to α -lipoic acid $(48.80 \pm 4.28$ pg/ml). The results indicate that oxidative stress is reduced significantly on treatment with the combination of antioxidants.

mGST with GSH controls NF- κ B (p65) level in lung nuclear extract

OVA-immunized and challenged mice showed significantly raised NF- κ B (p65) level (0.404 ± 0.037) in lung nuclear extract compared to PBS control (0.161 ± 0.02) ; Fig. 6). The NF- κ B (p65) level in nuclear extract of lung tissues was reduced significantly by intranasal administration of the antioxidants. Here mGST + GSH showed a twofold reduction in NF- κ B level (0.196 ± 0.04) , similar to α -lipoic acid (0.188 ± 0.02) . Mice treated with mGST and GSH alone showed a comparatively less reduction in NF- κ B level $(0.315 \pm 0.038$ and 0.255 ± 0.052 , respectively).

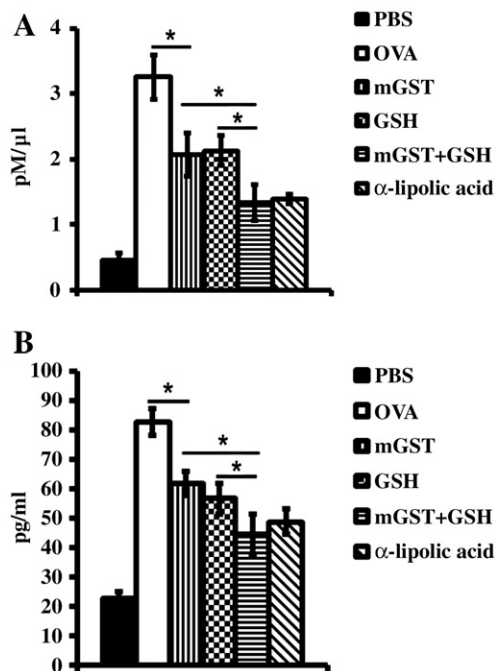


Fig. 5. (A) Oxidative stress in BALF of the treatment groups, OVA and PBS controls. Oxidative stress in BALF was determined by measuring the TBARS concentration spectrophotometrically. Data are presented as means $\pm$ SD; $N = 6$ mice per group; $*p < 0.01$. (B) 8-Isoprostane in BALF of the treatment groups and OVA and PBS controls. 8-Isoprostane, a potent pulmonary vasoconstrictor, was determined using an EIA kit (Cayman Chemical Co.). Data are presented as mean $\pm$ SD; $N = 6$ mice per group; $*p < 0.01$.

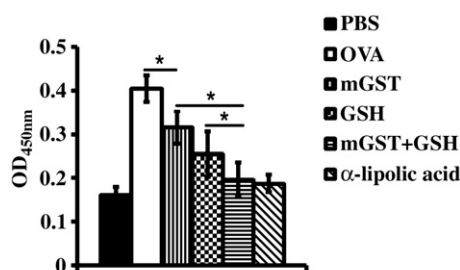


Fig. 6. NF- κ B levels in lung nuclear extract of the treatment groups, OVA and PBS controls. NF- κ B (p65) level in nuclear extract of lung tissues was determined using an ELISA kit (Cayman Chemical Co.). Data are presented as mean $\pm$ SD; $N=6$ mice per group; * $p<0.01$.

Discussion

The GSH redox system represents an important antioxidant system in bronchial and alveolar cells. In asthma, excessive generation of ROS results in depletion of intracellular GSH levels thus altering the ratio of GSH/GSSG in the lung epithelial cells [4]. The patients have a high level of reactive oxygen species in the blood that correlates with the severity of the disease [17–20]. Also, there is an increased level of oxidized glutathione in the BALF and elevated nitric oxide concentration in the exhaled air [21]. Furthermore, natural antioxidants, namely GST, glutathione peroxidase, superoxide dismutase, glutathione, and vitamins C and E, are reduced in the blood cells, plasma, and BALF of asthma patients [21]. Studies in animal models of airway inflammation have demonstrated that exogenous administration of antioxidants can reduce airway inflammation and hyperresponsiveness through the regulation of NF- κ B activity [8,22,23]. Previously, administration of mGST showed a better therapeutic effect than GST in suppressing allergic inflammation and oxidative stress [11]. However, the benefit was limited compared to α -lipoic acid, a standard antioxidant. This study evaluates the effects of mGST and its substrate GSH in combination for reducing oxidative stress and airway inflammation in a mouse model.

Cellular infiltration is the hallmark of asthma, and the eosinophil is a central effector cell in inflamed asthmatic airways [24,25]. Previous studies with different antioxidants using an oral route showed a reduction in inflammatory cells and mucus secretion in lung tissues [8,11,26]. In this study, OVA-challenged mice showed severe eosinophil infiltration that was reduced substantially on treatment with antioxidant(s) individually or in combination. Here, lung tissues of mGST + GSH-treated mice exhibited more reduction in inflammatory cells than those treated with the individual antioxidants.

IL-4 is required for differentiation of T cells and is a key factor for class switching to IgE in B cells [27]. Studies have demonstrated the effects of antioxidants such as vitamin E in reducing IL-4, IL-5, and IgE titers [28]. IL-5 and serum IgE also decreased in probucol-supplemented mice, but no change was seen in the IL-4 level [26]. In the present study, repeated exposure of sensitized mice to OVA caused a significant increase in IL-4 and IgE level compared to PBS control ($p<0.01$). Administration of mGST + GSH caused a significant reduction in IgE and IL-4 levels in BALF compared to individual antioxidants. The challenge with OVA also increased production of oxidants in the lungs as revealed by the high TBARS and 8-isoprostane levels in BALF. Treatment with mGST + GSH reduced these oxidants to almost half that of PBS-treated mice (Figs. 5A and 5B).

Oxidant injury can result in the activation of redox-sensitive transcription factors such as NF- κ B that induce numerous proinflammatory genes [5]. Antioxidants have been known to inhibit NF- κ B activation by preventing phosphorylation and/or degradation of inhibitory I κ B [29]. Furthermore, ROS modulate the activation of NF- κ B that further regulates the cytokines [30]. Mehta et al. have shown that the anti-inflammatory effect of choline reduces the NF- κ B level

and is mediated by the reduction in ROS production [31]. Consistent with these observations, the OVA-induced mouse model in this study showed a high level of NF- κ B in nuclear extracts of lung tissues. On treatment with mGST + GSH, the NF- κ B level was suppressed in the lung tissues, thereby reducing airway inflammation. Lee et al. have suggested a possible role for ROS-induced NF- κ B in regulating IL-18 gene expression [32]. IL-18, a proinflammatory cytokine, is expressed in human airway epithelium and can induce the production of IL-4, IL-13, and IgE. It also promotes the influx of allergen-induced eosinophils into airways [32]. Thus, antioxidants may exert their therapeutic potential by regulating NF- κ B-mediated IL-18 gene expression.

Dietary supplementation of antioxidants has been suggested as an adjunct therapy for management of asthma [26]. But allergic disorders such as asthma and rhinitis are multifactorial, and blocking oxidative stress alone may not lead to the complete resolution of the disease. However, the proper knowledge of the mechanisms involved in regulation of endogenous and exogenous antioxidants may open new therapeutic options for the treatment of inflammatory diseases such as asthma.

In conclusion, intranasal treatment with the complementary antioxidants mGST and GSH has a synergistic effect in reducing oxidative stress and airway inflammation and has potential for the management of asthma.

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