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Original article

Prophylactic effect of rosmarinic acid on tracheal responsiveness, white blood cell count and oxidative stress markers in lung lavage of sensitized rats

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ABSTRACT

Background: Rosmarinic acid (RA) as an active component of several medicinal plants, has shown anti-inflammatory and anti-oxidant effects. In this study, the effect of RA on tracheal responsiveness (TR), lung inflammatory cells, oxidant biomarkers in sensitized rats were evaluated.
Methods: TR to methacholine and ovalbumin (OVA) as well as total and differential white blood cell (WBC) count and levels of nitrogen dioxide, nitrate, malondialdehyde, thiol, superoxide dismutase, and catalase in bronchoalveolar lavage fluid were measured in control (group C) rats, sensitized animals to OVA and given drinking water alone (group S), S groups receiving drinking water containing three concentrations of RA (0.125, 0.250 and 0.500 mg/mL) and dexamethasone (1.25 μg/mL), (n = 6 in each group).
Results: Increased TR to methacholine and OVA, total WBC count, percentages of eosinophils, monocytes, neutrophils and levels of oxidant biomarkers but decreased other measured parameters were observed in group S compared to group C. Percentages of lymphocytes and antioxidant biomarkers were significantly increased but other measured parameters were significantly decreased in S group treated with dexamethasone and in rats treated with the two higher concentrations of RA compared to S group. The effect of RA medium concentration on percentage of eosinophils and RA high concentration on total WBC count and percentages of eosinophils and lymphocytes, were significantly higher than those of dexamethasone.
Conclusion: These results showed the concentration-dependent effect of RA on tracheal responses, lung inflammatory cells and oxidant-antioxidant parameters which was comparable to that of dexamethasone at used concentrations in sensitized rats.

Introduction

Asthma is mainly characterized by airway hyper-responsiveness (AHR) to different stimuli [1] which is due to airway inflammation [2]. Several inflammatory cells such as eosinophils are involved in the pathogenesis of airway inflammation in asthma [3]. Increased airway responsiveness as well as augmented total white blood cell (WBC) and eosinophil counts have been shown in animal models of asthma [4,5] and asthmatic patients [6,7]. Reactive oxygen species secreted by inflammatory cells can modulate contractility of airway smooth muscles [8]. There is evidences regarding an imbalance between oxidant, antioxidant biomarkers in favor of an oxidative state in asthma. Endogenous and exogenous reactive oxygen species, such as superoxide, hydroxyl, hypohalite, and hydrogen peroxide radicals, and reactive nitrogen species, such as nitric oxide, peroxynitrite, and nitrite, play a major role in the airway inflammation as asthma severity depends on these molecules. Asthma is also associated with decreased antioxidant defenses, such as superoxide dismutase, catalase and glutathione [9].

Rosmarinic acid (RA) is a phenolic compound which is found in many herbal plants such as *Ocimum basilicum* L. and *Rosmarinus officinalis* L. (both from the family Lamiaceae) [10]. Different pharmacological properties including anti-oxidant [11], anti-viral,

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anti-microbial, and anti-inflammatory [12] were described for RA. Furthermore, Liang and colleagues showed that RA reduced AHR and substantially inhibited ovalbumin (OVA)-induced eosinophilia in the lung tissue and mucus hypersecretion by goblet cells in the airways of OVA-sensitized mice [13].

With regard to existence of airway inflammation in asthma and due to anti-inflammatory and antioxidant properties of RA, in the present study, the effect of oral RA on tracheal responsiveness, total and differential WBC counts and levels of nitrogen dioxide (NO_2), nitrate (NO_3), malondialdehyde (MDA), thiol, superoxide dismutase (SOD), and catalase (CAT) were examined in bronchoalveolar lavage fluid (BALF) of sensitized rats were examined.

Materials and methods

Animal sensitization

Rats were sensitized by intra-peritoneal (*ip*) injections of 1 mg/kg of OVA (Sigma Chemical Ltd, UK, 98% pure) dissolved in 0.9% sterile saline containing 100 mg aluminium hydroxide, Al $(OH)_3$ as adjuvant, on days 1, 2 and 3 and exposed to 2% OVA aerosol on days 6, 9, 12, 15, 18 and 21 for 20 min/day. OVA aerosol was produced by a DeVilbiss PulmoSonic nebulizer with an air flow of 8 lit/min in a 0.8 m^3 chamber with animal normal-breathing. In control group, saline (*ip*) was used instead of OVA [14,15].

Experimental groups

Thirty six Wistar rats (weighing 200 ± 20 g) were randomly divided into six groups according a previous study [15], as fallows (n = 6 in each group):

I) Non-sensitized control animals (group C).

II) Untreated sensitized animals (group S).

III) Sensitized group treated with 1.25 μg/ mL dexamethasone (Sigma Chemical Ltd, UK, purity; 97%), (group D).

IV-VI) Sensitized groups treated with 3 concentrations (0.125, 0.250 and 0.500 mg/mL) of rosmarinic acid (Sigma Chemical Ltd, UK, purity; 96%) according a previous study [16], (groups RA 0.125, 0.250 and 0.500).

Dexamethasone and RA were added to animals' drinking water during sensitization period. On average, each rat drank 40 mL/day drinking water which was not significantly different among the animals of different groups.

Animals were kept in a stainless steel cage with clean filtered air (Maximiser, Thorens Caging System Inc), at 22 ± 2 °C with 12 h/12 h light/dark cycles and water and food available *ad libitum* during the experimental period [14].

Animal procedures were done in compliance with National Laws and National Institutes of Health guidelines for the use and care of laboratory animals and the study was approved by Ethics Committee of our institution (Ethical allowance No. 930842).

Tissue preparations

At the end of the experimental period (day 22), animals were sacrificed by ketamine 10%, 50 mg/kg (sigma Chemical Ltd, UK, purity: 99%), the chest was opened and trachea was removed and cut into 2 parts, each containing 5–6 cartilaginous rings.

Tracheal ring was hung between two Nichrome hooks inserted into the lumen, and placed in a 10-mL organ bath containing Krebs- Henseleit solution (KHS), with the following composition (mM): NaCl 120, KCl 4.72, KH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2{\cdot}2H_2O$ 2.5, $NaHCO_3$ 25 and Dextrose 11. KHS solution was maintained at 37 ± 0.5 °C and bubbled constantly with 5% CO_2-95% O_2. Tissue was suspended under isometric tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with KHS solution every 15 min. In all experiments, contraction responses were measured using an isometric transducer (MLT0202) which was connected to a PowerLab system (Power Lab 8/30, ML870).

Measurement of tracheal responsiveness (TR) to methacholine

Increasing concentrations (10^{-8} to 10^{-3} M) of methacholine hydrochloride (Sigma Chemical Ltd, UK, purity: 98%) were added to organ bath every 2 min and the contraction due to each concentration was recorded at the end of each 2 min to produce a cumulative log concentration–response curve, in each experiment [17]. The percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine in proportion to the maximum contraction obtained by methacholine final concentration, was plotted against log concentration of methacholine and the effect reached to a plateau in all experiments. The effective concentration of methacholine, causing 50% of maximum response (EC_{50}) was measured using methacholine-response curve in each experiment.

Measurement of tracheal responsiveness (TR) to ovalbumin

Here, 1 mL of 2% OVA solution was added to 10-mL organ bath to produce 0.2% solution of OVA. Tracheal smooth muscle contraction was measured after 10 min and expressed as gram contraction force according to a previously described method [17].

Bronchoalveolar lavage fluid (BALF) preparation

Trachea and lungs were dissected after sacrificing animals and external surfaces were washed with normal saline. The trachea was cannulated and the left lung was lavaged with 1 mL saline for five times (total = 5 mL) at room temperature. The BALF was centrifuged at 2500 g at 4 °C for 10 min. Supernatants were collected and stored at −80 °C for measurement of NO_2, NO_3, MDA, thiol, SOD and CAT levels [18].

Total and differential white blood cells count

Leukocyte count was determined in 1 mL of BALF stained with Turk's solution using a Neubauer counting chamber. For differential WBC counts, the smear of centrifuged BALF was prepared and stained with Wright-Giemsa. Differential WBC analysis was carried out according to staining and morphological criteria, under a light microscope by counting 100 cells and the percentage of each cell type was calculated [18].

Oxidant and anti-oxidant biomarkers measurement

All oxidant and antioxidant biomarkers were measured according to previously published reports [19–24].

Statistical analysis

The results were presented as means ± SEM. The results of different groups were compared using one way analysis of variance (ANOVA) with Tukey-Kramer's as *post-hoc* test. InStat (GraphPad Software Inc., La Jolla, USA) was used for statistical analysis and significance was considered at $p < 0.05$.

Results

Tracheal responses to methacholine and ovalbumin

A left-ward shift in concentration-response curve to methacholine was observed in group S as compared to that of group C but the curves of groups treated with dexamethasone and all concentrations of RA, had a right-ward shift as compared to group S (Figs. 1).

EC_{50} for methacholine in group S was significantly lower but maximum response to methacholine and TR to OVA were significantly higher than those of group C ($p < 0.01$ to $p < 0.001$,

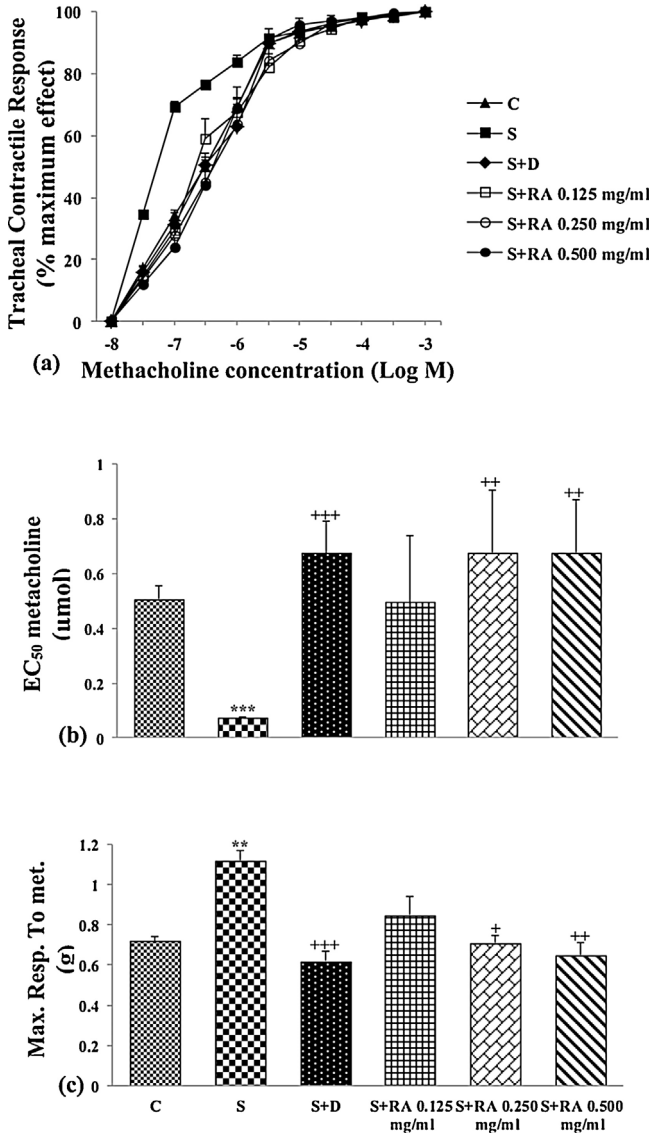

Fig. 1. Cumulative log concentration-response curves of methacholine-induced contraction of isolated tracheal smooth muscle (a), EC_{50} (the effective concentration of methacholine causing 50% of maximum response), (b) and maximum response to methacholine (c) in control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 in each group). ** $p < 0.$ 0.01, *** $p < 0.001$, comparison of groups S, S + D and S + RA *vs.* group C. $^{+}$ $p < 0.$ 05, $^{++}$ $p < 0.$ 01, $^{+++}$ $p < 0.001$, comparison of groups S + D and S + RA *vs.* group S. There was not any significant difference among three concentration of rosmarinic acid. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

Figs. 1 and 2). However, the values of EC_{50} in groups treated with dexamethasone and two higher concentrations of RA were significantly increased but maximum responses to methacholine and TR to OVA were significantly decreased compared to group S ($p < 0.05$ to $p < 0.001$, Figs. 1 and 2).

The effects of two lower concentrations of RA on TR to OVA were lower than those of dexamethasone-treated group ($p < 0.05$ and $p < 0.001$ for medium and low concentrations respectively, Fig. 1). There was no significant differences in TR to methacholine and OVA among the three concentrations of RA (Fig. 1).

White blood cell counts

Total WBC, eosinophils, neutrophils and monocytes percentages in BALF of S group were significantly higher but lymphocytes percentage was lower than those of control group ($p < 0.05$ to $p < 0.001$, Figs. 3 and 4).

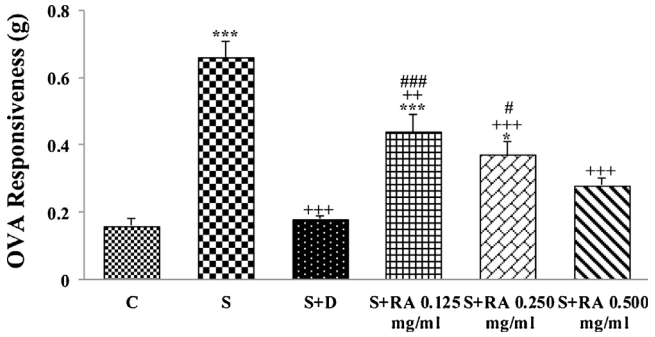

Fig. 2. Values of tracheal response to ovalbumin in control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 in each group). * $p < 0.05$, *** $p < 0.001$, comparison of groups S, S + D and S + RA *vs.* group C. $^{++}$ $p < 0.$ 01, $^{+++}$ $p < 0.001$, comparison of groups S + D and S + RA *vs.* group S. $^{\#}$ $p < 0.$ 05, $^{\#\#\#}$ $p < 0.01$, comparison of group S + RA *vs.* group S + D. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

Total WBC count in groups treated with dexamethasone and high concentration of RA and percentages of eosinophils, neutrophils and monocytes in groups treated with dexamethasone and two higher concentrations of RA were significantly decreased but lymphocytes percentage was increased compared to group S ($p < 0.05$ to $p < 0.001$, Figs. 3 and 4).

The effect of high concentration of RA on total WBC count and lymphocytes percentage and the effect of two higher concentrations of RA on eosinophils percentage were more marked than those of dexamethasone-treated group ($p < 0.05$ to $p < 0.001$, Figs. 3 and 4). The effect of low concentration of RA on the percentages of all types of WBC was lower than dexamethasone ($p < 0.01$ to $p < 0.001$, Fig. 4).

The effects of two higher concentrations of RA on the percentages of all types of WBC and the effect of the highest concentration of RA on total WBC count, were significantly higher than those of its lowest concentration ($p < 0.05$ to $p < 0.001$, Fig. 3). The effect of the highest concentration of RA on percentage of all types of WBC (except for monocytes) was also significantly higher than that of its medium concentration ($p < 0.05$ to $p < 0.001$, Fig. 4).

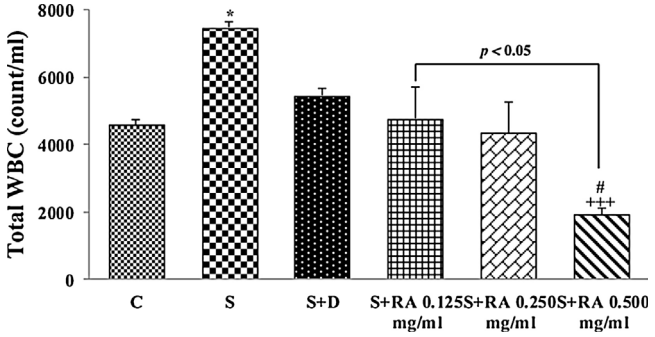

Fig. 3. Total WBC number (mean ± SEM) in one ml bronchoalveolar lavage fluid (BALF) of control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 for each group). * $p < 0.05$, comparison of groups S, S + D and S + RA *vs.* group C. $^{+++}$ $p < 0.001$, comparison of groups S + D and S + RA *vs.* group S. $^{\#}$ $p < 0.05$, comparison of group S + RA *vs.* group S + D. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

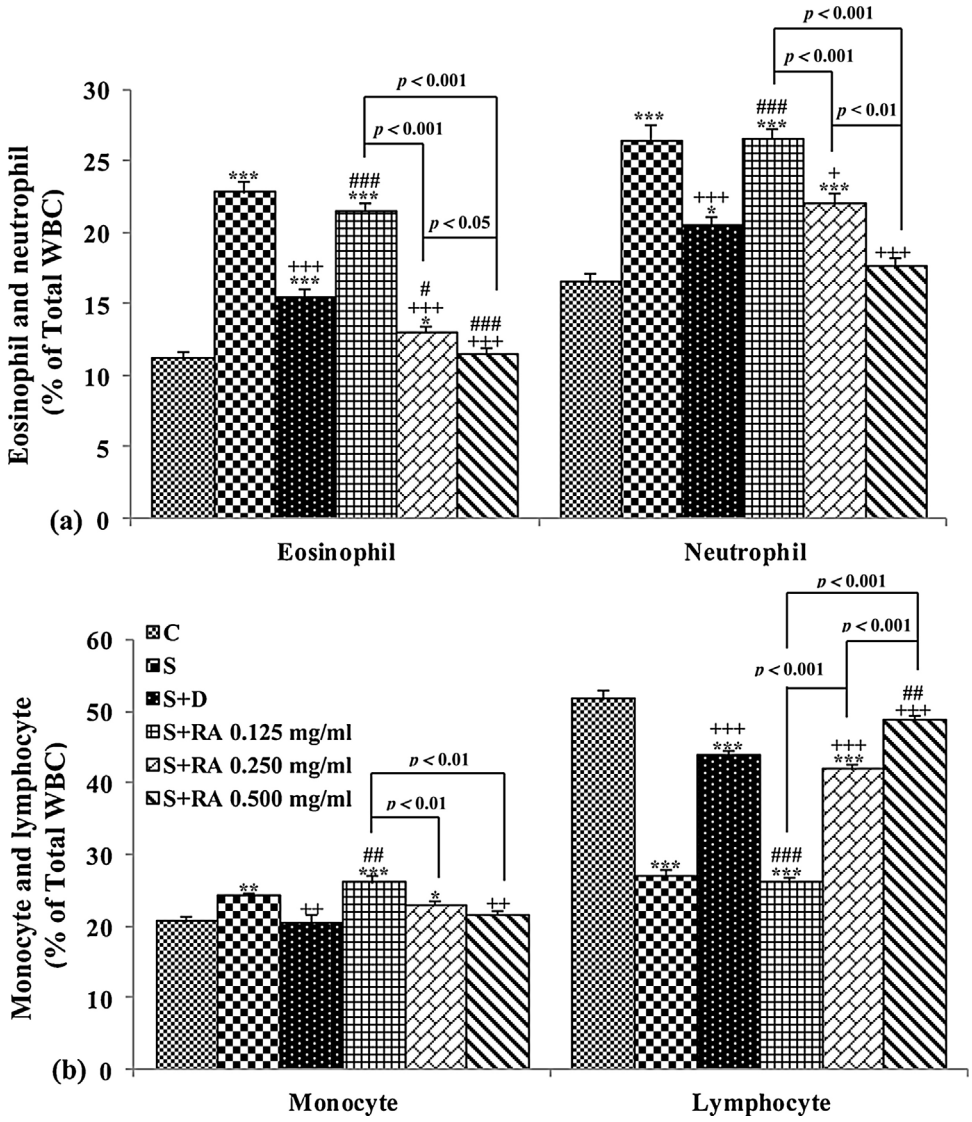

Fig. 4. The percentages (mean ± SEM) of eosinophils and neutrophils (a), monocytes and lymphocytes (b) of control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparison of groups S, S + D and S + RA *vs.* group C. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, comparison of groups S + D and S + RA *vs. vs.* group S. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, comparison of group S + RA *vs.* group S + D. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

Oxidant and anti-oxidant biomarkers

Thiol, SOD and CAT levels in the BALF of group S were significantly lower than those of group C ($p < 0.001$ for all cases, Fig. 5). All anti-oxidant biomarkers were significantly increased in groups treated with dexamethasone and two higher concentrations of RA compared to untreated sensitized group ($p < 0.01$ to $p < 0.001$, Fig. 5).

The effects of two lower concentrations of RA on thiol and RA lowest concentration on SOD and CAT, were lower than those of dexamethasone ($p < 0.05$ to $p < 0.001$, Fig. 5). The changes in all antioxidant biomarkers (except for SOD) due to treatment with two higher concentrations of RA were significantly more marked compared to RA lowest concentration as well as those of the highest concentration of RA compared to RA medium concentration, ($p < 0.05$ to $p < 0.001$, Fig. 5).

Oxidant markers in BALF of group S were significantly higher than those of control group ($p < 0.001$ for all cases, Fig. 6). BALF levels of NO_2 and NO_3 in sensitized animals treated with dexamethasone and all concentrations of RA as well as MDA level in groups treated with dexamethasone and two higher concentrations of RA were significantly lower than those of untreated sensitized group ($p < 0.001$ for all cases, Fig. 6).

The effects of all concentrations of RA on MDA level in BALF and those of its two lower concentrations on NO_3 were lower than the effect of dexamethasone ($p < 0.05$ to $p < 0.001$, Fig. 6). The effects of two higher concentrations of RA on BALF levels of all oxidant biomarkers were significantly higher than the effect of its lowest concentration ($p < 0.05$ to $p < 0.001$). The effect of the highest concentration of RA on NO_3 and MDA was also significantly higher than that of its medium concentration ($p < 0.01$ for both cases, Fig. 6).

Discussion

Increased TR to methacholine and OVA as well as augmented total and differential WBC counts, and oxidant biomarkers levels but decreased antioxidant biomarkers were observed in group S compared to group C, confirming the sensitization of animals. Asthma is a chronic airway inflammatory disease characterized by

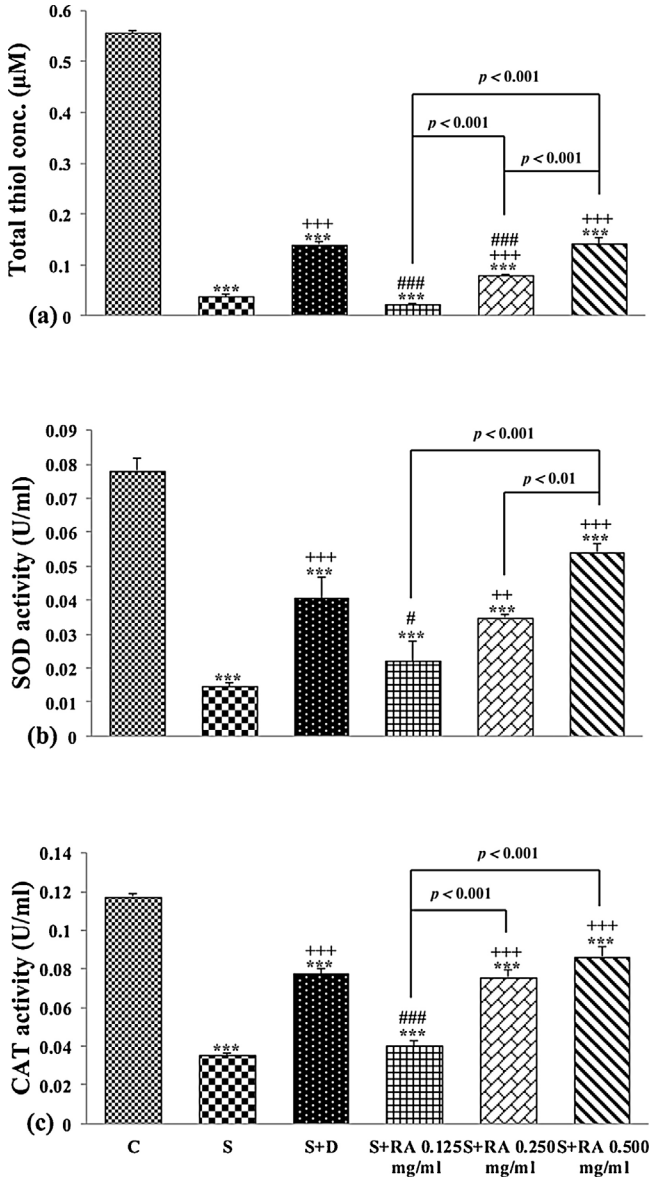

Fig. 5. Bronchoalveolar lavage fluid levels of thiol (a), superoxide dismutase (SOD) (b), and catalase (CAT) (c) in control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 in each group). *** $p < 0.001$, comparison of groups S, S + D and S + RA *vs.* group C. ++ $p < 0.01$, +++ $p < 0.001$, comparison of groups S + D and S + RA *vs.* group S. # $p < 0.05$, ### $p < 0.001$, comparison of group S + RA *vs.* group S + D. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

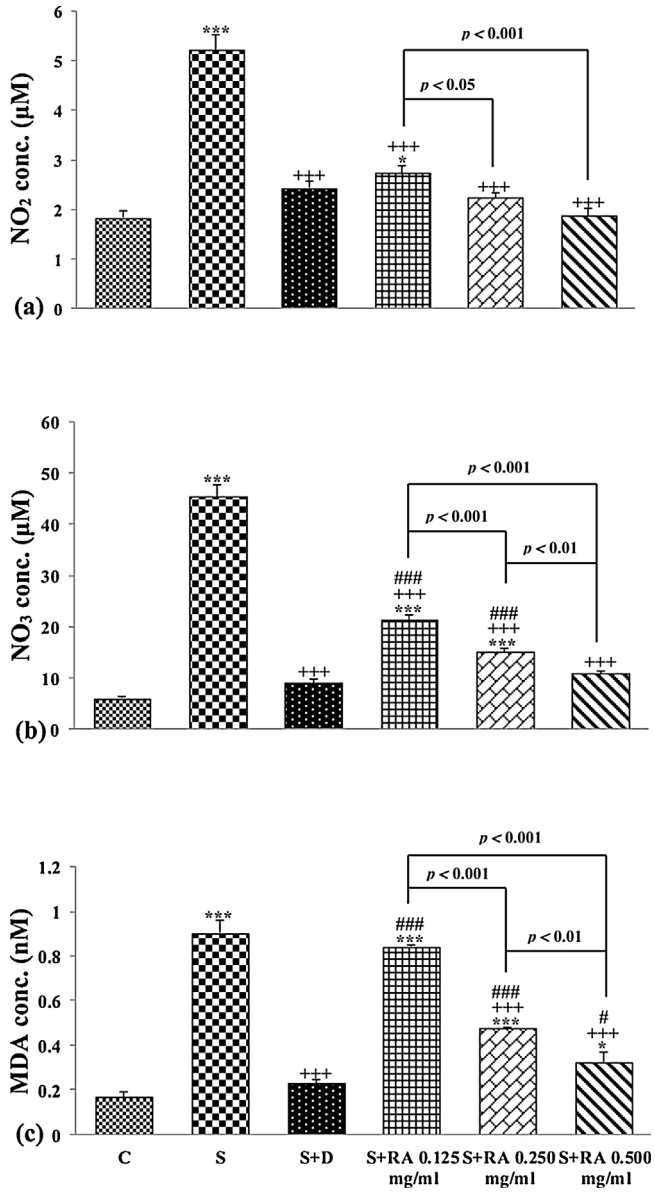

Fig. 6. Bronchoalveolar lavage fluid levels of NO_2 (a), NO_3 (b) and malondialdehyde (MDA) (c) in control rats (C), sensitized animals (S), S treated with dexamethasone (S + D) and three concentrations of rosmarinic acid (S + RA), (n = 6 in each group). * $p < 0.05$, *** $p < 0.001$, comparison of groups S, S + D and S + RA *vs.* group C. +++ $p < 0.001$, comparison of groups S + D and S + RA *vs.* group S. # $p < 0.05$, ### $p < 0.001$, comparison of group S + RA *vs.* group S + D. Data were presented as means ± SEM and the statistical comparisons were made using Tukey–Kramer multiple *post*-test.

infiltration of inflammatory cells in lung tissues, both in humans [25] and animal models [18]. Airway hyper-responsiveness is the most important feature of asthma which has been also shown in sensitized animals [1,17]. Also, oxidative stress aggravates airway inflammation in bronchial asthma, by inducing diverse pro-inflammatory mediators, enhancing airway hyper-responsiveness, increasing mucus secretion and inducing bronchospasm [26]. Additionally, increased oxidant markers (NO and MDA) and decreased antioxidant factors (thiol groups, SOD and CAT) in asthma have been reported previously [9,27].

TR to methacholine and OVA, percentages of eosinophils, monocytes, and neutrophils and the levels of oxidant biomarkers in BALF, were significantly decreased but lymphocytes and antioxidant biomarkers were significantly increased in groups treated with dexamethasone and two higher concentrations of RA compared to untreated S animals. Total WBC count was also decreased in animals treated with the highest RA concentration in the present study. The effect of RA on almost all measured values seems to be concentration-dependent. These results suggest the preventive effect of RA on TR, which is increased in asthma, as well as its effect on lung inflammation and oxidative stress in sensitized rats. It should be noted that increased lymphocyte percentage in RA-treated groups observed in this study is perhaps due to reduction of total WBC count in these groups which has been shown in previous studies [18].

The effects of RA on tracheal responsiveness as well as its anti-inflammatory and antioxidant properties have been previously shown which support the results of the present study. Liang and

colleagues showed that RA may effectively delay the development of airway inflammation in a murine model of asthma. They demonstrated that RA reduced airway hyper-responsiveness and substantially inhibited OVA-induced eosinophilia in the lung tissue and diminished mucus hypersecretion by goblet cells in the airways of OVA-sensitized mice [13]. However, the study of Liang et al. was performed in sensitized mice while the present study was done in sensitized rats. In addition, Liang et al. used only one dose of RA (20 mg/kg) while in the present study, the effects of three doses of RA (0.125, 0.250 and 0.500 mg/ml) were evaluated. Since each rat used 40 mL drinking water/day and considering the average weight of rats (200 g), the RA dose in the study of Liang et al., was equal to the lowest dose of RA used in the present study. In another study, Liang et al. also showed the effect of RA on tracheal responsiveness, total and differential WBC counts and cytokine levels in sensitized mice following administration of RA (5, 10 or 20 mg/kg, *ip*) 1 h prior to OVA challenge on days 25–27 [28] but in the present study RA (25, 50 and 100 mg/kg/day) was administered in animals' drinking water. In addition, in the present study, tracheal responsiveness to OVA as specific airway responsiveness and the effect of RA on oxidant and anti-oxidant levels in BALF of sensitized rats, were also examined.

Anticarcinogenic effect of *Perilla frutescens* extract was also reported which was partially due to anti-inflammatory and antioxidant properties of RA [29]. In addition, RA showed potent anti-inflammatory effect on acute lung injury induced by lipopolysaccharide in mice [30]. Lee et al. indicated that the major antioxidant compound of *Ocimum basilicum* was RA. The antioxidant activity of RA liposomes showed that one RA molecule can capture 1.52 radicals [31]. Another study also demonstrated the ameliorative effect of RA on cisplatin-induced oxidative stress, inflammation, and apoptosis in the kidneys [32]. All the above-described studies supported the findings of the present study and indicated the preventive effect of RA on specific and non-specific TR (TR to OVA and methacoline, respectively), lung inflammation and oxidative stress.

In the present study, RA showed a concentration-dependent preventive effect on almost all measured values. The effects of two higher concentrations of RA on the percentages of all types of WBC and all oxidant and antioxidant biomarkers, except for total WBC count and SOD, were significantly higher than those of its low concentration. The effect of the highest concentration of RA on the percentage of all types of WBC (except for monocytes) and NO_3, MDA, thiol and SOD values, were significantly higher than its medium concentration. The concentration-dependent preventive effect of RA could be regarded as further evidence for its antioxidant and anti-inflammatory effects.

The results also showed that the effects of high and medium concentrations of RA on the percentage of eosinophils and those of the highest concentration of RA on total WBC count and the percentages of eosinophils and lymphocytes, were significantly higher than dexamethasone effect. These findings also confirmed the preventive effect of RA in sensitized rats which was comparable or even greater than the effect of dexamethasone at studied concentrations. The inhibitory effect of dexamethasone on respiratory tract inflammation and its effect on lymphocytes in asthmatic mice have been previously shown which support the results of our study [33].

In conclusion, these results indicated a concentration-dependent preventive effect for RA on tracheal responsiveness, total and differential WBC counts and oxidative stress in lung lavage of OVA-sensitized rats which was comparable to the effect of dexamethasone at used concentrations. These results showed that RA could be valuable in treatment of inflammatory diseases such as asthma. However, further studies are needed to evaluate the effect of RA in asthmatic patients.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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