

ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol
December 2019; 18(6):624-638.

Rosuvastatin Affects Tracheal Responsiveness, Bronchoalveolar Lavage Inflammatory Cells, and Oxidative Stress Markers in Hyperlipidemic and Asthmatic Rats

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Received: 19 April 2019; Received in revised form: 12 May 2019; Accepted: 25 May 2019

ABSTRACT

Statins provide greater protection than predicted from just cholesterol-lowering effects, which is possibly mediated by other pleiotropic actions. This study aimed to examine the possible interaction effect of asthma on lipid profiles and evaluate the effect of rosuvastatin treatment on asthma.

The animals were assigned into (1) control, (2) asthmatic, (3) hyperlipidemic, (4) asthmatic-hyperlipidemic, (5) rosuvastatin (40 mg/kg/day intraperitoneally, for 3 weeks)-treated asthmatic, (6) rosuvastatin-treated hyperlipidemic and (7) rosuvastatin-treated asthmatic-hyperlipidemic groups. Tracheal responsiveness to methacholine and ovalbumin, total and differential WBC (white blood cell) counts, and oxidative stress markers in bronchoalveolar lavage fluid (BALF) were evaluated.

In the asthmatic and asthmatic-hyperlipidemic groups, tracheal responsiveness to ovalbumin, total WBC count, numbers of eosinophils, neutrophils, and monocytes were higher than the control group ($p < 0.001$). A left-ward shift in the concentration-response curves to methacholine, an increase in nitrite and malondialdehyde concentrations, and a decrease in total thiol content, superoxide dismutase and catalase activities were also observed in the asthmatic and asthmatic-hyperlipidemic groups compared to control group ($p < 0.01$ to $p < 0.001$). Beyond lipid-lowering effect in the treated hyperlipidemic and asthmatic-hyperlipidemic groups, rosuvastatin treatment decreased tracheal responsiveness to methacholine, reduced total WBC count, the numbers of eosinophils, neutrophils, and monocytes, as well as decreased malondialdehyde concentration, and increased total thiol content, superoxide dismutase and catalase activities in treated asthmatic and asthmatic-hyperlipidemic groups ($p < 0.05$ to $p < 0.001$).

The improving effect of rosuvastatin on asthmatic and asthmatic-hyperlipidemic animals was shown due to pleiotropic mechanisms including the effect on airway hyperresponsiveness, lung inflammation, and oxidative stress.

Keywords: Asthma; Hyperlipidemia; Oxidative stress; Rosuvastatin

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INTRODUCTION

There are shreds of evidence indicating the association of hyperlipidemia and increased the frequency of asthma.¹ Hyperlipidemia plays a pro-inflammatory role which is one possible mechanism linking to obesity and asthma.² Because hyperlipidemia and asthma are both inflammatory diseases, it is possible that pathophysiological interactions exist between the two conditions. Hyperlipidemia affects inflammation, and high plasma cholesterol levels may trigger pro-inflammatory cellular responses.²

Hyperlipidemia was found to be associated with a switch from T helper (Th)1 to Th2 response in an animal study.³ Although animal studies suggest that cholesterol trafficking and inflammation are coupled in the lungs^{4,5}, few epidemiological studies have examined the association of lipid profiles and asthma. While one study showed that hypercholesterolemia was associated with an increase in asthma prevalence,¹ another investigation revealed that asthmatic patients had higher triglyceride (TG) levels than subjects without asthma.⁶ Some studies conducted in murine model suggest that hypercholesterolemia promotes airway inflammation.^{5,7} By contrast, another recent large epidemiological study showed that total cholesterol (TC) and non-high density lipoprotein cholesterol (non-HDL) were inversely associated with asthma.⁸ The above studies reported inconsistent findings regarding the effect of hyperlipidemia on immune system which is perhaps due to differences in sexes, ages, and races of the studied subjects. Therefore, up to date, studies exploring the relationship between lipid profiles and asthma were few and inconsistent.

In the past decade, mounting evidence has shown that statins and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors provide greater protection than predicted from just cholesterol-lowering effect, which is possibly mediated by other pleiotropic actions.⁹ These pleiotropic actions include anti-inflammatory, antioxidant, immunomodulatory, improved endothelial cell function, and anti-thrombotic effects. The exploitation of these pleiotropic statin-induced protective effects could be therapeutically used to manage chronic lung diseases with inflammatory and oxidative stress components such as asthma, chronic obstructive pulmonary disease (COPD), and pulmonary hypertension.⁹ Statins may reduce airway inflammation

in asthmatics, although there is not sufficient evidence to make a conclusion that statins can improve the lung function. There have been several published studies reporting the use of statins in the treatment of asthma, but their results are not consistent.¹⁰

Rosuvastatin is a synthetic statin that exhibits various pharmacologic effects. The drug has a high affinity for the active site of HMG-CoA reductase and exhibits greater potency in inhibiting enzyme activity and cholesterol synthesis *in vitro* than other statins.¹¹ Rosuvastatin has been known to have more powerful pleiotropic effects than other statins¹² such as anti-atherosclerotic, anti-atherothrombotic, metabolic, cardioprotective, neuroprotective, cytoprotective and renoprotective effects, vascular remodeling, endothelial function regulation, plaque stabilization, and anti-inflammatory, immunomodulatory and antioxidant properties.¹³

The effects of rosuvastatin on airway inflammation and its inhibitory mechanism in mucus hypersecretion were previously investigated in a murine model of chronic asthma.¹⁴ It has been debated, but not yet established, whether rosuvastatin can improve increased airway responsiveness in asthma. Airway hyperresponsiveness is a characteristic feature of asthma and consists of an increased sensitivity of the airways to an inhaled constrictor agent, a steeper slope of the dose-response curve, and a greater maximal response to the allergen.¹⁵ Whether rosuvastatin can affect airway hyperresponsiveness is still unclear.

The present study aimed to examine the possible interaction effect of hyperlipidemia on asthma, and evaluate the effect of rosuvastatin treatment on asthma through measurement of tracheal responsiveness to methacholine and ovalbumin, inflammatory cells, and oxidative stress markers in asthmatic, hyperlipidemic, and asthmatic-hyperlipidemic rat models. This study was conducted in three different conditions which have not been done before.

MATERIALS AND METHODS

Animals

Forty-nine male Wistar rats (140–160 g) were purchased from Animal House, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, and were maintained in Plexiglas cages with 3 or 4 rats in each, under standard conditions of 12 h light/dark cycle, 22±2°C and humidity of 54±2%. Food

and water were freely available during the experimental period. The study was approved by the ethics committee of Mashhad University of Medical Sciences for Animal Experiments (code 940997).

The animals were randomly assigned into seven groups (n=7 in each group) as control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR) and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Treated groups received 40 mg/kg/day rosuvastatin (Sigma Chemical Ltd, UK) intraperitoneally for 3 weeks.^{16,17} (Figure 1)

For hyperlipidemia induction, rats received normal diet plus 10% ethanol (Sanaeh Shemicali Daro Hamon Teb Markazi, Zarandieh, Iran) and 10% fructose (Sigma Chemical Ltd, UK) in drinking water during 9 weeks.¹⁸ Each rat in all groups averagely drank 40 ml/day drinking water which was significantly not different among groups.

Rats were sensitized by intraperitoneal injections of 1 mg/kg of ovalbumin (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% ovalbumin aerosol on days 6, 9, 12, 15, 18, and 21 for 20 min/day (Figure 1). Ovalbumin aerosol was produced by a nebulizer with an airflow of 8 lit/min in a 0.8 m³ chamber with animal normal-breathing.^{19,20} In the control group, saline was used instead of ovalbumin.

Biochemical Measurements

At the end of the experimental period, the rats were

anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Blood samples (5 mL per rat) were withdrawn from the heart and centrifuged at 2000 revolution per minute (rpm) for 10 minutes. The Serum was collected; using pasture pipette and stored at -20°C and thawed just before use for the determination of blood lipids.

Serum TC, TG, low-density lipoprotein (LDL-C) and high-density lipoprotein (HDL-C) were measured on the basis of colorimetric method for single-point measurement; using colorimetric kits (Pars Azmoon Co, Iran) according to the manufacturer’s instructions.

Total and Differential White Blood Cells Counts

For bronchoalveolar lavage fluid (BALF) preparation, a cannula was placed into the trachea and the right lung was washed with one mL normal saline for five times (total 5 mL).^{21,22} The BALF was centrifuged at 2500 rpm at 4°C for 10 min. The supernatant was collected and stored at -80°C for oxidant and anti-oxidant biomarkers measurement.

Leukocyte count was determined in one 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.²¹ After determining the total cell count and differential cell count, the absolute cell count for each cell type was calculated by multiplying the percentage of each subset in an individual sample by the total number of cells in that sample.²⁰

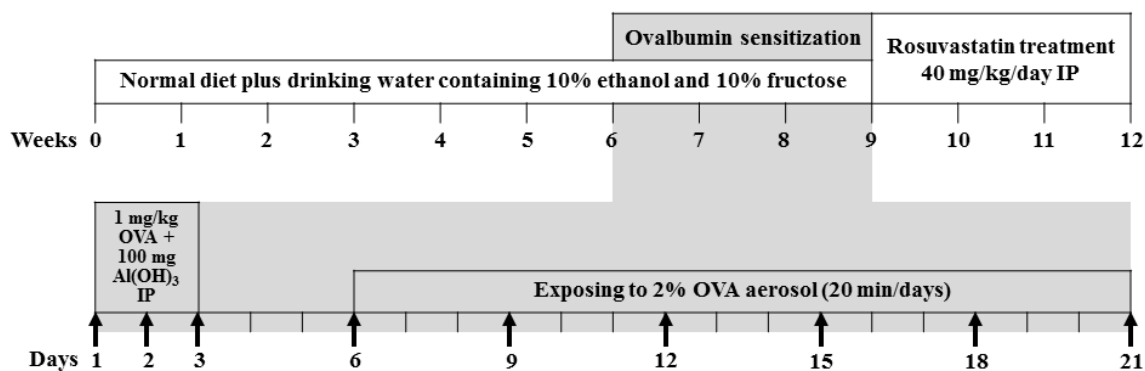


Figure 1. Time table of the study and protocol of animal sensitization with ovalbumin (OVA) and treatment

Measurement of Tracheal Responsiveness to Methacholine and Ovalbumin

Rat tracheal ring preparation and the method of measuring tracheal response was carried out as previously described.²³ 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.²³ 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 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.

One mL of 2% ovalbumin solution was added to 10 mL organ bath to produce 0.2% solution of ovalbumin. Tracheal smooth muscle contraction was measured after 10 min and expressed as gram contraction force according to a previously described method.²³

Oxidant and Anti-Oxidant Biomarkers Measurement

Total nitrite concentration was measured in BALF by Griess reagent method; using a standard enzyme-linked immunosorbent assay (ELISA) kit (Promega Corp., USA, Cat#G2930). In brief, 100 μ L BALF was added to a 96-well flat-bottomed microplate. Then, sulfanilamide solution and N-1-naphthylethylenediamine dihydrochloride were added to all collected samples under acidic conditions. The absorbance was detected by a microplate reader (Biotek, USA) in 520–550 nm wavelengths. The limit detection was 2.5 μ M nitrite.²⁴

Malondialdehyde (MDA), a biological marker of lipid peroxidation, was assayed in BALF based on the reaction between MDA and thiobarbituric acid (TBA) as previously described.¹⁹

Total thiol content was also assayed in BALF; using a previously established method.¹⁹ Briefly, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) interact with SH groups, forming a highly colored anion with the maximum peak at 412 nm.

Superoxide dismutase (SOD) activity was assayed

in BALF according to the previously described method.¹⁹ The method is based on the generation of superoxide through auto-oxidation of pyrogallol and dependent revived inhibition of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) to formazan.¹⁹

Catalase (CAT) activity was assayed based on its ability to decompose hydrogen peroxide (H_2O_2), which is reflected in the reduction of absorption at 240 nm.¹⁹

Statistical Analysis

Data were analyzed by the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and results were presented as mean \pm SEM. Values of $p < 0.05$ were considered statistically significant. In the present study, the operator and data analyst were blinded. The percentage changes for each variable was calculated in non-treated groups, using the following equations:

$$A/C, H/C \text{ or } AH/C = [(value \text{ in } A, H \text{ or } AH \text{ groups} - value \text{ in } C \text{ group}) \times 100] / value \text{ in } C \text{ group}$$

And in non-treated groups are as follows:

$$AR/A, HR/H \text{ or } AHR/AH = [(value \text{ in } AR, HR \text{ or } AHR \text{ groups} - value \text{ in } A, H \text{ or } AH \text{ groups}) \times 100] / value \text{ in } A, H \text{ or } AH \text{ groups}$$

RESULTS

Plasma Lipid Profile

The lipid profiles of TC, TG, and LDL-C in H and AH groups were significantly higher than controls ($p < 0.05$ to $p < 0.001$). There was no significant difference in the lipid profile test among H and AH groups (Figure 2).

A significant reduction in the serum level of TC was seen in HR and AHR groups compared to H and AH groups ($p < 0.01$ for both cases), (Figure 2a). Rosuvastatin treatment decreased serum level of TG in AR ($p < 0.05$), HR, and AHR groups ($p < 0.001$ for both cases) compared to A, H, and AH groups (Figure 2b). A significant reduction in the serum level of LDL-C was seen in AHR group compared to AH group ($p < 0.05$), (Figure 2c).

Tracheal Responsiveness to Methacholine and Ovalbumin

A left-ward shift in the concentration-response curves to methacholine was observed in A and AH groups compared to controls.

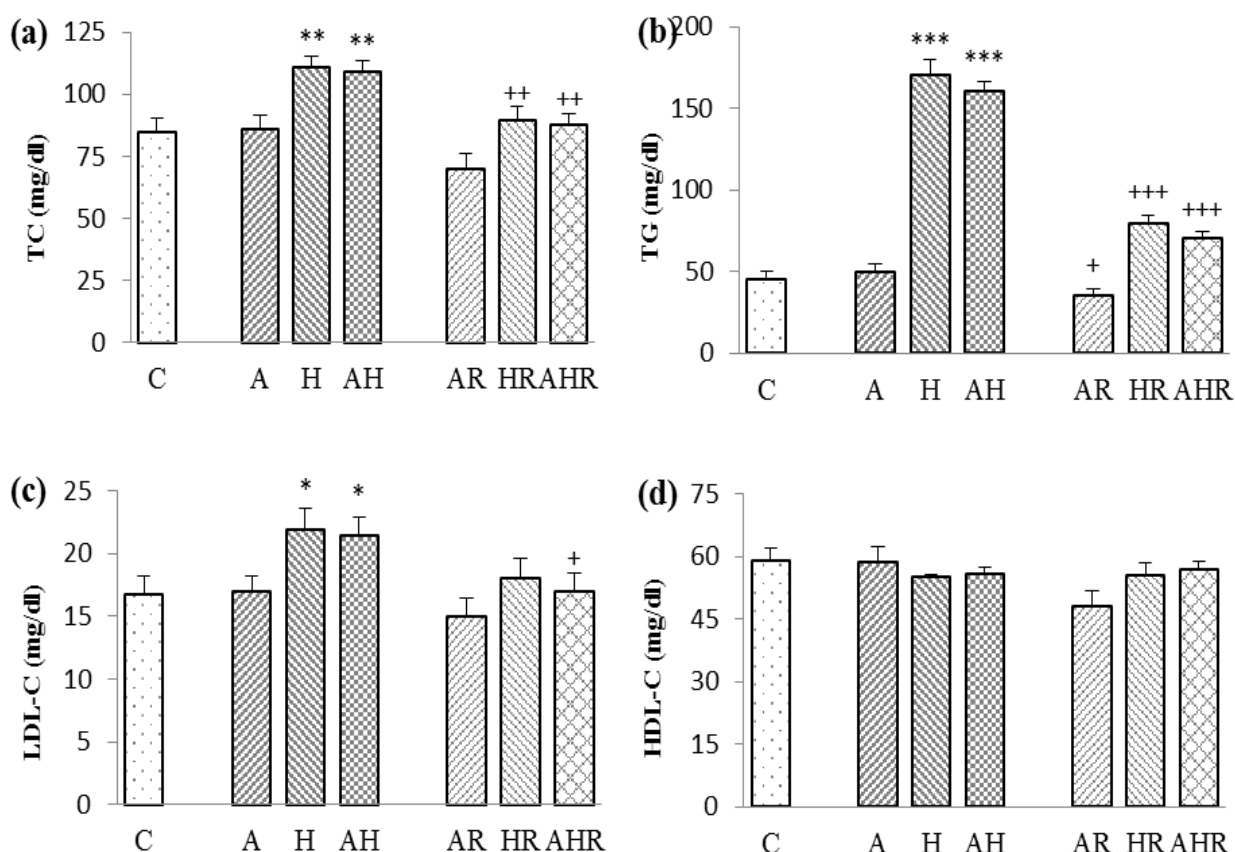


Figure 2. The lipid profiles of total cholesterol (TC, a), triglyceride (TG, b), low-density lipoprotein (LDL-C, c), and high-density lipoprotein (HDL-C, d) in the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Data are shown as mean±SEM (n=7 in each group). **p*<0.05, ***p*<0.01 and ****p*<0.001 compared to control group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

Furthermore, the concentration-response curve in AH group showed leftward shifts compared to A group (Figure 3). However, in comparison to A and AH groups, the curve in AR and AHR groups were shifted to the right (Figure 3).

The mean values of EC₅₀ for methacholine in A and AH groups were significantly lower than that of controls (*p*<0.001 for both cases). In the AH group, the mean value of EC₅₀ was also significantly lower than that of A group (*p*<0.05). Compared with A and AH groups, the mean values of EC₅₀ were significantly increased in AR and AHR groups (*p*<0.01 for both cases), (Figure 4a). The percent change of EC₅₀ for methacholine in AH/C was significantly lower than

A/C (*p*<0.05) and H/C (*p*<0.001) (Figure 5a). The percent change of EC₅₀ for methacholine in AHR/AH was also significantly higher than those of AR/A (*p*<0.05) and HR/H (*p*<0.001) (Figure 5a).

Tracheal responsiveness to ovalbumin in A and AH groups was significantly higher than that of controls (*p*<0.001 for both cases) (Figure 4b). The percent change of tracheal responsiveness to ovalbumin in AH/C was significantly higher than H/C (*p*<0.001), (Figure 5b). There was no significant difference in the percent change of tracheal responsiveness to ovalbumin among rosuvastatin-treated and untreated groups (Figure 5b).

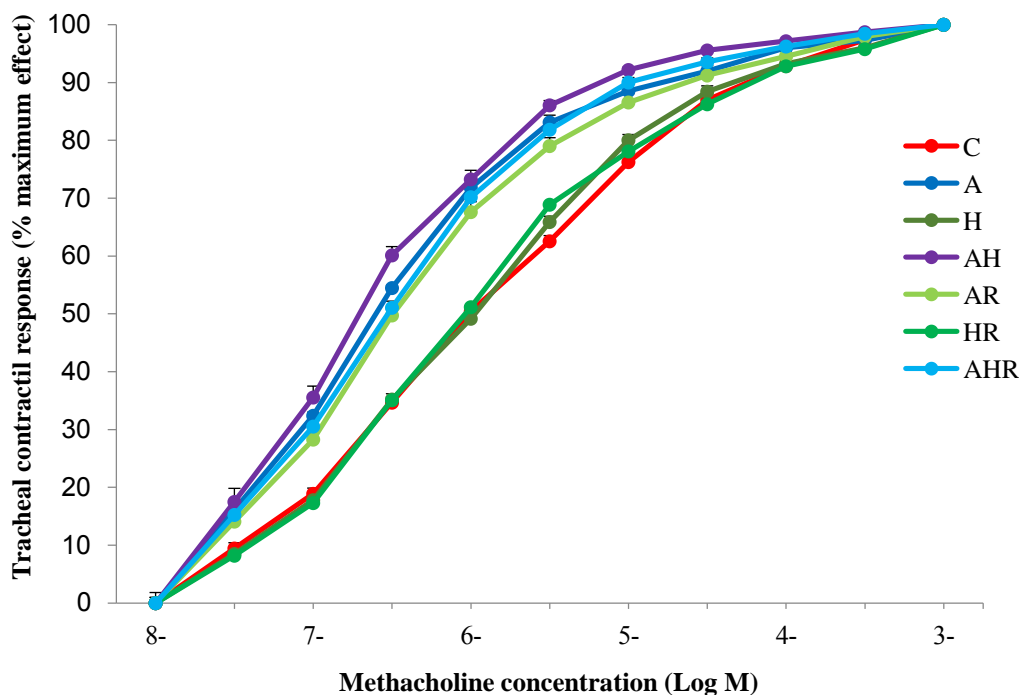


Figure 3. Cumulative log concentration-response curves of methacholine-induced contraction of the isolated trachea in the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR). Groups. Data are shown as mean±SEM (n=7 in each group).

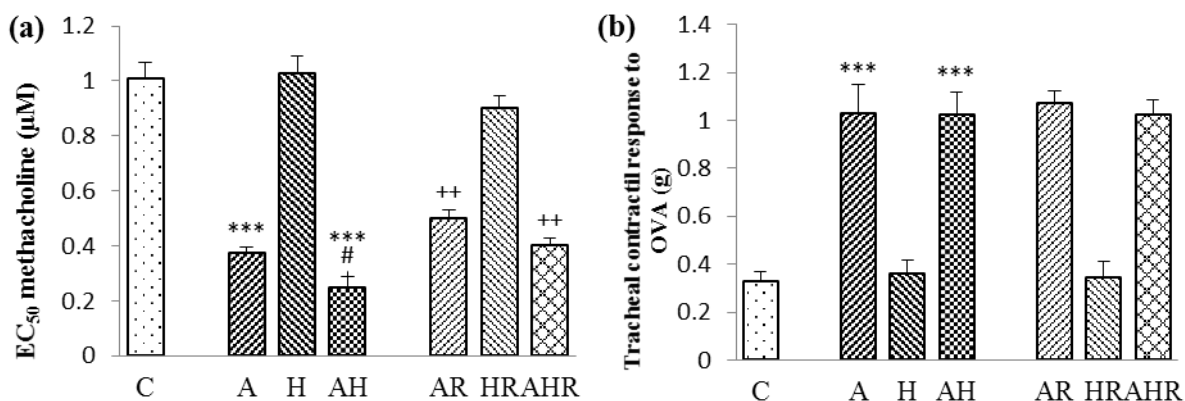


Figure 4. The effective concentration of methacholine, causing 50% of maximum response (EC_{50}), (a) and response to ovalbumin (OVA), (b) in the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Data are shown as mean±SEM (n=7 in each group). *** $p < 0.001$ compared to control group, # $p < 0.05$ compared to the asthmatic group, and ++ $p < 0.01$ compared to untreated groups. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

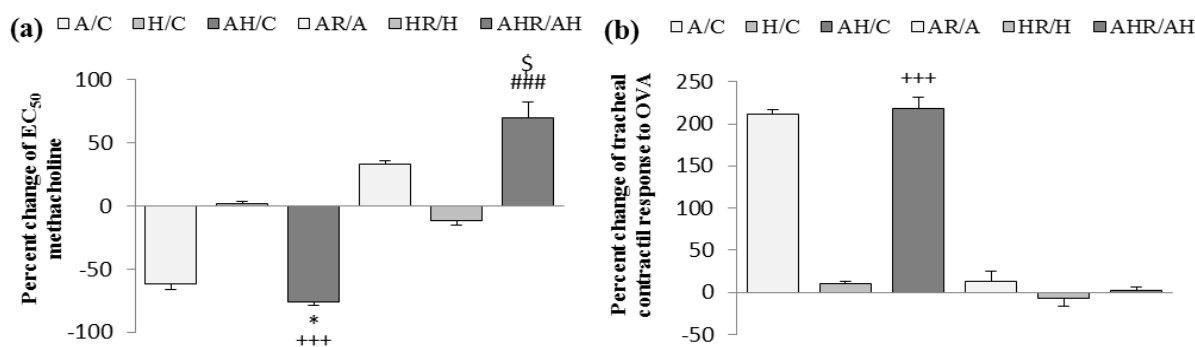


Figure 5. Percent change of the effective concentration of methacholine, causing 50% of maximum response (EC₅₀), (a) and response to ovalbumin (OVA), (b) in the asthmatic group relative to the control group (A/C), hyperlipidemic group relative to the control group (H/C), asthmatic-hyperlipidemic group relative to the control group (AH/C), rosuvastatin-treated asthmatic group relative to the asthmatic group (AR/A), rosuvastatin-treated hyperlipidemic group relative to the hyperlipidemic group (HR/H), and rosuvastatin-treated asthmatic-hyperlipidemic group relative to the asthmatic-hyperlipidemic group (AHR/AH). Data are shown as mean±SEM (n=7 in each group). **p*<0.05 compared to A/C group, +++*p*<0.001 compared to H/C group, \$; *p*<0.05 compared to AR/A group, ###*p*<0.001 compared to HR/H group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

Total and Differential White Blood Cells in BALF

Total WBC count, the numbers of eosinophils, neutrophils, and monocytes of A and AH groups were significantly higher than those of controls (*p*<0.001 for all cases) (Figures 6a-e). Significant reduction in total WBC, the numbers of neutrophils, eosinophils, and monocytes were seen in AR and AHR groups compared to A and AH groups (*p*<0.05 to *p*<0.001), (Figures 6a-d). The percent change of total WBC count, the numbers of eosinophils, neutrophils, and monocytes in AH/C were significantly higher than H/C (*p*<0.001 for all cases) (Figure 7a-d). The percent change of total WBC count, the numbers of eosinophils, neutrophils, and monocytes in AHR/AH were also significantly lower than those of HR/H (*p*<0.05 to *p*<0.001), (Figure 7a-d).

Oxidant and Anti-Oxidant Biomarkers in BALF

The significant increase in nitrite concentration was seen in A and AH groups compared to controls (*p*<0.001 for both cases) (Figure 8a). The percent change of nitrite concentration in AH/C was significantly higher than A/C and H/C (*p*<0.001 for both cases), (Figure 9a). There was no significant difference in nitrite concentration or the percent change of nitrite concentration between rosuvastatin-treated and untreated groups (Figure 8a).

MDA concentration in A and AH groups was significantly increased compared to controls (*p*<0.001 for both cases), (Figure 8b). The percent change of MDA concentration in AH/C was significantly higher than H/C (*p*<0.001) (Figure 9b). Rosuvastatin treatment decreased MDA concentration in AR (*p*<0.01), HR, and AHR (*p*<0.05 for both cases) groups (Figure 8b). The percent change of MDA concentration in AHR/AH was significantly lower than those of HR/H (*p*<0.01) (Figure 9b).

Total thiol content was significantly decreased in A and AH groups compared to controls (*p*<0.01 and *p*<0.001, respectively) (Figure 10a). The percent change of total thiol content in AH/C was significantly lower than A/C (*p*<0.05) and H/C (*p*<0.001) (Figure 11a). Rosuvastatin treatment increased total thiol content in AR and AHR groups compared to A and AH groups (*p*<0.05 for both cases) (Figure 10a). The percent change of total thiol content in AHR/AH was also significantly higher than those of HR/H (*p*<0.001), (Figure 11a).

SOD and CAT activities were significantly decreased in A and AH groups compared to controls (*p*<0.01 for all cases) (Figures 10b and c). The percent change of SOD and CAT activities in AH/C was significantly lower than H/C (*p*<0.001 for both cases) (Figures 11b and c).

Influence of Rosuvastatin Treatment on Asthma

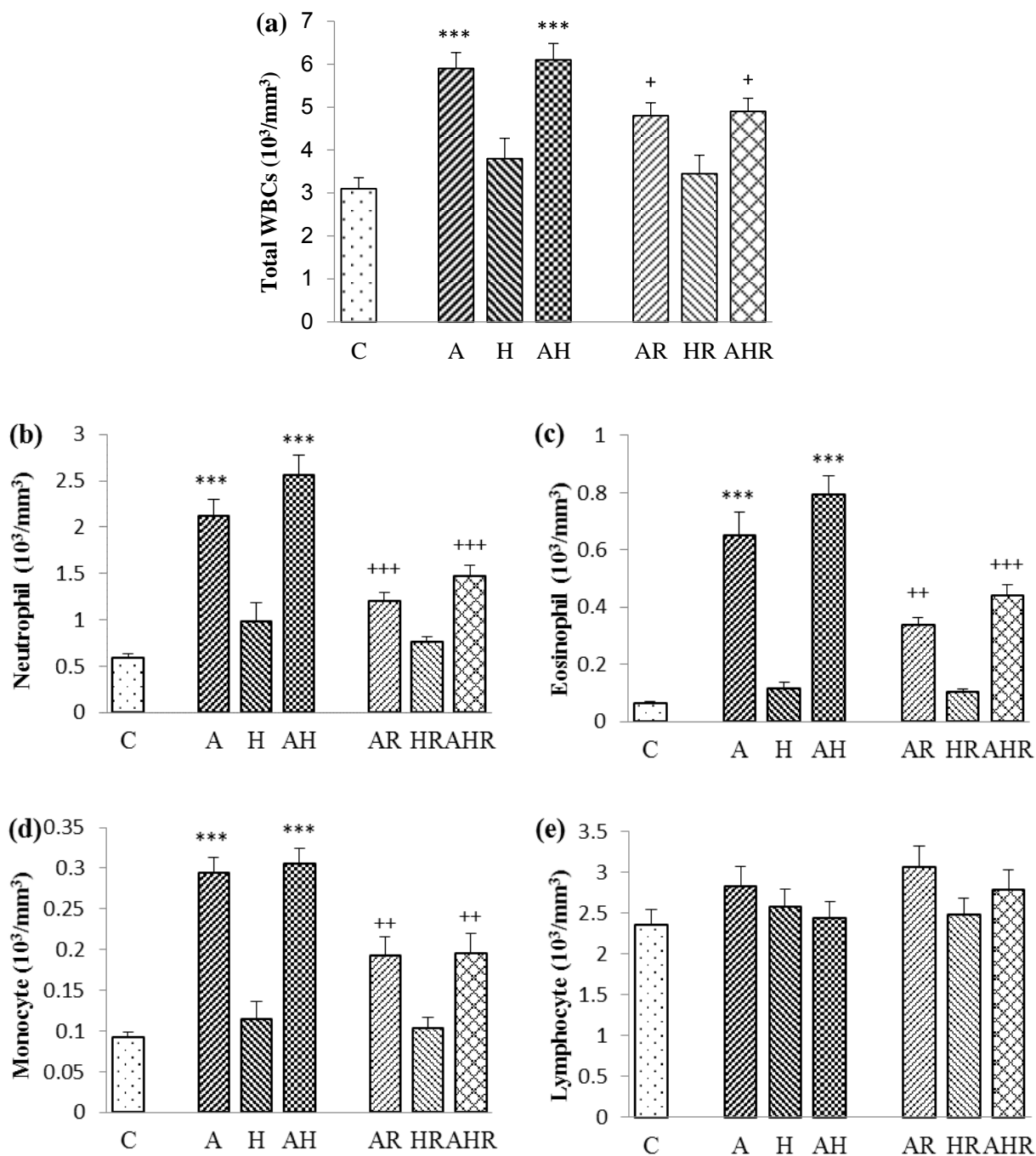


Figure 6. Total (a) and differential (b-e) white blood cell (WBC) counts in BALF of the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Data are shown as mean \pm SEM (n=7 in each group). *** p <0.001 compared to control group, + p <0.05, ++ p <0.01 and +++ p <0.001 compared to untreated groups. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

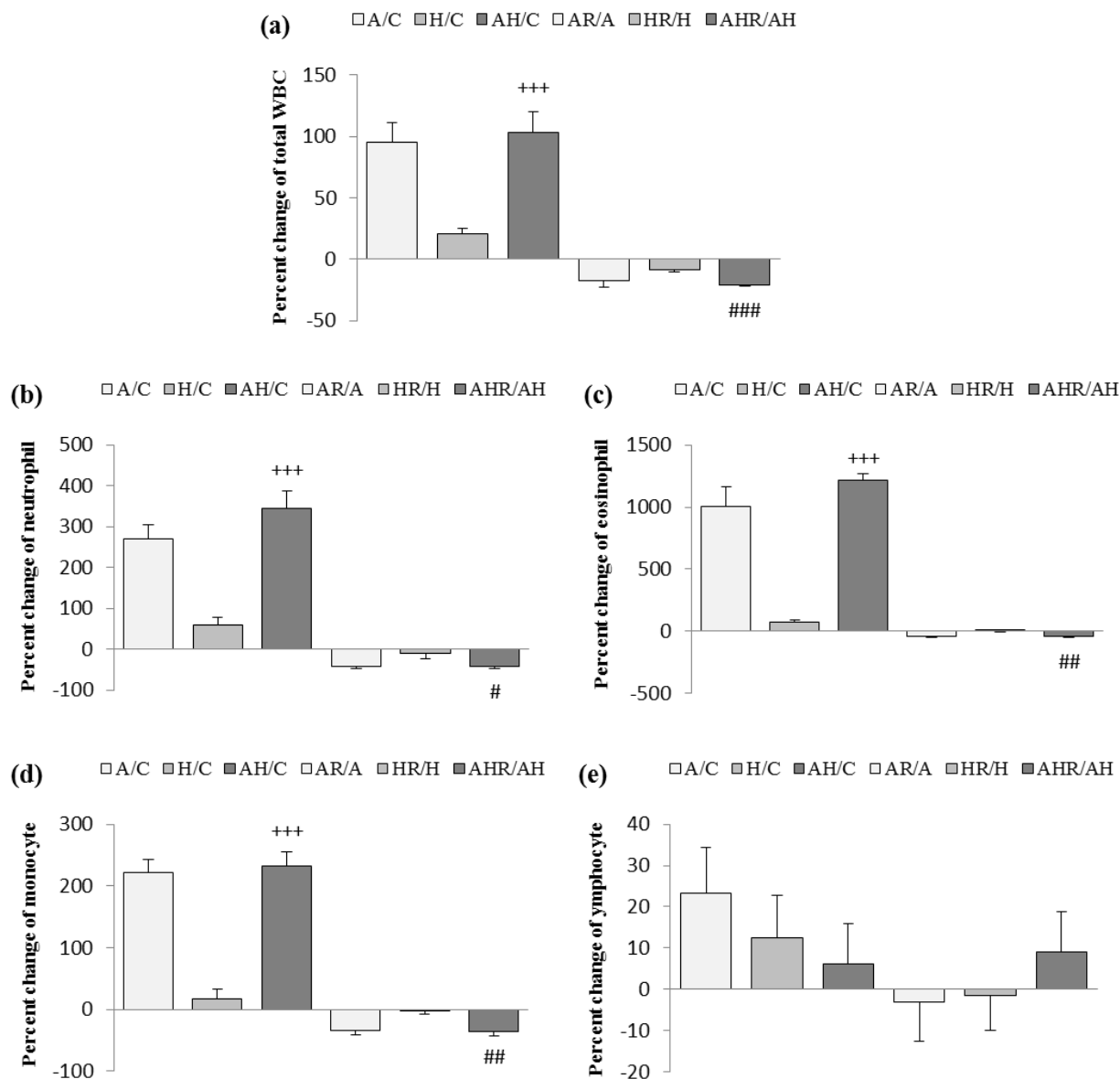


Figure 7. Percent change of total (a) and differential (b-e) white blood cell (WBC) counts in the asthmatic group relative to the control group (A/C), hyperlipidemic group relative to the control group (H/C), asthmatic-hyperlipidemic group relative to the control group (AH/C), rosuvastatin-treated asthmatic group relative to the asthmatic group (AR/A), rosuvastatin-treated hyperlipidemic group relative to the hyperlipidemic group (HR/H), and rosuvastatin-treated asthmatic-hyperlipidemic group relative to the asthmatic-hyperlipidemic group (AHR/AH). Data are shown as mean±SEM (n=7 in each group). +++*p*<0.001 compared to H/C group, #*p*<0.05, ##*p*<0.01 and ###*p*<0.001 compared to HR/H group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

Rosuvastatin treatment increased SOD and CAT activities in AR and AHR groups compared to A and AH groups (*p*<0.01 for AR group and *p*<0.05 for AHR group), (Figures 10 b and c). The percent change of

SOD and CAT activities in AHR/AH was also significantly higher than those of AR/A (*p*<0.05 for both cases) and HR/H (*p*<0.05 and *p*<0.001, respectively) (Figures 11 b and c).

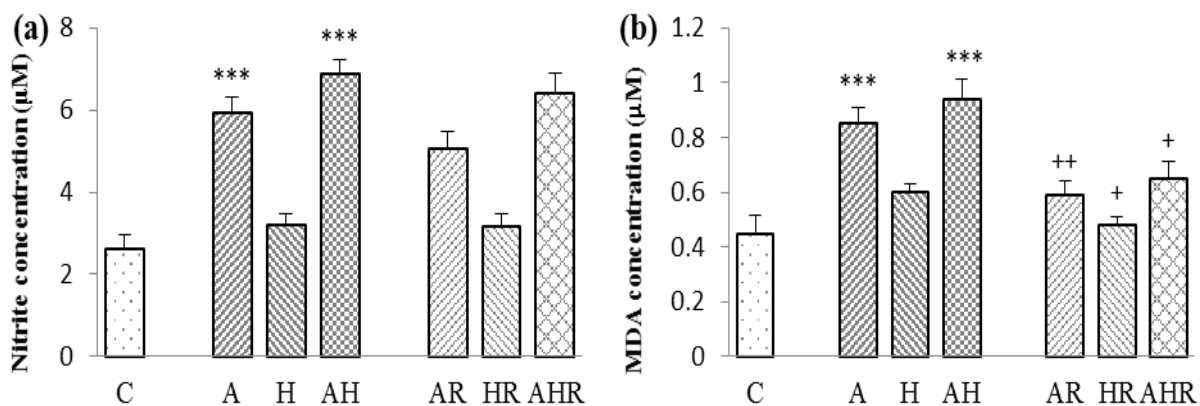


Figure 8. Nitrite (a) and malondialdehyde (MDA); b) concentrations in BALF of the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Data are shown as mean ± SEM (n=7 in each group). *** $p < 0.001$ compared to control group, + $p < 0.05$ and ++ $p < 0.01$ compared to untreated groups. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

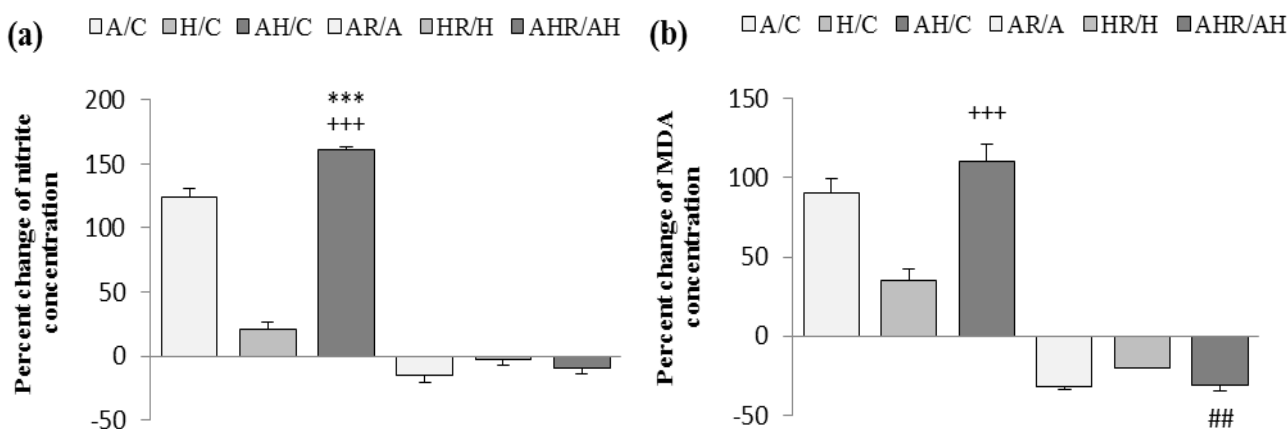


Figure 9. Percent change of nitrite (a) and malondialdehyde (MDA); b) concentrations in the asthmatic group relative to the control group (A/C), hyperlipidemic group relative to the control group (H/C), asthmatic-hyperlipidemic group relative to the control group (AH/C), rosuvastatin-treated asthmatic group relative to the asthmatic group (AR/A), rosuvastatin-treated hyperlipidemic group relative to the hyperlipidemic group (HR/H), and rosuvastatin-treated asthmatic-hyperlipidemic group relative to the asthmatic-hyperlipidemic group (AHR/AH). Data are shown as mean ± SEM (n=7 in each group). *** $p < 0.001$ compared to A/C group, +++ $p < 0.001$ compared to H/C group, ## $p < 0.01$ compared to HR/H group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

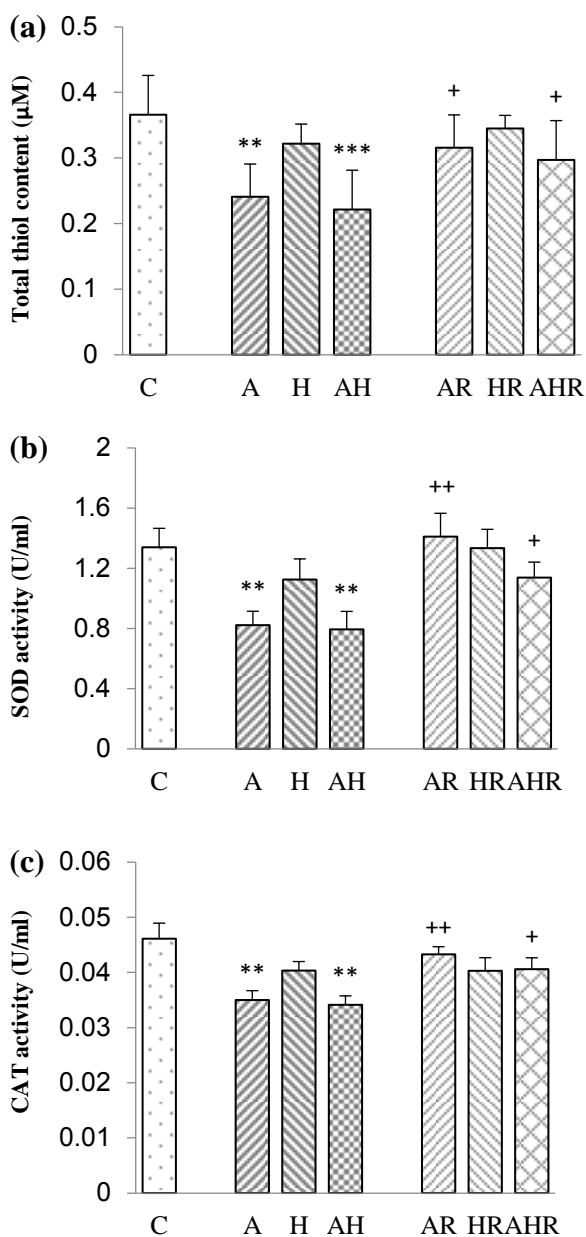


Figure 10. Total thiol content (a), superoxide dismutase (SOD); b) and catalase (CAT); c) activities in BALF of the control (C), asthmatic (A), hyperlipidemic (H), asthmatic-hyperlipidemic (AH), rosuvastatin-treated asthmatic (AR), rosuvastatin-treated hyperlipidemic (HR), and rosuvastatin-treated asthmatic-hyperlipidemic (AHR) groups. Data are shown as mean ± SEM (n=7 in each group). ***p*<0.01 and ****p*<0.001 compared to control group, +*p*<0.05 and ++*p*<0.01 compared to untreated groups. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

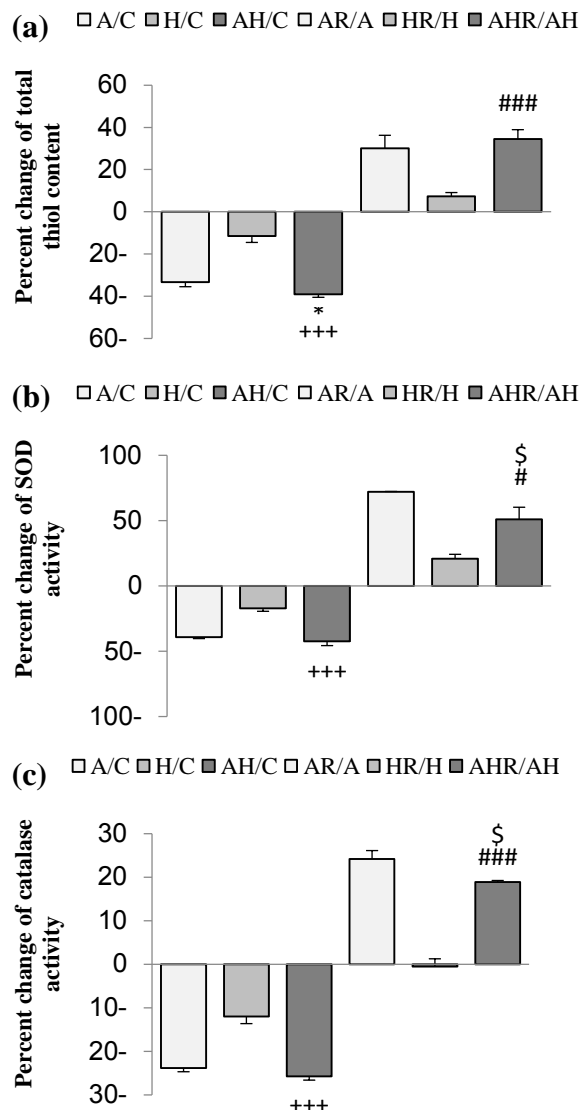


Figure 11. Percent change of total thiol content (a), superoxide dismutase (SOD); b) and catalase (CAT); c) activities in the asthmatic group relative to the control group (A/C), hyperlipidemic group relative to the control group (H/C), asthmatic-hyperlipidemic group relative to the control group (AH/C), rosuvastatin-treated asthmatic group relative to the asthmatic group (AR/A), rosuvastatin-treated hyperlipidemic group relative to the hyperlipidemic group (HR/H), and rosuvastatin-treated asthmatic-hyperlipidemic group relative to the asthmatic-hyperlipidemic group (AHR/AH). Data are shown as mean±SEM (n=7 in each group). **p*<0.05 compared to A/C group, +++*p*<0.001 compared to H/C group, \$; *p*<0.05 compared to AR/A group, #*p*<0.05 and ###*p*<0.001 compared to HR/H group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

DISCUSSION

Several previous studies have shown the induction of an animal model of asthma by ovalbumin exposure; using similar method used in the present study.^{19,25-27} The results of the present study showed significantly increased tracheal responsiveness to methacholine and ovalbumin, increment the BALF counts of total WBC, eosinophils, neutrophils, and monocytes as well as increased levels of nitrite and MDA, and decreased levels of total thiol, SOD, and CAT which were confirmed to cause asthma induction in the present study as stated in previous studies.^{19,21,26,27}

The results indicated that the induction of hyperlipidemia caused a significant increase in the serum levels of TC, TG, and LDL-C compared to control group. The ethanol-fructose combined diet significantly worsened plasma lipid profiles in rats which were in line with the previous study.¹⁸

The dyslipidemic murine strains were reported to display increased airway hyperresponsiveness.²⁸ However, this study did not indicate airway hyperresponsiveness to methacholine in hyperlipidemic rats compared to controls. In a high-fat diet-induced obesity rat model, airway contractility to methacholine was not changed in hyperlipidemic rats compared to controls²⁹ which was in line with the present study.

Hyperlipidemia is a systemic inflammatory state in which increased oxidative stress is seen. In this study, oxidative stress markers in BALF remained unchanged in hyperlipidemic rats compared to controls.

Beyond lipid-lowering, systemic treatment with rosuvastatin decreased tracheal responsiveness to methacholine and increased the mean values of EC₅₀ for methacholine as well as reduced BALF total WBC, neutrophils, eosinophils, and monocytes counts. Rosuvastatin treatment also reduced lung oxidative stress by decreasing MDA concentration and increasing total thiol content as well as SOD and CAT activities in asthmatic and asthmatic-hyperlipidemic groups. This finding gives a novel insight into the pleiotropic effects of rosuvastatin.

In this study, a rightward shift of the concentration-response curves to methacholine and an increase in the mean values of EC₅₀ methacholine was observed in the treated groups with rosuvastatin which indicates a decrease in the responsiveness to methacholine in the rosuvastatin treated groups. The percent change of EC₅₀ methacholine in treated asthmatic-hyperlipidemic

group relative to untreated asthmatic-hyperlipidemic group (AHR/AH) was significantly higher than those of treated asthmatic group relative to untreated asthmatic group (AR/A) and treated hyperlipidemic group relative to untreated hyperlipidemic group (HR/H) which shows that the response to treatment in treated asthmatic-hyperlipidemic group was due to the improvement of asthma and hyperlipidemia conditions by rosuvastatin. In some mouse model of ovalbumin-induced asthma, treatment and pretreatment with inhaled and intraperitoneal simvastatin significantly reduced the airway responsiveness to methacholine.^{30,31} In another similar model of asthma, intratracheal instillation of pravastatin had no statistically significant effect on airway hyperreactivity to methacholine.³² A case-control study among asthmatic patients showed that rosuvastatin improved pulmonary function tests but did not show significant effect on mean methacholine provocation dose.¹² Statins can inhibit the mevalonate pathway and the synthesis of downstream intermediates including farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which post-translationally modify small guanosine triphosphatases (GTPases). GTPases may play a role in the pathophysiology of asthma because they could enhance airway smooth muscle contraction and proliferation, and increase airway hyperresponsiveness.¹⁰ Therefore, the possible mechanism of rosuvastatin in reducing the tracheal responsiveness to methacholine is perhaps the inhibition of the production of mevalonate pathway metabolites. The absence of the effect of rosuvastatin treatment on tracheal responsiveness to ovalbumin despite its effect in reducing tracheal responsiveness to methacholine, is not known. These findings may suggest that the rosuvastatin treatment only affect non-specific airway responsiveness (responsiveness to methacholine) and does not affect specific airway responsiveness (responsiveness to ovalbumin). However, further studies should be performed to examine this suggestion.

Increase in total inflammatory cells; especially BALF eosinophilia is another characteristic of asthma.²⁹ In the present study, rosuvastatin reduced total inflammatory cell and eosinophilia in the BALF. The percent change of total WBC count, the numbers of eosinophils, neutrophils, and monocytes in treated asthmatic-hyperlipidemic group relative to untreated asthmatic-hyperlipidemic group (AHR/AH) was significantly higher than those of treated

hyperlipidemic group relative to untreated hyperlipidemic group (HR/H) which shows that the reduction of inflammatory cells infiltration in the lung of asthmatic-hyperlipidemic group was due to the improvement of asthma condition by rosuvastatin. Rosuvastatin improved peripheral eosinophilia in asthmatic patients.¹² In the mice models of ovalbumin-induced asthma, simvastatin³⁰ and rosuvastatin treatment dose-dependently reduced the numbers of total inflammatory cells, lymphocytes, macrophages, neutrophils, and eosinophils recruited into BALF¹⁴ which supported the results of this study.

Statins can inhibit stimuli-induced NO formation and iNOS induction to different extents. This inhibition occurs at the transcriptional level, and displays potency in the order of lovastatin > atorvastatin > fluvastatin >> pravastatin.³³ In the current study, BALF level of nitrite remained unchanged in AR and AHR groups compared to untreated groups. However, administration of rosuvastatin reduced iNOS expression in ligature-induced periodontitis with/without hyperlipidemia.³⁴ To reduce the BALF level of nitrites, it may need long-term treatment or inhalation therapy for local treatment.

Rosuvastatin treatment decreased lung oxidative stress by decreasing MDA concentration, and increasing total thiol content, SOD, and CAT activities in treated asthmatic and asthmatic-hyperlipidemic groups. The decreased percent change of MDA concentration and the increased percent change of total thiol content in treated asthmatic-hyperlipidemic group relative to untreated asthmatic-hyperlipidemic group (AHR/AH) compared to those of treated hyperlipidemic group relative to untreated hyperlipidemic group (HR/H) shows that the reduction of lung oxidative stress also contribute in the treatment of asthmatic-hyperlipidemic group. However, the percent change of total thiol content, SOD and CAT activities in treated asthmatic-hyperlipidemic group relative to untreated asthmatic-hyperlipidemic group (AHR/AH) was significantly higher than those of treated hyperlipidemic group relative to untreated hyperlipidemic group (HR/H) which also support this suggestion that the reduction of lung oxidative stress in the lung of asthmatic-hyperlipidemic group was due to the improvement of asthma condition by rosuvastatin. In cigarette smoke-induced pulmonary oxidative stress, treatment with atorvastatin, pravastatin, simvastatin, and rosuvastatin showed a reduction in SOD activity,

but only the simvastatin induced a reduction in CAT activity.³⁵ It is suggested that superoxide anion is rapidly scavenged, whereas hydrogen peroxide is more stable and may cross the cell membrane.³⁶ From this perspective, the ability of statins to prevent the production of hydrogen peroxide is more important than their ability to prevent the production of superoxide anion. Thus, considering the results of concerning CAT activity in this study, rosuvastatin demonstrated very promising antioxidant agent.

There are a few limitations to this study that need to be addressed in further studies. First, only one dose of rosuvastatin has been studied. It has already been shown that the pleiotropic effect of rosuvastatin is dose-dependent. Rosuvastatin 40 mg/kg showed an anti-hyperlipidemic effect in previous studies, whereas higher dose (≥ 80 mg/kg) of rosuvastatin induced adverse effects.³⁷ Second, currently there is no reference drug that can be administrated simultaneously to treat hyperlipidemia and asthma. Third, there was no significant change in the tracheal responsiveness to ovalbumin between the groups after the treatment with rosuvastatin. Longer duration or higher dose of rosuvastatin in larger sample size may provide more improvement in the tracheal responsiveness to ovalbumin. Fourth, although hyperlipidemia is a potential risk factor for asthma independent of obesity and high body weight,¹ it might be better if the weight of the animals was reported. Fifth, airway inflammation and/or airway remodeling was not studied in this study.³⁸ Therefore, the histopathological evidence of the effectiveness of rosuvastatin on asthma needs to be evaluated in further studies.

However, in the present study, the effect of rosuvastatin on non-specific tracheal (airway) responsiveness (response to methacholine) and specific tracheal responsiveness (response to ovalbumin) which is the main hallmark features of asthma³⁸ was examined. In addition, the effect of rosuvastatin on lung inflammation (total and differential WBC) and lung oxidative stress was shown. In fact, the results of the present study indicated the ameliorative effects of rosuvastatin on lung inflammation and oxidative stress which resulted in a reduction of tracheal responsiveness to methacholine. In addition, the results showed that the therapeutic effect of rosuvastatin on asthma is not only due to its lipid-lowering effect. If the therapeutic effect of rosuvastatin on asthma was only due to its lipid-lowering effect, it shouldn't affect non-hyperlipidemic

asthmatic group.

The results of this study indicated the anti-oxidative and anti-inflammatory effects of rosuvastatin on asthmatic rat which perhaps leads to reduction in tracheal responsiveness to methacholine. Important pleiotropic mechanisms may be responsible for the rosuvastatin-induced reduction of airway hyperresponsiveness, inflammatory cells, and oxidative stress.

ACKNOWLEDGEMENTS

The study was approved by the ethics committee of Mashhad University of Medical Sciences for Animal Experiments (code 940997).

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