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Combination of ascorbic acid and calcitriol attenuates chronic asthma disease by reductions in oxidative stress and inflammation

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ABSTRACT

Airway inflammation and oxidative stress are the two major characteristics of asthma pathogenesis. Therefore, this study evaluated the protective effects of ascorbic acid in combination with calcitriol on the oxidative damages and inflammation in asthma model. All animals, except in the control group, were sensitized and challenged with ovalbumin. One day after the last challenge, samples of bronchoalveolar lavage fluid was collected for the assessment of total white blood cell counts and differential count of white blood cell and plasma was used for the measurement of pro-oxidant/antioxidant balance level. Lung tissue samples were also stored for examining peribronchial inflammatory cell infiltration, phosphorylated nuclear factor-kappa B expression and measurement of malondialdehyde level. Induction of asthma caused significant increases in total white blood cell counts, percentage of neutrophils and eosinophils and a decrease in the percentage of lymphocytes. Moreover, asthma resulted in significant increases of peribronchial inflammatory cell infiltration, phosphorylated nuclear factor-kappa B expression and malondialdehyde level. However, no significant changes were observed in pro-oxidant/antioxidant balance level with the induction of asthma. Co-administration of low doses of ascorbic acid and calcitriol returned all to the levels measured before sensitization and challenge. Combination of low doses of ascorbic acid with calcitriol improves mouse asthma model by a possible additive effects through the decrease of oxidative stress and inflammation.

1. Introduction

Asthma, as a complex chronic respiratory disease, is characterized by recurrent episodic symptoms including wheezing and coughing (Oraka et al., 2013). As estimated, it affects over 300 million people of all ages, worldwide, with great social impact and huge burden on healthcare costs (Shaikh et al., 2016). Airway inflammation is a major characteristic of asthma pathogenesis (Shaikh et al., 2016).Upon allergen exposure, T helper (Th) 2 lymphocytes, neutrophils and eosinophils are attracted into the airway resulting in inflammation (Edwards et al., 2009). Then, inflammation causes the production of excessive reactive oxygen species (ROS) that lead to oxidative stress (Rochelle et al., 1998). Inflammation also reduces antioxidant capacity defensing against ROS (Cross, 2003). Nowadays, corticosteroids are among the widely used drugs in asthma therapy. However, they have several adverse effects such as osteoporosis and infections which highlight a need to improve the understanding of treatment of this disease (Manson et al., 2009). Considering the critical role of inflammation and oxidative stress in asthma pathophysiology, it is possible that exogenous supplementation of anti-inflammatory and antioxidant agents can be valuable in the treatment and/or management of asthma. It is well known that ascorbic acid and calcitriol have antioxidant, anti-inflammatory and immunomodulatory activities (Chambial et al., 2013; Kerley et al., 2015; Rodriguez-Lecompte et al., 2016). On the other hand, there is a common belief that a combination of antioxidants could be more effective for treatment due to the additive or synergistic effects (Tripathi et al., 2010). Therefore, in the present study, the protective effects of ascorbic acid combined with calcitriol were investigated in a mouse asthma model.

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2. Materials and methods

2.1. Ethics statement

The animal experimental procedures were approved by the Animal Ethics Committee of the Faculty of Medicine, Tehran University of Medical Sciences.

2.2. Animal grouping and treatment

Male BALB/c mice (6–8 weeks) were randomly divided into eight groups, each containing 4–5 mice. 1) Control: no sensitization and no challenge. 2) Asthma: sensitization and challenge with ovalbumin (OVA) (grade V; Sigma-Aldrich, USA). 3) Ineffective C: each mouse orally received ineffective dose (39 mg/kg in normal saline) of ascorbic acid (Sigma-Aldrich, USA)

(Salahudeen and Nishtala, 2017). 4) Ineffective D: animals were intraperitoneally given ineffective dose (1.5 μg/kg in 0.9% ethanol) of calcitriol (Sigma-Aldrich, USA) (Salahudeen and Nishtala, 2017). 5) Effective C: mice were orally treated with effective dose of ascorbic acid (130 mg/kg in normal saline) (Chang et al., 2009). 6) Effective D: animals intraperitoneally received effective dose of calcitriol (5 μg/kg in 0.9% ethanol) (Zhang et al., 2015). 7) Combination: mice were treated with combination of oral administration of ascorbic acid and intraperitoneal administration of calcitriol in ineffective doses. In all treatment groups, animals were sensitized and challenged with OVA and the vitamins were administrated 30 min before each challenge from day 21 to 74. Effective doses of ascorbic acid and calcitriol were selected from studies that found the protective effects of these vitamins in asthma model (Chang et al., 2009; Zhang et al., 2015). To determine ineffective doses of ascorbic acid and calcitriol, semi log of effective doses was calculated (Salahudeen and Nishtala, 2017). Animals were obtained from the Department of Pharmacology, Tehran University of Medical Sciences. They were adapted for one week under standard experimental conditions and had free access to food and water at the same condition of 20 ± 2 °C room temperature and 12 h light-12 h dark cycle. For induction of chronic model of asthma in mice, a protocol of immunization with OVA was used (Mohammadian et al., 2016). Briefly, on days 0 and 14, animals were intraperitoneally sensitized with 20 μg OVA plus 2 mg aluminum hydroxide (Sigma-Aldrich, USA). In the control group, mice were intraperitoneally injected normal saline instead of OVA. One week later, animals were challenged with OVA (3% in normal saline) in a Plexiglas chamber (dimensions 40 × 40 × 70 cm) connected to an ultrasonic nebulizer (Beurer, Germany) for 30 min once a day, 3 times per week for 8 consecutive weeks. Mice in the control group were challenged with normal saline alone (Fig. 1).

2.3. Bronchoalveolar lavage fluid (BALF), blood and lung tissue collection

24 h after the last challenge, animals were intraperitoneally anesthetized by ketamine (80 mg/kg) and xylazine (8 mg/kg) and the tracheas were cannulated. Then, 0.4 ml sterile normal saline was injected into the lungs for three times by syringe. BALF samples were collected into microtubes and centrifuged at 2000 *g* for 3 min at 4 °C. The cell pellet was resuspended in 500 μl normal saline and total white blood cells (WBC) counts were carried out with a hemocytometer. The remaining cells were again centrifuged at 4000 *g* for 5 min at 4 °C, smeared on a slide and stained with Wright-Giemsa for differential cell count. Based on standard morphological criteria, 50 cells were counted and classified as neutrophils, eosinophils or lymphocytes. Blood samples were taken from the heart, centrifuged at 3000 *g* for 10 min at 4 °C and plasma was stored at −70 °C for the measurement of pro-oxidant/antioxidant (PAB). The lungs were excised and washed in cold saline on ice. The upper lobe of right lung was fixed in 10% formalin for histological assessments and upper lobe of left lung was kept at −70 °C for measurement of malondialdehyde level (MDA).

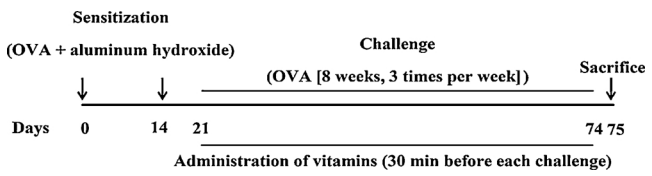

Fig. 1. Experimental protocol of the study. OVA: ovalbumin.

2.4. Lung histopathological and immunofluorescence analyses

The lung tissues were fixed in formalin and embedded in paraffin. Then, they were sectioned at 4 μm thickness and were stained with hematoxylin-eosin (H&E) solution. Five airway sections were randomly selected throughout the lung and analyzed for each animal. The severity of lung inflammation was calculated according to the degree of inflammatory cell infiltration to the peribronchiolar area. The scoring system of peribronchiolar inflammation was 0, no cells; 1, a few cells; 2, a ring of cells (1 cell layer deep); 3, a ring of cells (2–4 cells deep); 4, a ring of cells (4–6 cells deep); and 5, a ring of cells (6 cells deep) (Cao et al., 2011). For fluorescent immunocytochemistry, briefly, the paraffin sections were deparaffinized by xylene and then hydrated by immersion into reducing concentrations of ethanol. At least 30 sections were provided and collected onto poly-L-lysine coated cover slips for each independent experiment. After that, for antigen retrieval, slides were pretreated by trypsin solution (Sigma-Aldrich, USA) which can significantly reopen the cross-linked epitopes so that antibodies can quickly stick to target antigens. Non-specific protein binding was blocked by 2 h incubation in 1% BSA + FBS 10% in tris-buffered saline (TBS)-Tween (Sigma-Aldrich, USA). Next, the sections were incubated (4 °C) overnight with one primary antibody including mouse monoclonal antibody against phosphorylated nuclear factor-kappa B (p-NF-κB) as inflammatory marker (Santa Cruz Biotechnology, USA). Thereafter, the sections were incubated 6-h (room temperature) with the secondary antibodies (Santa Cruz Biotechnology, USA) including fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin G (IgG) to detect p-NF-κB. All sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA) to visualize nuclei. Using a fluorescence microscopy, the images were captured and quantified using NIH Image *J* analysis program (NIH, Bethesda, MD, USA) (Sanadgol et al., 2018). p-NF-κB positive immunoreactivity was measured after background subtraction. Similar threshold level was set for every image, on dark background and then positive signals were quantified.

2.5. Measurement of plasma PAB level

In order to measure the PAB values, a modified method was applied based on Alamdari method (Alamdari et al., 2008). Briefly, after preparation of tetramethylbenzidine (TMB) cation sample (Sigma-Aldrich, USA), peroxidase enzyme solution was added to it. Then, TMB solution was prepared and the working solution was prepared by mixing TMB cation with TMB solution. 200 μL of this solution was added to 10 μl of each sample, standard or blank (distilled water) in each well of a 96 well plate. After incubation at 37 °C for 12 min in a dark place, 100 μL of hydrochloric acid (HCl) (Sigma-Aldrich, USA) was added to each well and measured in an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instrument, ELX 800, Inc, USA) at 450 nm.

2.6. Measurement of lung MDA level

Lung MDA level was estimated according to Esterbauer and Cheeseman method (Cheeseman, 1994). On the basis of this method, briefly, the lung tissue was mixed with 2 volumes of 10% trichloroacetic acid (TCA) (Sigma-Aldrich, USA) for precipitation of proteins. After centrifugation of the mixture at 3000 cycles for 15 min, supernatant was separated and reacted with thiobarbituric acid (TBA) (Sigma-Aldrich, USA) in boiling water for 15 min followed by a cooling period. Reaction of MDA with TBA creates a pink pigment which has a

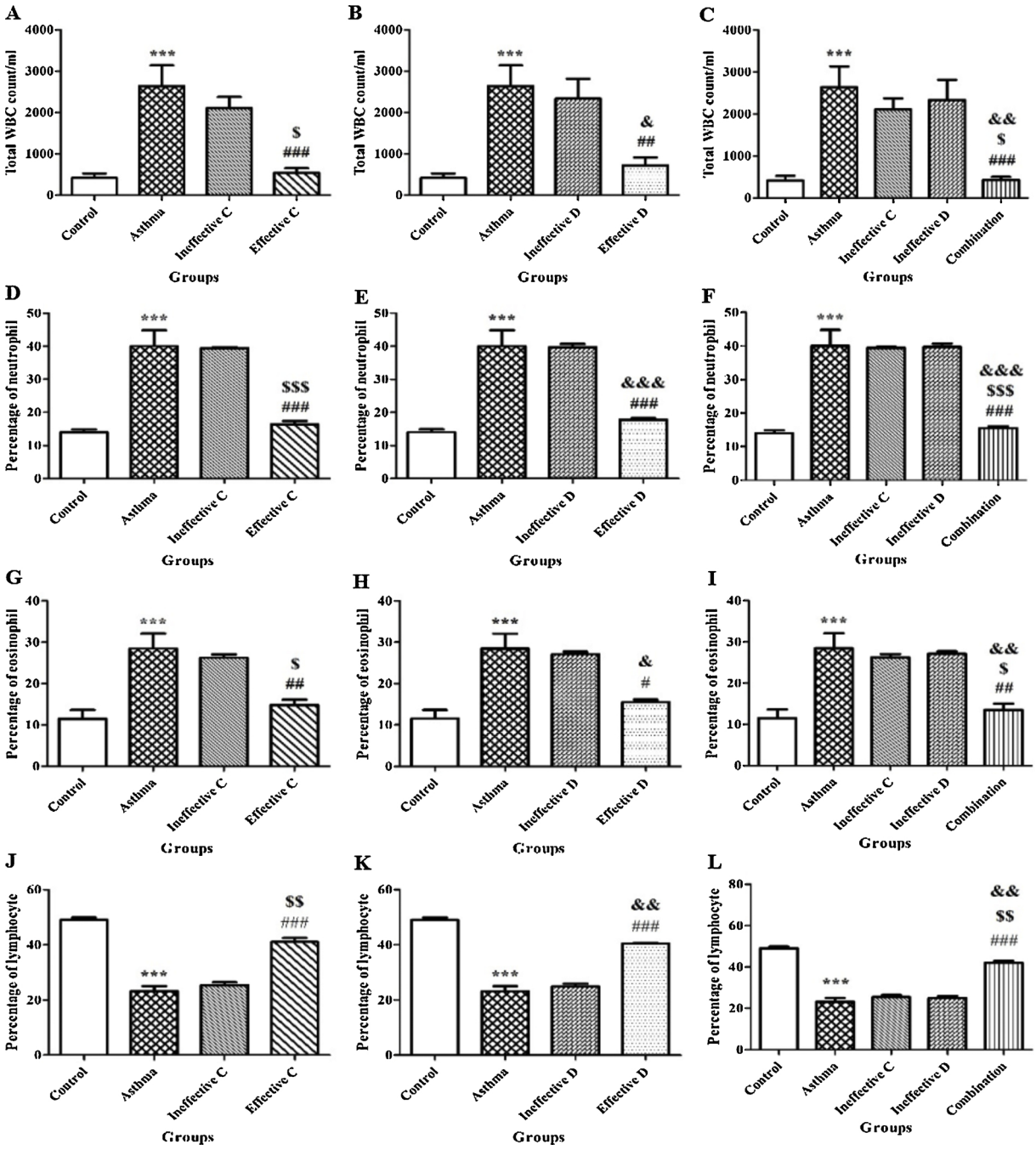

Fig. 2. Changes of total white blood cell (WBC) count (A–C) and differential count of WBC in bronchoalveolar lavage fluid (BALF) supernatant (D–L) with ineffective and effective doses of ascorbic acid (A, D, G, *J*), with ineffective and effective doses of calcitriol (B, E, H, K) and with combination of ineffective doses of ascorbic acid and calcitriol (C, F, I, L). Data are expressed as mean ± SEM. *** $P < 0.001$ versus the control group. # $P < 0.05$ versus the asthma group. ## $P < 0.01$ versus the asthma group. ### $P < 0.001$ versus the asthma group. $ $P < 0.05$ versus the ineffective C group. $$ $P < 0.01$ versus the ineffective C group. $$$ $P < 0.001$ versus the ineffective C group. & $P < 0.05$ versus the ineffective D group. && $P < 0.01$ versus the ineffective D group. &&& $P < 0.001$ versus the ineffective D group. Ineffective C: ascorbic acid with ineffective dose, Ineffective D: calcitriol with ineffective dose, Effective C: ascorbic acid with effective dose, Effective D: calcitriol with effective dose.

maximum absorption at 532 nm.

2.7. Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). Comparisons between groups were calculated by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Kruskal Wallis analysis of variance was used to analyze histology scores. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Effect of ascorbic acid and calcitriol and their combination on total WBC counts and differential counts of WBC in the BALF

There was a significant increase in total WBC counts in the asthma group compared to the control group ($P = 0.000$) (Fig. 2). Administration of ascorbic acid and calcitriol in effective doses significantly reduced total WBC counts in comparison with the asthma group

(P = 0.000 and P = 0.003, respectively) (Fig. 2). Administration of ascorbic acid and calcitriol in effective doses significantly decreased total WBC counts compared to the groups of ineffective C and ineffective D (P = 0.013 and P = 0.017, respectively) (Fig. 2). There were no significant differences in total WBC counts in the groups of ineffective C and ineffective D in comparison with the asthma group (Fig. 2). However, combined administration of ascorbic acid and calcitriol in ineffective doses compared to the groups of asthma, ineffective C and ineffective D significantly reduced total WBC counts (P = 0.000, P = 0.012 and P = 0.003, respectively) (Fig. 2). There was a significant increase in the percentage of neutrophils (P = 0.000) and eosinophils (P = 0.000) and a decrease in the percentage of lymphocytes (P = 0.000) in the asthma group compared to the control group (Fig. 2). Administration of ascorbic acid and calcitriol in effective doses significantly reduced the percentage of neutrophils (P = 0.000 and P = 0.000, respectively) and eosinophils (P = 0.003 and P = 0.010, respectively) and increased the percentage of lymphocytes (P = 0.000 and P = 0.000, respectively) in comparison with the asthma group (Fig. 2). Administration of ascorbic acid and calcitriol in effective doses significantly decreased the percentage of neutrophils (P = 0.000 and P = 0.000, respectively) and eosinophils (P = 0.019 and P = 0.026, respectively) and increased the percentage of lymphocytes (P = 0.001 and P = 0.002, respectively) compared to the groups of ineffective C and ineffective D (Fig. 2). There were no significant differences in the percentage of neutrophils, eosinophils and lymphocytes in the groups of ineffective C and ineffective D in comparison with the asthma group (Fig. 2). However, combined administration of ascorbic acid and calcitriol in ineffective doses compared to the asthma, ineffective C and ineffective D groups reduced the percentage of neutrophils (P = 0.000, P = 0.000 and P = 0.000, respectively) and eosinophils (P = 0.002, P = 0.011 and P = 0.006, respectively) and increased the percentage of lymphocytes (P = 0.000, P = 0.001 and P = 0.001, respectively) (Fig. 2).

3.2. Effect of ascorbic acid and calcitriol and their combination on peribronchial inflammatory cell infiltration

There was a significant increase in the score of peribronchial inflammatory cell infiltration in the asthma group compared to the control group (P = 0.000) (Fig. 3). Administration of ascorbic acid and calcitriol in effective doses significantly reduced the score of peribronchial inflammatory cell infiltration in comparison with the asthma group (P = 0.001 and P = 0.002, respectively, (Fig. 3). Administration of ascorbic acid and calcitriol in effective doses significantly decreased the score of peribronchial inflammatory cell infiltration compared to the groups of ineffective C and ineffective D (P = 0.029 and P = 0.038, respectively, (Fig. 3). There were no significant differences in the score of peribronchial inflammatory cell infiltration in the groups of ineffective C and ineffective D in comparison with the asthma group (Fig. 3). However, combined administration of ascorbic acid and calcitriol in ineffective doses compared to the asthma, ineffective C and ineffective D groups significantly reduced the score of peribronchial inflammatory cell infiltration (P = 0.000, P = 0.015 and P = 0.010, respectively, Fig. 3). Lung histopathology photographs in different groups were also provided (Fig. 3).

3.3. Effect of ascorbic acid and calcitriol and their combination on lung p-NF-κB

There was a significant increase in p-NF-κB positive cells in the asthma group compared to the control group (P = 0.000) (Fig. 4). Administration of ascorbic acid and calcitriol in effective doses significantly reduced p-NF-κB positive cells in comparison with the asthma group (P = 0.000 and P = 0.000, respectively) (Fig. 4). Administration of ascorbic acid and calcitriol in effective doses significantly decreased p-NF-κB positive cells compared to the groups of ineffective C and ineffective D (P = 0.000 and P = 0.000, respectively). There were no significant differences in p-NF-κB positive cells in the groups of ineffective C and ineffective D in comparison with the asthma group (Fig. 4). However, combined administration of ascorbic acid and calcitriol in ineffective doses compared to the groups of asthma, ineffective C and ineffective D significantly reduced p-NF-κB positive cells (P = 0.000, P = 0.000 and P = 0.000, respectively, Fig. 4). Lung histopathology photographs in different groups were also provided (Fig. 4).

3.4. Effect of ascorbic acid and calcitriol and their combination on plasma PAB level

There was a significant decrease in PAB level in the effective C group compared to the asthma group (P = 0.022, Fig. 5). However, there were no significant differences in PAB level between other groups (Fig. 5).

3.5. Effect of ascorbic acid and calcitriol and their combination on lung MDA level

There was a significant increase in MDA level in the asthma group compared to the control group (P = 0.000, Fig. 6). Administration of ascorbic acid and calcitriol in effective doses significantly reduced MDA level in comparison with the asthma group (P = 0.000 and P = 0.011, respectively, Fig. 6). Administration of ascorbic acid and calcitriol in effective doses significantly decreased MDA level compared to the groups of ineffective C and ineffective D (P = 0.000 and P = 0.004, respectively, Fig. 6). There were no significant differences in MDA level in the groups of ineffective C and ineffective D in comparison with the asthma group (Fig. 6). However, combined administration of ascorbic acid and calcitriol in ineffective doses compared to the asthma, ineffective C and ineffective D groups significantly reduced MDA level (P = 0.000, P = 0.000 and P = 0.000, respectively, (Fig. 6).

4. Discussion

The murine OVA sensitization and challenge model has been widely used to evaluate the pathophysiology of asthma (Janssen-Heininger et al., 2009). This is the model that was used in the present study for the induction of asthma.

The precise molecular mechanisms underlying asthma are not fully elucidated. However, several causal factors have been suggested to contribute to its pathogenesis. Upon allergen exposure, innate immune cells process them and contribute to the initial commitment of naive Th cells into Th2 subsets (Lalla et al., 2010). Then, Th2 cells release various cytokines that finally recruit granulocytic cells (e.g. neutrophils and eosinophils) resulting in the inflammation (Lalla et al., 2006; Lee et al., 2008). Moreover, these granulocytic cells are the major source of reactive oxygen species (ROS) that induce oxidative stress. In this condition, ROS formation leads to lung tissue damage by membrane lipid peroxidation, protein dysfunction and deoxyribonucleic acid (DNA) breakdown (Nounou et al., 2010).

One important characteristic of asthma is the infiltration of neutrophils and eosinophils into the airways (Cheng et al., 2018). The current study demonstrated that infiltration of neutrophils and eosinophils to BALF were significantly higher in the asthma group compared to the control group. Peribronchial inflammatory cell infiltration was also significantly increased in asthmatic animals. These findings are in accordance with another study that reported a significant increase in the infiltration of neutrophils and eosinophils to BALF as well as peribronchial inflammatory cell infiltration as a result of asthma induction (Mohammadian et al., 2016).

Several lines of evidence indicate an increased NF-κB nuclear localization in airway tissue of asthmatics (Hart et al., 1998; Gagliardo et al., 2003). For example, in a murine asthma model, bronchial

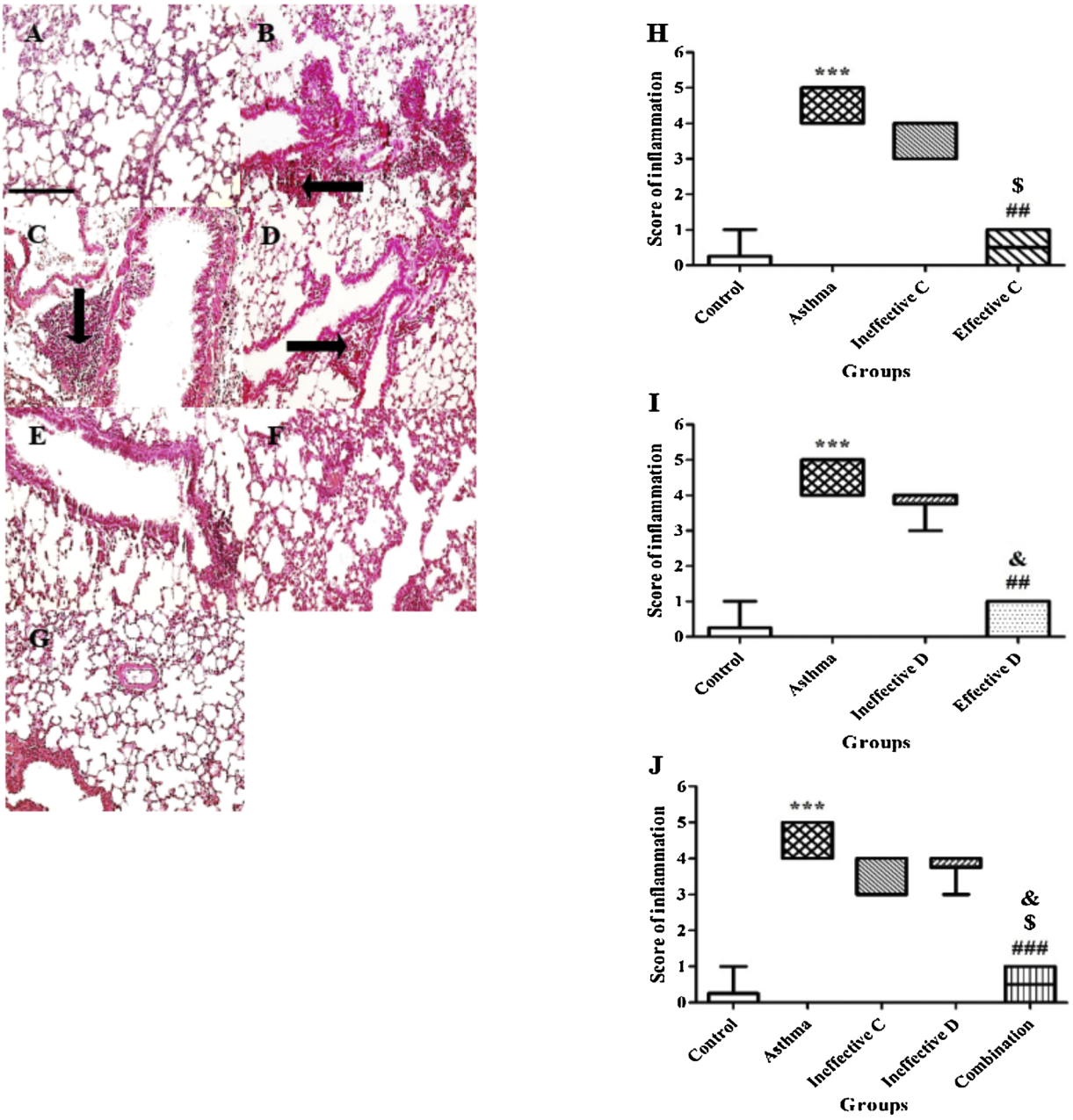

Fig. 3. The peribronchial inflammatory cell infiltration score with ineffective and effective doses of ascorbic acid (H), with ineffective and effective doses of calcitriol (I) and with combination of ineffective doses of ascorbic acid and calcitriol (*J*). Data are expressed as mean ± SEM. (A):Control, (B): Asthma, (C): Ineffective C, (D): Ineffective D, (E): Effective C, (F): Effective D, (G): Combination group. Arrows show peribronchial inflammatory cell infiltration. Bar: 100 μm.
*** $P < 0.001$ versus the control group. ## $P < 0.01$ versus the asthma group. ### $P < 0.001$ versus the asthma group. $ $P < 0.05$ versus the ineffective C group. & $P < 0.05$ versus the ineffective D group. Ineffective C: ascorbic acid with ineffective dose, Ineffective D: calcitriol with ineffective dose, Effective C: ascorbic acid with effective dose, Effective D: calcitriol with effective dose.

epithelium exhibits robust and rapid p-NF-κB nuclear translocation in comparison with controls (Poynter et al., 2002). Pro-inflammatory cytokines are released by inflammatory cells which increase inflammation in asthmatic airways and result in the activation of NF-κB (Barnes and Adcock, 1997). NF-κB is a transcription factor which plays a key role in the expression of many pro-inflammatory genes and contributes to a variety of acute and chronic inflammatory diseases (Rothwarf and Karin, 1999). In this study, OVA-induced asthma caused a significant increased expression of NF-κB in the lung tissues.

Tissue MDA level is a valuable indicator of lipid peroxidation (Mahfoudh-Boussaid et al., 2012).The present study indicated that lung MDA level was significantly higher in asthmatic animals. This observation is in good agreement with El-Kashef study that found a significant increase of MDA level in OVA-challenged mice (El-Kashef, 2018).

Excessive ROS production causes changes in the levels of both enzymatic (e.g. superoxide dismutase) and non-enzymatic (e.g. ascorbic acid) antioxidants resulting in oxidant-antioxidant airway imbalance (Bowler and Crapo, 2002). Therefore, in the current study, we evaluated PAB level that measures the total prooxidants and total antioxidants at the same time in samples. However, this study did not show any significant difference between the asthma and control groups. One possible explanation is that PAB may be an acute indicator of oxidative stress and not a long term index (Alamdari et al., 2008). Thus, since we used a chronic model for the induction of asthma in which the sampling was performed in the last day of the protocol, a significant difference was not detected.

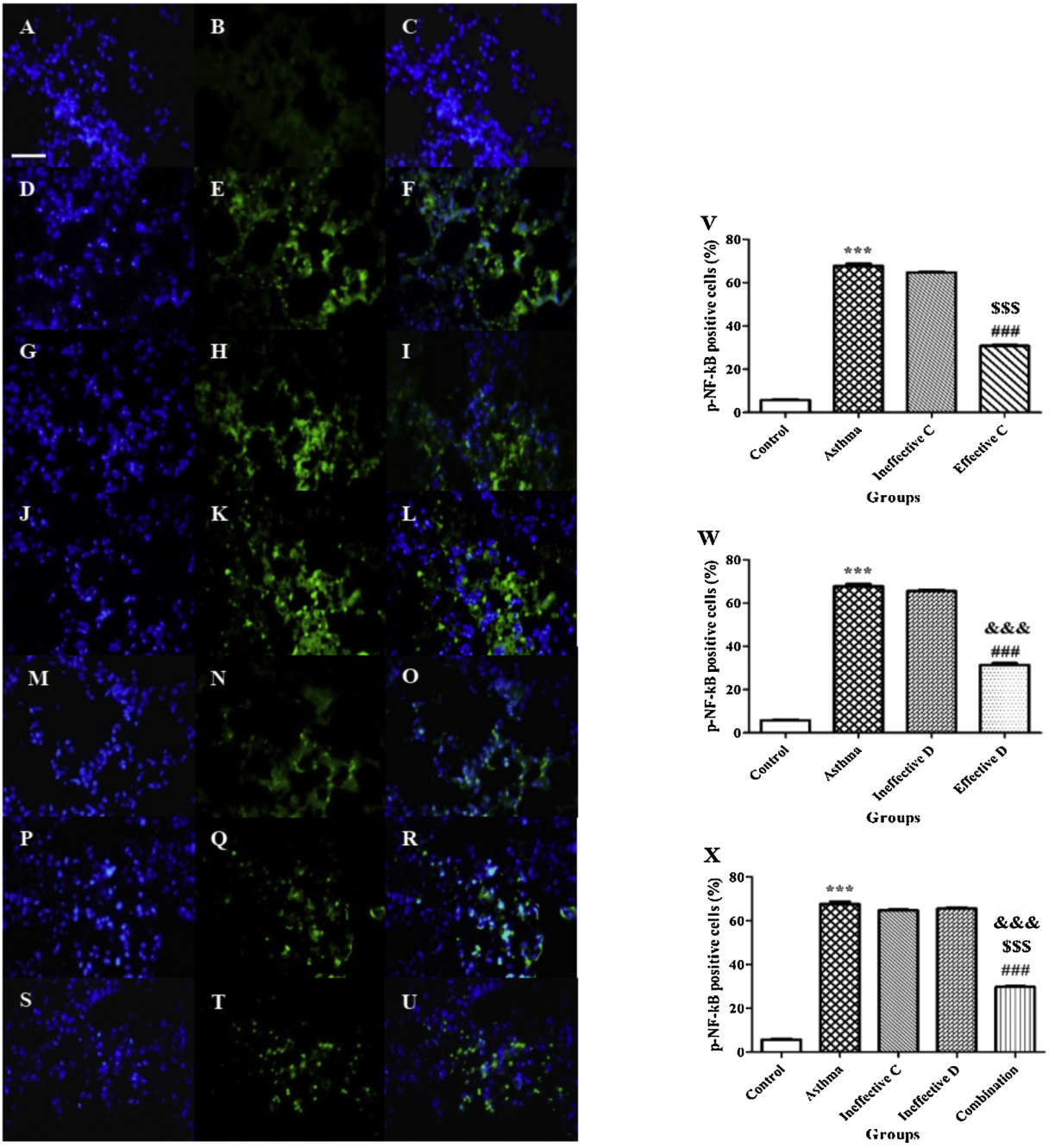

Fig. 4. Lung phosphorylated nuclear factor-kappa B (p-NF-κB) positive cells with ineffective and effective doses of ascorbic acid (V), with ineffective and effective doses of calcitriol (W) and with combination of ineffective doses of ascorbic acid and calcitriol (X). Fluorescence photographs in different groups were also provided. (A–C): Control, (D–F): Asthma, (G–I):Ineffective C, (J–L): Ineffective D, (M–O): Effective D, (P–R): Effective C, (S–U): Combination group. In the fluorescence photographs, the left column is lung tissues with 4′,6-diamidino-2- phenylindole (DAPI) staining of deoxyribonucleic acid (DNA) (blue), the middle column is lung tissues with antibodies against p-NF-κB (green) and the right column is merged lung tissue photographs of the left and middle columns. Bar: 20 μm. Data are expressed as mean ± SEM. *** $P < 0.001$ versus the control group. $^{###}$ $P < 0.001$ versus the asthma group. $^{\$\$\$}$ $P < 0.001$ versus the ineffective C group. $^{\&\&\&}$ $P < 0.001$ versus the ineffective D group. Ineffective C: ascorbic acid with ineffective dose, Ineffective D: calcitriol with ineffective dose, Effective C: ascorbic acid with effective dose, Effective D: calcitriol with effective dose.

Ascorbic acid is a hydrophilic vitamin that has different beneficial properties. This vitamin functions as an antioxidant agent that scavenges ROS leading to the prevention of oxidative damage to important biological macromolecules such as lipids, proteins and DNA (Frei et al., 1990). In this study, administration of effective dose of ascorbic acid significantly decreased plasma PAB level compared with the asthma group. This may be as a result of increases in anti-oxidant capacity. Our results also showed that lung tissue MDA level was significantly decreased in the effective C group. In addition to anti-oxidative effect, ascorbic acid has also anti-inflammatory activity. One study found that ascorbic acid reduces the number of inflammatory cells in BALF (Jeong et al., 2010). Similarly, the present study showed that there was fewer total WBC count in the effective C group. Moreover, some studies have indicated that this vitamin decreases the release of pro-inflammatory cytokines (Mikirova et al., 2012; Kong et al., 2015). It has been reported that ascorbic acid exerts this anti-inflammatory activity via intracellular suppression of NF-κB (Santanam et al., 2013). In this study, administration of effective dose of ascorbic acid was able to decrease lung p-NF-κB expression.

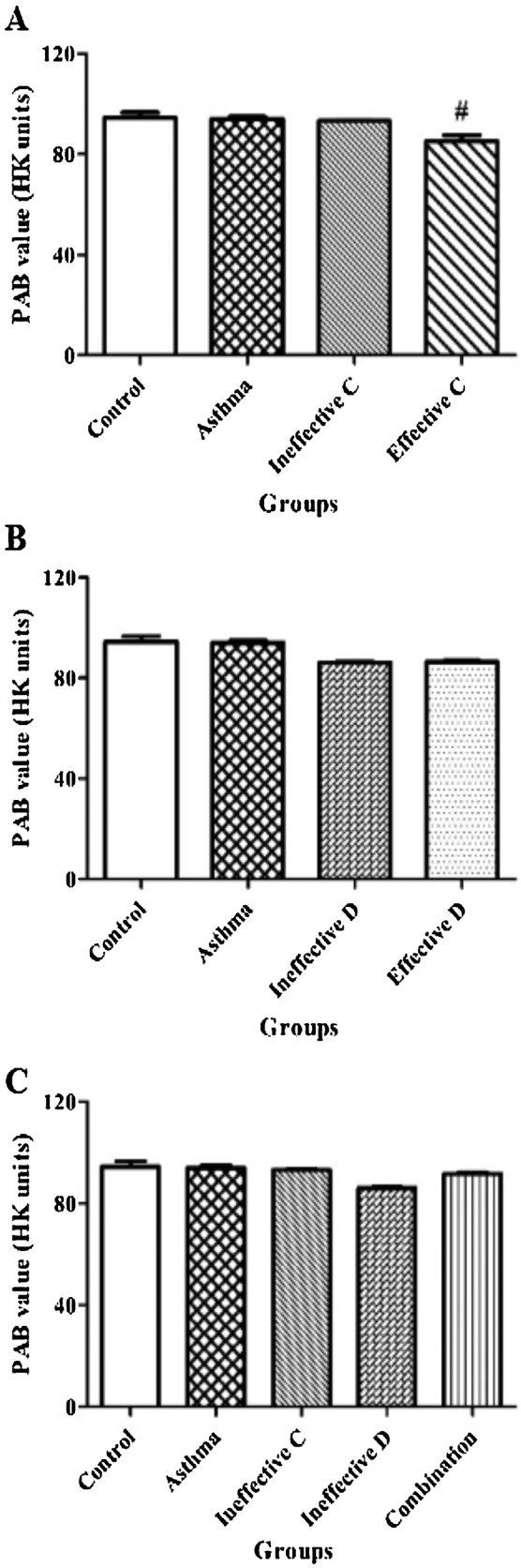

Fig. 5. Changes in plasma prooxidant-antioxidant balance (PAB) level with ineffective and effective doses of ascorbic acid (A), with ineffective and effective doses of calcitriol (B) and with combination of ineffective doses of ascorbic acid and calcitriol (C). Data are expressed as mean ± SEM. $^{\#}$ $P < 0.05$ versus the asthma group. Ineffective C: ascorbic acid with ineffective dose, Ineffective D: calcitriol with ineffective dose, Effective C: ascorbic acid with effective dose, Effective D: calcitriol with effective dose.

Calcitriol, a lipophilic vitamin, has been suggested to have antioxidant activities (Lin et al., 2005). Along with this, our results found that calcitriol administration in effective dose significantly reduced

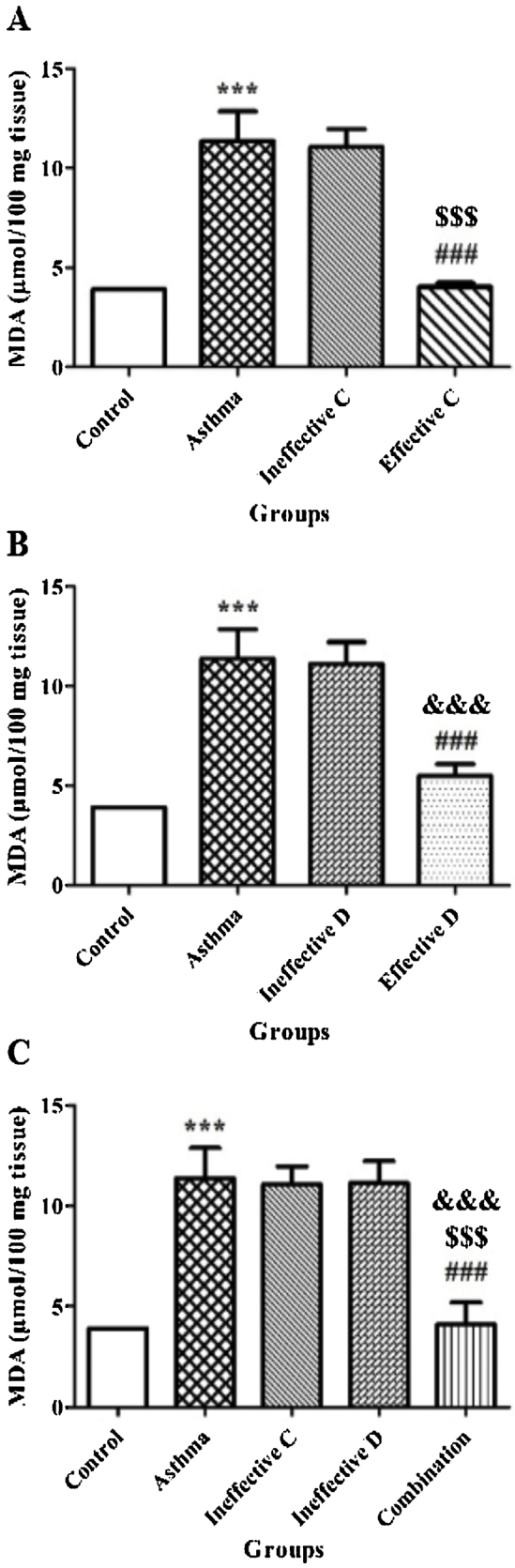

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Fig. 6. Changes in lung malondialdehyde (MDA) level with ineffective and effective doses of ascorbic acid (A), with ineffective and effective doses of calcitriol (B) and with combination of ineffective doses of ascorbic acid and calcitriol (C). Data are expressed as mean ± SEM. ***.
$P < 0.001$ versus the control group. ### $P < 0.001$ versus the asthma group. $$$ $P < 0.001$ versus the ineffective C group. &&& $P < 0.001$ versus the ineffective D group. Ineffective C: ascorbic acid with ineffective dose, Ineffective D: calcitriol with ineffective dose, Effective C: ascorbic acid with effective dose, Effective D: calcitriol with effective dose.

lung MDA level in comparison with the asthma group. This vitamin may also play an important role in pulmonary health through inhibition of inflammation (Zhou et al., 2015). Calcitriol has been shown to function as an anti-inflammatory agent through modulating both innate and adaptive immune systems and inhibiting the production of pro-inflammatory cytokines () and activation of NF-kB (Deluca and Cantorna, 2001; Zhang et al., 2012; Yu et al., 1995). In agreement with several studies (Mann et al., 2014; Wang et al., 2016; Zhang et al., 2018), our results showed that administration of effective dose of calcitriol significantly decreased inflammation as it was shown by the reduction of neutrophil and eosinophil infiltration, peribronchial inflammatory cell infiltration and lung p-NF-κB expression.

There are many studies suggesting that a combination of antioxidants may be more effective than the individuals, owing to additive or synergistic effects (Tripathi et al., 2010). In this regard, our results showed that combined administration of lower doses of ascorbic acid and calcitriol is able to reduce the infiltration of neutrophils and eosinophils, peribronchial inflammatory cell infiltration, lung MDA level and lung p-NF-κB expression.

5. Conclusion

The current study indicates that although ascorbic acid and calcitriol alone in ineffective doses do not have any protective effects, combined administration of both vitamins attenuates oxidative damages and inflammation and improves histopathological damages due to additive effects.

Declaration of Competing Interest

The authors declare no conflict of interest.

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