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Study Effect of Azithromycin and Doxycycline in Mucus Producing and Inflammatory Signaling Pathways of Allergic Asthma

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ABSTRACT

Asthma is a respiratory disease; involving millions of people worldwide. The main cause of asthma is allergy and immune response dysregulation. The effects of azithromycin and doxycycline as asthma-controlling drugs were evaluated in this study.

Mice asthma model was produced and asthmatic mice were treated with azithromycin (75 mg/kg, orally) and doxycycline (20 mg/kg, orally). Eosinophils and neutrophils count, interleukin (IL)-4, IL-5, IL-12, IL-13, and total immunoglobulin E (IgE) levels were measured. Histological study and evaluation of the gene's expression of *Muc5ac*, *Muc5b*, *IL-33*, *COX2*, *MYD88,* and *TRAF6* were performed.

Azithromycin and doxycycline did not affect eosinophil and neutrophil percentage, IL-4, IL-5, IL-12, total IgE levels, peribronchial and perivascular inflammation, goblet cell hyperplasia, and gene expression of *MYD88*, *TRAF6,* and *COX2*. Treatment with azithromycin significantly decreased IL-13 level, mucus secretion, and gene expression of *IL-33*, *Muc5ac,* and *Muc5b*; compared to the non-treated asthma group.

Azithromycin administration controls mucus secretion and inflammation. Azithromycin therapy and not doxycycline might be an effective adjuvant option in asthma by reducing mucus in the airway.

Keywords: Allergy and immunology; Antibiotic prophylaxis; Inflammation; Signal transduction

INTRODUCTION

One of the main respiratory diseases is asthma which involves millions of people worldwide. Inflammation and obstruction of the airway are beginning during an asthma attack and are recognized by cough, dyspnea, wheezing, inflammation, smooth muscle spasm, and mucus hyper-secretion that leads to bronchial obstruction.[1,2]

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The main cause of asthma is an allergic reaction and immune response dysregulation. The imbalance of Th1 and Th2 is a critical factor in asthma pathogenesis. Th2 cytokines initiate an allergic mechanism. Interleukin (IL)-4 forces B cells to produce immunoglobulin E (IgE) as the main allergic biofactor which can force mast cells and basophils to release allergic mediators that lead to airway smooth muscle spasms, hyper-responsiveness, and bronchial obstruction; handling allergy and asthma symptoms. IL-5 initiates the eosinophilic inflammation in bronchi and IL-13 stimulated mucus secretion in the airway; leading to airway obstruction.[3,4]

Azithromycin is a macrolide antibiotic that is used for the control of infection. Previous studies have shown that azithromycin has a beneficial effect on asthma and reduces clinical symptoms of asthma but its effect on asthma exacerbations has been inconclusive due to the lack of large long-term trials. However, azithromycin controls neutrophilic asthma and can reduce IL-13-induced mucus secretion. Doxycycline is another antibiotic that may have asthma-controlling effects.[5,6] The pathophysiology of allergic diseases is complicated and manipulation of signaling pathways would introduce a new controlling mechanism for asthma. In this study, the anti-asthmatic effects of azithromycin and doxycycline on mucus production and inflammatory signaling pathways were evaluated in asthmatic mice.

MATERIALS AND METHODS

Animal Model

Frothy female 8-week-old mice (BALB/c) were kept under Laboratory Animal Care ethical guidelines with free access to food and water. Mice sensitization and challenge procedures have been previously described and shown in Figure 1.[7] Briefly, mice were initially sensitized via the intraperitoneal (IP) injection of ovalbumin (OVA) with alum on day 1 and repeated on day 14. Then, these mice were challenged via inhalation administration (IT) of 1% OVA solution by a nebulizer, for 30 minutes on days 24, 26, 28, and 30. All mice were euthanized by CO2 on day 31 and blood, bronchoalveolar lavage fluid (BALf), and lung tissue samples were taken.

All study protocols have been approved by the ethical committee of Ani.X.Hos.Pro. (No: A.HO.MeP.2021.2128009).

Treatment Schedule

The mice were divided into 4 experimental groups (n=10 in each group): negative control (normal mice) that were treated with phosphate-buffered saline (PBS), positive control (asthmatic mice) with no treatment, and two asthmatic groups treated with azithromycin (75 mg/kg via oral gavage), and doxycycline (20 mg/kg via oral gavage) on days 24-29.[5,6]

BALf Cells

BALf samples were collected from the trachea of mice via intubation. BALf samples were centrifuged, and then the supernatant was stored to determine the cytokines levels. BALf samples slides were prepared via cytospin and stained with Giemsa to determine the percentages of eosinophils and neutrophils.

Cytokines

The IL-4, IL-5, and IL-13 levels as Th2 cytokines and IL-12 as Th1 cytokines were measured in BALf supernatant according to the manufacturer's instructions by specific enzyme-linked immunosorbent assay (ELISA) kits.

Serum IgE

After centrifuging blood samples, the total IgE level was measured in serum by the ELISA method.

Real-time PCR

In BALf cells, after RNA extraction; using the TRI reagent, cDNA was synthesized by a cDNA synthesis kit. The expression of target genes was measured; using SYBR Green Master Mix and specific primers (Table S1). *GAPDH* as an internal housekeeping gene was used in this study.

Histological Study

Lung tissues after fixation by formalin were used to produce slide sections and staining with Haemotoxylin and Eosin (H&E), Periodic Acid-Schiff PAS, and H&E-PAS. The pathology slides were evaluated with light microscopy for inflammation in the peribronchial and perivascular, hyperplasia of goblet cells, and mucus secretion.[7]

Statistical Analysis

For statistical analyses, version 20 of the SPSS was performed. ANOVA test and Pearson's method were used for data and correlation analysis and $p<0.05$ was considered significant. The data were presented as the mean±SD of at least three independent experiments, and the graphs were drowned by GraphPad prism.

RESULTS

BALf Cells

The eosinophil percentage in BALf of the non-treated asthmatic group had no significant differences compared with azithromycin- and doxycycline-treated groups ($p>0.05$). Also, the percentage of neutrophils in BALf of the non-treated asthmatic group had no

significant differences compared to azithromycin- and doxycycline-treated groups (p>0.05) (Figure 2).

Cytokines

The IL-4, IL-5, and IL-13 levels were significantly higher in the asthmatic group compared with healthy animals (p<0.05); while reverse results were obtained for IL-12 (Figure 3).

There was no significant difference in levels of IL-4, IL-5, and IL-12 (p>0.05) between the asthma group with azithromycin- and doxycycline-treated groups. The level of IL-13 had no significant difference between the asthma group and the doxycycline-treated group (p>0.05), but the level of IL-13 was significantly decreased (p<0.05) in the azithromycin-treated group compared with the non-treated asthma group (Figure 3).

IgE

The Asthma group had a significantly enhanced total IgE level (1925.14±46.77 ng/mL) compared to healthy group (193.3±44.98 ng/mL) (p<0.05). The two treated asthma groups (azithromycin and doxycycline) had no significant difference (1787.27±216.69 and 1897.76±106.98 ng/mL respectively) (p>0.05) compared with non-treated asthma group (Figure 4).

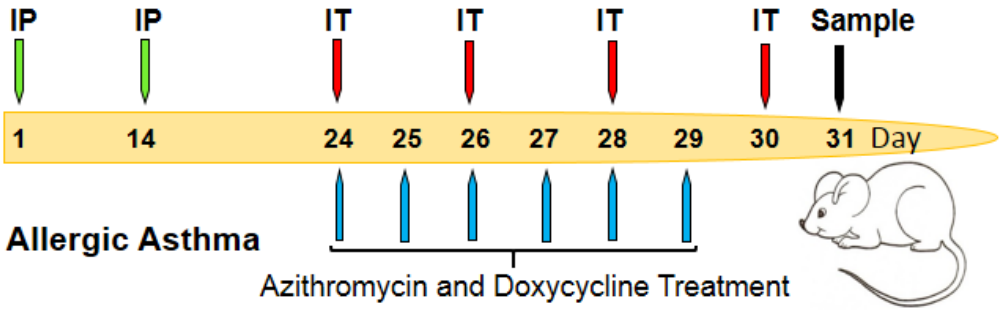

Figure 1. Mouse model of allergic asthma. The mice were sensitized and challenged by ovalbumin (OVA) to produce an asthma model. Then, treatment with azithromycin and doxycycline was done on days 24-29.

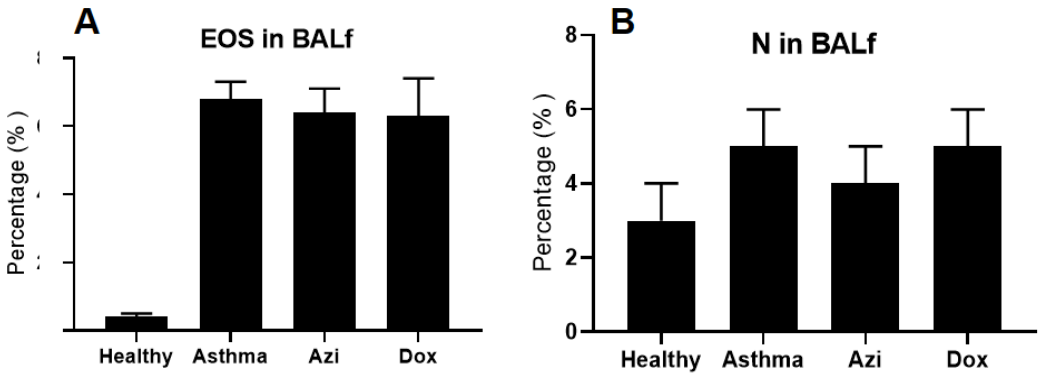

Figure 2. Bronchoalveolar lavage fluid (BALf) cells. The percentage of eosinophils and neutrophils was counted in BALf cells. There was no significant difference between the treated (Azi and Dox) and non-treated asthma groups (p>0.05). A: Percentage of the eosinophils in BALf, B: Percentage of the neutrophils in BALf.

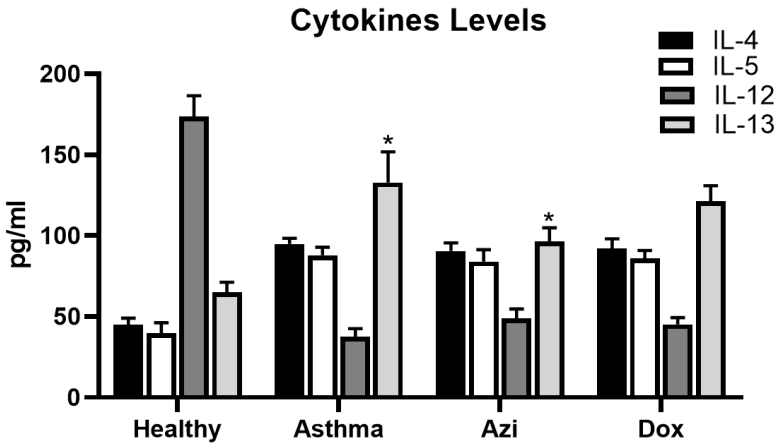

Figure 3. Cytokines levels. The interleukin (IL)-4, IL-5, IL-12, and IL-13 levels were measured in bronchoalveolar lavage fluid (BALf) by ELISA and presented in all groups. There were no significant differences in IL-4, 5, and 12 levels between treated (Azi and Dox) and non-treated asthma groups (p>0.05). Treatment with Azi could significantly (p<0.05) control IL-13 level compared with the non-treated asthma group that was shown with *, but Dox had no significant (p>0.05) effect.

Gene Expression

Treated asthma groups with azithromycin and doxycycline had no significant effect on gene expression of *MYD88*, *TRAF6*, and *COX2* (p>0.05) compared to the non-treated asthma group (Figure 5). The expression of *IL-33*, *Muc5ac*, and *Muc5b* was significantly decreased only in the azithromycin-treated asthma group (p<0.05) compared to the non-treated asthma group (Figure 5).

Lung Pathology

Peribronchial inflammation, perivascular inflammation, and hyperplasia of goblet cells and mucus over-producing were significantly increased in the bronchi of the asthmatic group (p<0.05) compared to the healthy group. Treatment with azithromycin and doxycycline had no significant effect on peribronchial inflammation, perivascular inflammation, and goblet cell hyperplasia (p>0.05). Mucus hypersecretion was significantly decreased in the azithromycin-treated group (p<0.05) compared to the non-treated asthma group but treatment with doxycycline had no significant effect (p>0.05) on mucus secretion (Figure 6).

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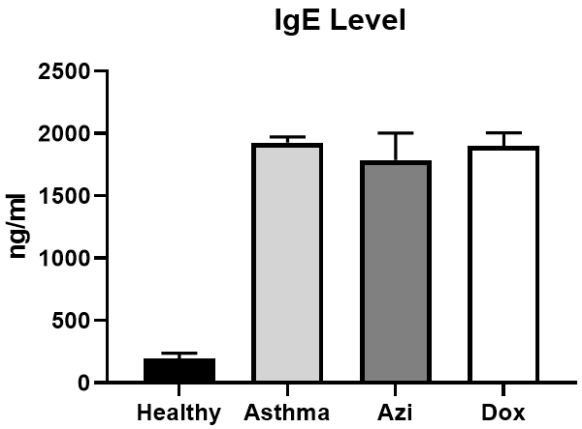


Figure 4. The level of total IgE was measured in the serum of all treated and non-treated mice. There was no significant difference between the treated (Azi and Dox) and non-treated asthma groups (p>0.05).

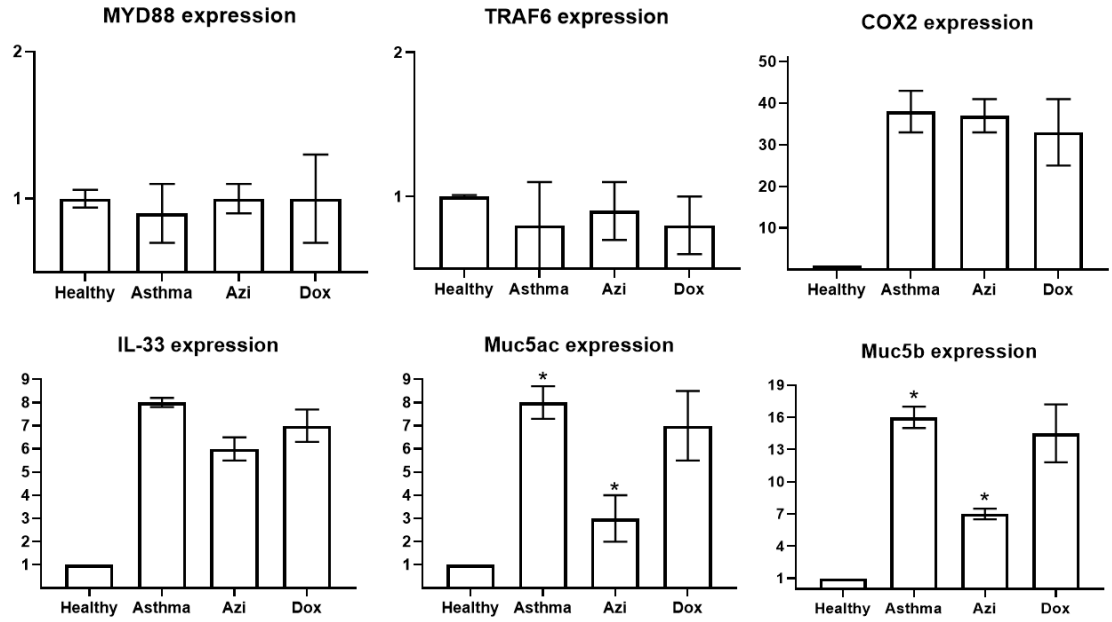
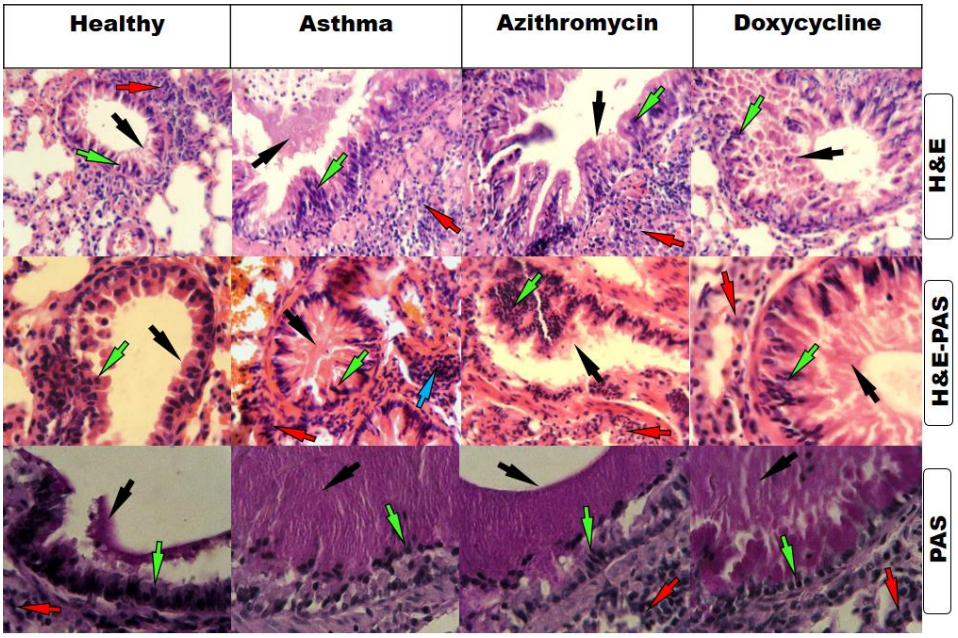


Figure 5. Real-time PCR. The genes expression of *MyD88*, *TRAF6*, *COX2*, *IL-33*, *Mus5ac*, and *Muc5b* were measured and shown in all groups. There was no significant difference between treated (Azi and Dox) and non-treated asthma groups ($p>0.05$) in the gene expression of MyD88, TRAF6, COX2, and IL-33. Treatment with Azi could significantly ($p<0.05$) decrease gene expression of Muc5ac and Muc5b compare with the non-treated asthma group that was shown with *, but Dox had no significant ($p>0.05$) effect.



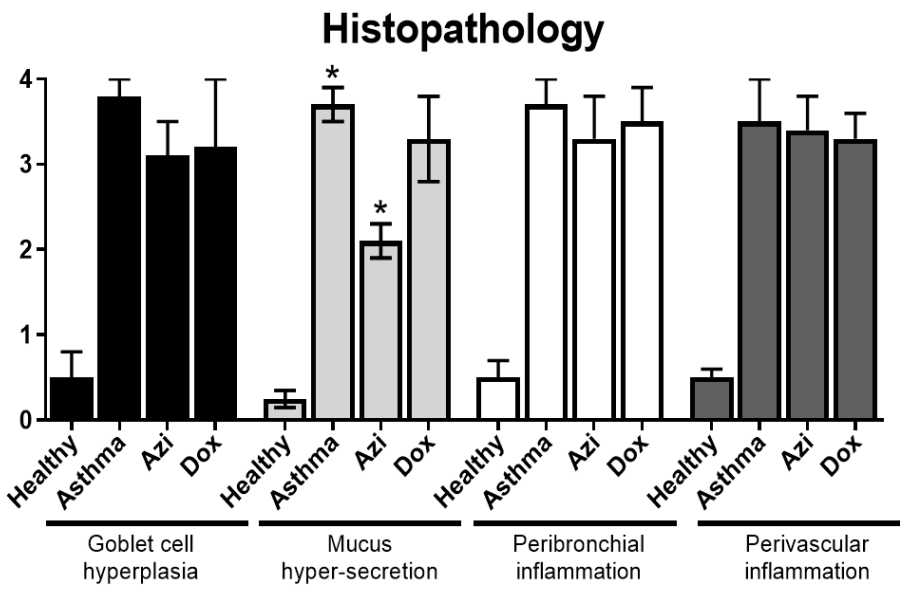


Figure 6. Histopathological study. The slides of the lung tissues were stained and then, the inflammation of lung tissue (in around vessels and bronchi), hyperplasia of goblet cells, and hypersecretion of mucus were evaluated. There was no significant difference between treated (Azi and Dox) and non-treated asthma groups ($p>0.05$) in the goblet cell hyperplasia, and perivascular and peribronchial inflammation. Treatment with Azi could significantly ($p<0.05$) decrease mucus hypersecretion compared with the non-treated asthma group that was shown with *, but Dox had no significant ($p>0.05$) effect on mucus hypersecretion.

DISCUSSION

Respiratory tract infections are important triggers for an asthma attack and treatment with macrolides such as azithromycin is beneficial to control airway inflammation.[8]

Azithromycin inhibits mTOR activity and it might prevent viral-induced episodes in asthma.[9] Azithromycin suppressed MDC (Th2 chemokine) and IP-10 (Th1 chemokine) expression in monocytes through the NFkBep65 and the MAPKeJNK/ERK pathways and modulates the MAPK pathway.[8] In our study, azithromycin and doxycycline treatment had no significant effect neither on eosinophilic percentage in BALf nor in controlling eosinophilic inflammation. Also, the number of neutrophils was less than 5% in all treated and non-treated groups. Because we kept the mice in the pathogen–allergen-free condition, neutrophils were not increased in response to the pathogen.

Studies showed that azithromycin reduces clinical symptoms of asthma.[10-12] In addition, ERK and NF-kB pathways are involved in MUC5AC production in airways.[11] Azithromycin dramatically decreases the phosphorylation of the MAPK pathway (JNK, ERK1, and p38).[11]

MUC5AC and MUC5B are two major secreted mucins that are closely related to the viscoelasticity of the sputum. Intra-tracheal administration of lipopolysaccharide (LPS) activates the signaling cascades such as MAPKs and PI3K-Akt pathways. Members of the MAPK are the critical signaling molecules that are involved in inflammation and regulation of MUC5AC synthesis; including p38 MAPKs, JNK, and ERK1/2. Besides, Akt-mTOR-STAT3 is downstream of PI3K and regulates inflammatory response and expression of the *MUC5AC*. Therefore, regulation of the MAPKs and PI3K-Akt signaling pathways may have the therapeutic potential for control of mucus hyper-secretion.[13] In this study, peribronchial and perivascular inflammation in doxycycline and the azithromycin-treated asthma groups had no significant changes compared with the non-treated asthma group. We observed that azithromycin and doxycycline treatment did not affect in control of inflammation eosinophilic. Goblet cell hyperplasia was decreased in the azithromycin-treated asthma group and mucus hypersecretion was decreased

significantly in the azithromycin-treated asthma group compared with the non-treated asthma group and also doxycycline-treated asthma group. Doxycycline treatment did not affect the reduction of mucus hypersecretion. Also, the gene expression of two main mucus-related genes including *Muc5ac* and *Muc5b* were harnessed by azithromycin and not by doxycycline.

Inhaled doxycycline can decrease eosinophilic inflammation in BALf and peribronchial areas, as well as in patients with airway hyperresponsiveness (AHR). In the lung, short-term inhaled doxycycline increased IL-10 levels, decreased levels of the IL-5, IL-13, and diminished matrix metalloproteinase (MMP)-related proteolysis and the activated MMP-9 proportion.[14,15] Leemans et al., 2012, demonstrated that doxycycline inhibits MMPs that are belonged to the collagenase (MMP-1, -8, and -13) and gelatinase (MMP-2 and -9) subfamilies.[16] The levels of IL-4, IL-5, and IL-12 had no significant difference between treated and non-treated asthma groups. The IL-13 level was insignificantly decreased in the doxycycline-treated asthma group. In the azithromycin-treated asthma group, the IL-13 level was significantly decreased compared with the non-treated asthma group. It may be explained that azithromycin could strongly reduce mucus hypersecretion in the histopathological lung section compared with doxycycline treatment. IL-13 is the main trigger of mucus hypersecretion in bronchi, especially in the asthmatic airway. Therefore, azithromycin decreased mucus secretion by controlling *IL-13* and suppressing *Muc5ac* and *Muc5b* gene expression which leads to reduced and controlled mucus secretion.

Antibiotics treatment of atypical bacteria, specifically *C. pneumoniae* has beneficial effects on asthmatic patients. Doxycycline suppresses *C. pneumoniae*-mediated IgE and IL-4 in patients with asthma and acts as an anti-inflammatory drug that can control asthma. Lung lymph nodes are local centers of immune responses and have an important role in allergic reactions. But some infections such as parasites may change the responses in lymph nodes.[17,18] In our study, we did not observe any change in the amount of total IgE in treated and non-treated groups. On the other hand, it was mentioned that azithromycin reduces Th2 cell proliferation and increases apoptosis in children with asthma, decreases IL-5 production, but does not affect IL-13.[19,20]

TLRs are downstream intracellular signaling pathways like NF-κB and MAPK pathways and are involved in the inflammatory response. The activation of NF-κB and MAPKs via TLR signal contributes highly to cytokine secretion and inflammation. Prolonged excessive inflammation results in diseases such as asthma.[21,22] Moreover, attenuating COX-2 as an inflammatory mediator via the MAPK and NF-κB signaling pathway can present anti-inflammatory potential. Also, the MAPK-dependent signaling pathway has a key role in IL-33 activation. MAPK suppression by activation of TRAF6, MyD88, and TNF regulates IL-33 secretion.[20] IL-33 also has an important role as the upper hand of Th2 cytokines and asthma pathophysiology. Therefore, the TLR–MyD88–TRAF6 axis of MAPK is a key target for the control of inflammation in asthma. In this study, azithromycin and doxycycline treatment had no significant effect on gene expression of the inflammation-related signaling molecules including *MyD88*, *TRAF6*, and *COX-2*. It may be stated that azithromycin and doxycycline treatment could not control eosinophilic inflammation around bronchi and lung vascular, because they did not affect modulatory signaling pathways of inflammation. While azithromycin treatment can control mucus secretion by regulating *IL-33* gene expression which is the upper hand of *IL-13*, doxycycline cannot have such effects.

This study provides some pieces of evidence that the administration of azithromycin in the murine model of asthma controls only mucus secretion and we did not observe any effect on airway inflammation, eosinophil infiltration, and the other related signaling pathways. This result suggests that azithromycin therapy might be an effective adjuvant option in asthma by reducing mucus in the airway. On the other hand, the gut microbiota is linked to airway disease through immune response modification (gut-lung axis). Moreover, altered gut microbiota augments allergic sensitization in allergic asthma and allergic inflammation in asthma may change gut microbiota, and azithromycin administration could alter the gut microbial composition. Therefore, it is suggested that the interaction between gut microbiota and airway inflammation in allergic asthma should be noted. Azithromycin is recommended as a valuable regimen for treating asthma but in the long-term therapy further evaluation of microbial resistance and diarrhea is required.

There were some limitations in this study. We used azithromycin at a concentration of 75 mg/kg and other increased concentrations were not studied. Further research on humans with asthma is helpful to verify the effect of azithromycin. We did not test the same dose in patients with asthma due to the development of side effects and also drug resistance. In addition, we could not study the AHR in this study. Also, the mechanism of the doxycycline effect on asthma was not evaluated and measurement of MMPs was not done.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ZW, LX, and WZ have participated in the design, evaluation, and drafting of the manuscript. ZW and WZ supervised the study.

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