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Effects of Quercetin Treatment on Epithelium-derived Cytokines and Epithelial Cell Apoptosis in Allergic Airway Inflammation Mice Model

Sule Caglayan Sozmen[1], Meral Karaman[2], Serap Cilaker Micili[3], Sakine Isik[1], Alper Bagriyanik[4], Zeynep Arikan Ayyildiz[1], Nevin Uzuner[1], Ozden Anal[1], and Ozkan Karaman[1]

[1] Department of Pediatrics, Division of Pediatric Allergy and Immunology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey
[2] Department of Medical Microbiology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey
[3] Department of Histology and Embryology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey
[4] Department of Histology and Embryology, Izmir International Biomedicine and Genome Institute (iBG-izmir), Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey

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ABSTRACT

Quercetin is a dietary flavonoid which has anti-inflammatory effects. This study aimed to evaluate the influence of quercetin on histopathological aspects and airway epithelium in allergic airway inflammation mice model.

Twenty-eight BALB/c mice were randomly divided into four groups: Group I (control), Group II (untreated mice with allergic airway inflammation), Group III (allergic airway inflammation quercetin-treated [16mg/kg/day]), Group IV (allergic airway inflammation dexamethasone-treated [1mg/kg/day]). Ovalbumin was administered intraperitoneally and via inhalation to achieve allergic airway inflammation mice model and treatments were also given intraperitoneally. Epithelium thickness, subepithelial smooth muscle thickness, number of mast and goblet cells, and basement membrane thickness were examined on samples isolated from lung. Immunohistochemical evaluationof lung tissues was performed using IL-25, IL-33, thymic stromal lymphopoietin (TSLP), terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling (TUNEL) and cysteine-dependent aspartate-specific proteases(caspase)-3 antibodies. IL-4, IL-25, IL-33, TSLP were quantified in bronchoalveolar lavage (BAL) and OVAspecific IgE levels was measured in serum by standard ELISA protocols. IL-25, IL-33, thymic stromal lymphopoietin (TSLP) and cysteine-dependent aspartate-specific proteases (caspase)-3.

Quercetin treatment led to lower epithelial thickness, subepithelial smooth muscle thickness, goblet and mast cell numbers compared to untreated mice with allergic airway inflammation ($p<0.05$). However, quercetin treatment was not effective on improving basal membane thickness. Immunohistochemical scores of IL-25, IL-33, TSLP, caspase-3 and

Corresponding Author: Sule Caglayan Sozmen, MD; Department of Pediatrics, Division of Pediatric Allergy and Immunology, Dokuz Eylul University Faculty of Medicine, 35340, Inciralti, Izmir, Turkey. Tel: (+90 232) 4126 061, Fax: (+90 232) 4123 649, E-mail: sulecaglayan07@yahoo.com

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TUNEL were lower in quercetin-treated mice t compared to untreated mice with allergic airway inflammation (p<0.05). IL-4, IL-25, IL-33, TSLP levels in BAL and OVA-specific IgE in serum were lower in quercetin treated mice compared to untreated mice (p<0.05).

These findings suggest that quercetin improves chronic histopathological changes except basal membrane thickness in lung tissue and its beneficial effects on inflammation might be related to modulating epithelium derived cytokines and epithelial apoptosis.

Keywords: Allergic airway inflammation; Antiinflammatory effects; Mouse model; Quercetin

INTRODUCTION

Asthma is a chronic inflammatory disease of the lung characterized by the recurrent airway obstruction and increased bronchial hyperresponsiveness.[1] This chronic inflammation is aggravated by acute asthma exacerbations which lead to unfavorable changes in the lung known as "airway remodeling". The components of the airway remodeling are subepithelial fibrosis, smooth muscle hypertrophy, goblet cell hyperplasia and basement membrane thickening.[2] Most of the researches in this field have aimed to prevent or treat these chronic changes in asthma. Another issue is to determine the immunopathological process which initiates these histological changes in asthma. Although, immunopathogenesis of asthma is still obscure, researchers suggest that, many different cell types accompany to chronic inflammation. T-helper type 2 (Th2) cells and secretion of some cytokines, such as IL-4, IL-5 and IL-13 play a major role in the pathogenesis of asthma. Other sources of these cytokines are basophils, eosinophils, mast cells and epithelial cells.[3] In recent years, there has been an increasing evidence for the molecular relationship between epithelial cells and immune cells, suggesting that bronchial epithelial cells act as a target for the immunologic injury associated with asthma.[4] Various environmental factors can activate epithelial cells to release cytokines like IL-33, IL-25, thymic stromal lymphopoietin (TSLP). These epithelium-derived cytokines can initiate and enhance the asthmatic airway inflammation.[5]

Apoptosis of damaged epithelial cells in the normal respiratory tract has beneficial effects.[6] However, excessive and inappropriate apoptosis contributes to the shedding of airway epithelium. Therefore, the exposure of basement membrane to toxic mediators may aggravate inflammation in the airways of patients with asthma.[7,8] Apoptotic process includes DNA fragmentation that can be detected by terminal deoxynucleotidyl transferase-mediated dnTP nick end-labeling (TUNEL) staining and cleavage of a number of proteins, which can be shown by cysteine-dependent aspartate-specific proteases (caspase)-3 staining.[9]

Corticosteroids are usually administered to control all these pathological process in asthma. However, long-term treatment with steroids might cause side effects such as hypertension, cataract, and osteoporosis and growth failure in childhood.[10] Thus, various attempts have been made in order to find safe and alternative therapies. Flavonoids are a large group of polyphenolic compounds which are widely-distributed secondary metabolites in plants. They are primarily known as pigments responsible for the colors of leaves. Quercetin is a flavonoid that is found in many fruits and vegetables including apple, broccoli, onion, black tea, red wine and berries as well as many seeds, flowers and leaves.[11-13] Several reports showed beneficial effects of quercetin on allergic airway inflammation, hyper-responsiveness and bronchial hyperactivity in experimental asthma models.[14-16] In this study, we aimed to investigate the effects of quarcetin on histopathological changes, airway remodeling, epithelium derived cytokines and epithelial apoptosis in a murine model of allergic airway inflammation in comparison with the conventional dexamethasone treatment.

MATERIALS AND METHODS

Experimental Animals

A total of twenty-eight, conventionally raised, 6- to 8-week-old male BALB/c mice (weighing 18-20 g) were included in the study. Animals were kept in hygienic macrolane cages in air-conditioned rooms at a

constant temperature of 22˚C on a 12 hour light/dark cycle. Mice were fed with commercial diet ad libitum for the experiment. All experimental procedures complied with the requirements of the Dokuz Eylul University Animal Care and Ethics Committee.

Study Design

Mice were divided into four groups: (I) Control (n=7), (II) Allergic airway inflammation (untreated) (n=7), (III) Allergic airway inflammation (quercetin-treated) (n=7) and (IV) Allergic airway inflammation (dexamethasone-treated) (n=6). At the beginning of the experiment each group consisted of 7 mice. However, one of the mice in dexamethasone group died in the 5th week of experiment. Control group was exposed to saline only. An allergic airway inflammation model was used in the groups II, III, and IV through the administration of intraperitoneal (IP) and inhaled ovalbumin (OVA) exposure. During the last five days of OVA inhalation, groups II, III, and IV received following injections respectively : IP saline injection; IP injection of quercetin (≥95% of purity, Sigma Aldrich, St. Louis, MO, USA) at 16 mg/kg once a day; IP injection of dexamethasone (Dekort; Deva Holding AS, Istanbul, Turkey) at 1 mg/kg once a day.[3,4] Although we used a chronic asthma model from the literature, we did not perform a spirometric lung volume evaluation to prove that our model was established asthma. Therefore the model in this study is defined as "allergic airway inflammation mice model".

Sensitization and Inhalation Procedure

We used chronic murine model of asthma described by Temolovski, et al.[5] Groups II, III, and IV were sensitized via IP OVA (grade V,≥98% pure; Sigma-Aldrich, St. Louis, MO, USA) (10 μg/0.1 mL) and they were exposed with alum adjuvants on the day 0 and day 14 of the experiment. On the 21st day of experiment, these groups were also exposed with 2.5% aerosolized OVA (dissolved in sterile saline) for 30 minutes per day for 3 days of every week up to 8 weeks (Figure 1). Exposures were carried out in a whole body inhalation exposure system in a plexiglass chamber with 40×60×120 cm volume. Aerosolized OVA was delivered via side stream jet nebulizer system (MedicAid, UK) achieving a diameter of 4 μm in 80% f the particles. The particle concentration was maintained 10-20 mg/m^3. Thus, it was predicted 90% of the particles would remain in the mouse airways, which would be enough for the development allergic airway inflammation mice model.[5] The mice in the control group (group I) received normal saline solution IP on day 0 and 14 of the experiment. Besides, aerosolized saline was given for 30 minutes per day for 3 days of the week up to 8 weeks since the 21st day of the experiment. The same system was used to deliver the saline via inhalation in the control group. An IP injection of ketamine hydrochloride (200 mg/kg) was administered on the first day following the application of the final medication. Then mice were sacrificed via exsanguination cardiac arrest.

Histomorphological Analysis

All histomorphological analyses described below were performed blindly by two investigators. Photomicrographs were taken by JVC TK-890-E camera (Japan), which was adapted on Olympus BH-2 RFCA model microscope (Olympus Optical, Tokyo, Japan). The histological analyses were carried out using UTHSCSA Image Tool software for Windows (Version 3.00, The University of Texas, San Antonio, TX, USA).

Samples were obtained from the midzone of the left lung and were placed in buffered formalin for light microscopic evaluation. Another sample from an adjacent region was placed in 2.5% glutaraldehyde for the electron microscopic evaluation. Upon fixation, samples were embedded in paraffin for light microscopic evaluation and 5 μm serial sections were obtained with rotary microtome (Leica RM2125, Germany). Thirty consecutive sections from each mouse were randomly selected and stained via 1 of 3 different staining processes. The first 10 samples were stained with hematoxylin and eosin (H&E). In these samples, general tissue features were examined and thicknesses of epithelium and subepithelial smooth muscle layers of the medium and small airways were measured. In order to evaluate the thicknesses of epithelium and subepithelial smooth muscle layers, the measurements were performed from 4 points of each airway. Considering that each section contained approximately 2 to 3 airways, nearly 20 airways were evaluated for each mouse.

The consecutive sections were stained with toluidine blue (10 sections) or periodic acid-Schiff (PAS; 10 sections). Photomicrographs of samples which were stained with toluidine blue were taken randomly from 5 fields of each section. Mast cells were

counted in 8 fields (20,000/μm^2 each) for each mouse. Goblet cells were stained with PAS and were counted in 10 sections of each mouse. In each section, 3 to 5 randomly selected airways were photographed. Circumferences of all airways were measured and goblet cell numbers in these areas were recorded. For standardization, goblet cell numbers in 100 μm^2 were analyzed by dividing the total goblet cell number to the total length of airway circumferences and multiplying the result by one hundred.

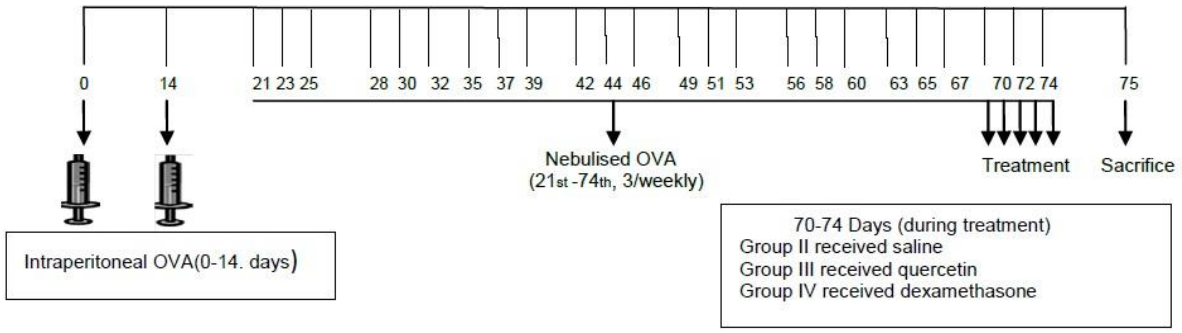

Figure 1. Schematic presentation of sensitization, inhalation exposure, and the drug administration times in the murine model ofallergic airway inflammation
OVA:Ovalbumin

Ultrastructural Analyses

For ultrastructural investigation, lung sections were fixed in 2.5% glutaraldehyde for 24 hours. The lung tissues were fixed with osmium tetroxide, dehydrated in a graded series of alcohol and then embedded in Araldite CY212. For each mouse, 5 to 7 ultra thin (60-90 nm) sections were obtained with the help of the ultramicrotome (Leica, Germany). Samples were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Carl Zeiss Libra 120, Germany). After that, 8 to 10 areas were photographed with a Trondle (2048 pixels, Germany) digital camera attached to the electron microscope. Thicknesses of the basement membrane of the respiratory epithelium were measured from 20 points of preparations at equal distances to each other and the data were recorded in sequence.

Measurement of Cytokines in Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) fluids were immediately collected from euthanized mice by instillation and recovery of sterile phosphate buffered saline through the tracheal cannula. BAL fluids were centrifuged at 3000×g for 10 min and supernatants were removed and stored at −80◦C. Levels of IL-4, IL-25, IL-33, and TSLP in BAL samples were measured using ELISA commercial kit (Bender MedSystems, MedSystems Diagnostics GmbH, Vienna, Austria).

Detection levels were 2.0 pg/mL for IL-4, 11.2 pg/ml for IL-33, 16 pg/ml for TSLP, 16 pg/ml for IL-25 (IL-17E). Serum levels of OVA specific IgE were measured using ELISA commercial kit (Sun Red Biological Technology, Shanghia, China).

Immunohistochemical Detections

After deparaffinization and rehydration, sections of right lung tissue were treated with 10 mM citrate buffer (Cat No.AP-9003-125 Labvision, UK) for 5 minutes. Sections were incubated in a solution of 3% H_2O_2 for 5 minutes in order to inhibit the endogenous peroxidase activity. Then, samples were incubated with normal serum blocking solution. Sections were again incubated in a humid chamber for 18 hours at +4˚C with anti-IL-33 antibody 1:100 (anti-IL-33 mouse monoclonal antibody,NBP1-75516,Novus Biologicals, USA), anti-IL-25 antibody 1:100 (anti-IL-25 mouse monoclonal antibody,NBP1-72027, Novus Biologicals, USA), anti-TSLP antibody 1:100 (anti-TSLP mouse monoclonal antibody,NBP1-76754, Novus Biologicals, USA), and anti-caspase-3 antibody 1:100 (AB3623, polyclonal antibody, Millipore, Temecula, CA, USA) . Upon

primary antibody incubation, samples were kept with biotinylated IgG and subsequently with streptavidin conjugated with horseradish peroxidase for 30 min according to kit instructions (Invitrogen-Plus Broad Spectrum 85-9043, Carlsbad, CA, USA). Sections were finally stained with 3, 3'-diaminobenzidine (DAB) substrate (1718096, Roche, Mannheim, Germany) and counter-stained with Mayer hematoxylin. Samples were analyzed with a light microscope.[6]

In order to detect DNA fragmentation in cell nuclei, TUNEL reaction was applied to paraffin sections. DeadEnd Colorimetric TUNEL system kit (Roche, Germany) was used for apoptotic cell detection. Serial five µm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and pretreated in proteinase K for 15 min. After washing in phosphate-buffered saline (PBS), specimens were incubated with fluorescein-labeled deoxy-uridine triphosphate (UTP) and TdT at 37 ˚C for 60 min. Then, converter conjugated with peroxidase (POD) solution was applied to slides. Sections were stained with DAB and counterstained with Mayer hematoxylin and analyzed with a light microscope.[7]

Semi-quantification of immunostaining data

Semi-quantitative scores were defined as follows: 0= no immunoreactivity; 1= very little positive staining and the staining was mild; 2= positive staining was moderate (between grade 1 and grade 3); and 3= strong positive staining was evenly distributed across the whole images. Each of the sections were graded blind by two histologists in order to maintain the consistency of the scoring system. The mean values were calculated for immunoscorings.[8]

Statistical Analysis

Statistical analyses were performed using SPSS statistical software, version 15.0 (SPSS Inc, Chicago, Illinois, USA). The Kruskal-Wallis test was used in order to compare the results of all four groups. Mann Whithey test was used for dual comparisons. '*p*' values lower than 0.05 were regarded as statistically significant ($p<0.05$).

RESULTS

Each group was composed of seven mice in the beginning of the experiment, and only one mouse expired in the dexamethasone-treated group during the study.

Comparison of Histomorphological Findings

The light microscopic findings were completely normal in control group (Table 1) (Figures 1-A1, B1, C1, D1). The numbers of mast cells and goblet cells as well as thicknesses of epithelium and smooth muscle layers were significantly higher in untreated mice with allergic airway inflammation compared to control group (Table 1) (Figures 2- A2, B2, C2,D2). Subepithelial smooth muscle and epithelial thicknesses were significantly lower in quercetin-treated group compared to untreated mice with allergic airway inflammation (Table 1) (Figures 2-B2, B3).

Mean of mast cell and goblet cell numbers were significantly lower in mice treated with quercetin compared to untreated mice with allergic airway inflammation. (Table 1) (Figures 2-C2, C3, D2, D3). All histological parameters were significantly lower in dexamethasone-treated group compared to untreated mice with allergic airway inflammation. (Table 1) (Figures 2- A4, B4, C4, D4). There was no difference between quercetin-treated and dexamethasone-treated groups in terms of epithelial thickness, subepithelial smooth muscle, mast and goblet cell numbers (Table 1).

Comparison of Electron Microscopic Findings

Electron microscopic findings demonstrated regular basement membrane in control group (Table 1) (Figure 2-E1). Basement membrane thickness was significantly lower in dexamethasone-treated group compared to untreated mice with allergic airway inflammation (Table 1) (Figure 2-E4). However, no statistical difference was observed between quercetin-treated mice and both untreated mice with allergic airway inflammation and mice treated with dexamethasone-regarding basement membrane thicknesses (Table 1) (Figures 2-E2, E3).

Comparison of Immunohistochemical Staining

Immunoscoring of IL-25, IL-33, TSLP, TUNEL and caspase-3 were found to be lower in control group compared to the values of untreated mice with allergic airway inflammation group (Table 2 and Figure 3). Quercetin- and dexamethasone- treated groups showed lower immunoscoring of IL-25, IL-33, TSLP, TUNEL, and caspase-3 than untreated mice with allergic airway inflammation group (Table 2) (Figure 3). We also observed lower immunoscorings of IL-33 in quercetin-treated group compared to the dexamethasone-treated group.

Comparison of Cytokines in BAL and OVA-Specific IgE in Serum

IL-4, IL-25, IL-33, TSLP levels in BAL liquids and OVA-specific IgE levels in serum were significantly lower in control group than untreated mice with allergic airway inflammation group. Quercetin and dexamethasone treatments led to significantly lower levels of IL-4, IL-25, IL-33, TSLP in BAL and OVA specific IgE in serum when compared to the levels of untreated mice with allergic airway inflammationgroup (Table 3).

Table 1. Comparisons of the histological characteristics in the murine model of allergic airway inflammation between groups

	Group I Mean±SD (Control)	Group II Mean±SD (Allergic airway inflammation)	Group III Mean±SD (Quercetin)	Group IV Mean±SD (Dexamethasone)	p^a
Epithelial thickness(μm)	15.5±4.58	26.3±8.65[b,c,d]	21.5±11.3[b]	17.3±3.21	<0.05
Subepithelial smooth muscle thickness(μm)	1.27±0.61	2.78± 0.60[b,c,d]	1.70 ±0.73 [b]	1.54 ±0.54	<0.05
Number of goblet cells/100 (μm)	0.32± 0.61	2.35± 2.00 [b,c,d]	1.21±0.95[b]	1.07±0.9	<0.05
Number of mast cells/20000 (μm^2)	0.60± 0.68	2.21±1.37 [b,c,d]	1.39±1.37 [b]	1.39±1.16	<0.05
Basement membrane thickness (nm)	87.49± 14.41	660.82±164.68[d]	638.3±32.67[b]	459.82±161.27 [c]	<0.05

[a]Kruskal-Wallis statistical analysis [b]Significantly higher than Group I
[c]Significantly higher than Group III [d]Significantly higher than Group IV

Table 2. Comparisons of immunoscoring of lung tissues between study groups in the murine model of allergic airway inflammation

	Group 1 Mean±SD (Control)	Group 2 Mean±SD (Allergic Airway Inflammation	Group 3 Mean±SD (Quercetin)	Group 4 Mean±SD (Dexamethasone)	p^a
IL-25	0.71±0.53	2.17±0.61[bc,d]	1.17±0.61	1.39±0.58	0.001
IL-33	0.78±0.68	2.21±0.56[b,c,d]	1.10±0.49	1.52±0.51[c]	0.001
TSLP	0.57±0.50	1.92±0.66[b,c,d]	1.21±0.56	1.43±0.50	0.001
Capase-3	0.28±0.46	2.25±0.58[b,c,d]	1.14±0.59	1.34±0.48	0.001
TUNEL	0.67±0.54	2.46±0.69[b,c,d]	1.32±0.81	1.47±0.51	0.001

IL: interleukin; TSLP: thymic stromal lymphopoietin; Caspase: cysteine dependent aspartate specific proteases; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling

aKruskal-Wallis statistical analysis [b]Significantly higher than Group I
[c]Significantly higher than Group III [d]Significantly higher than Group IV

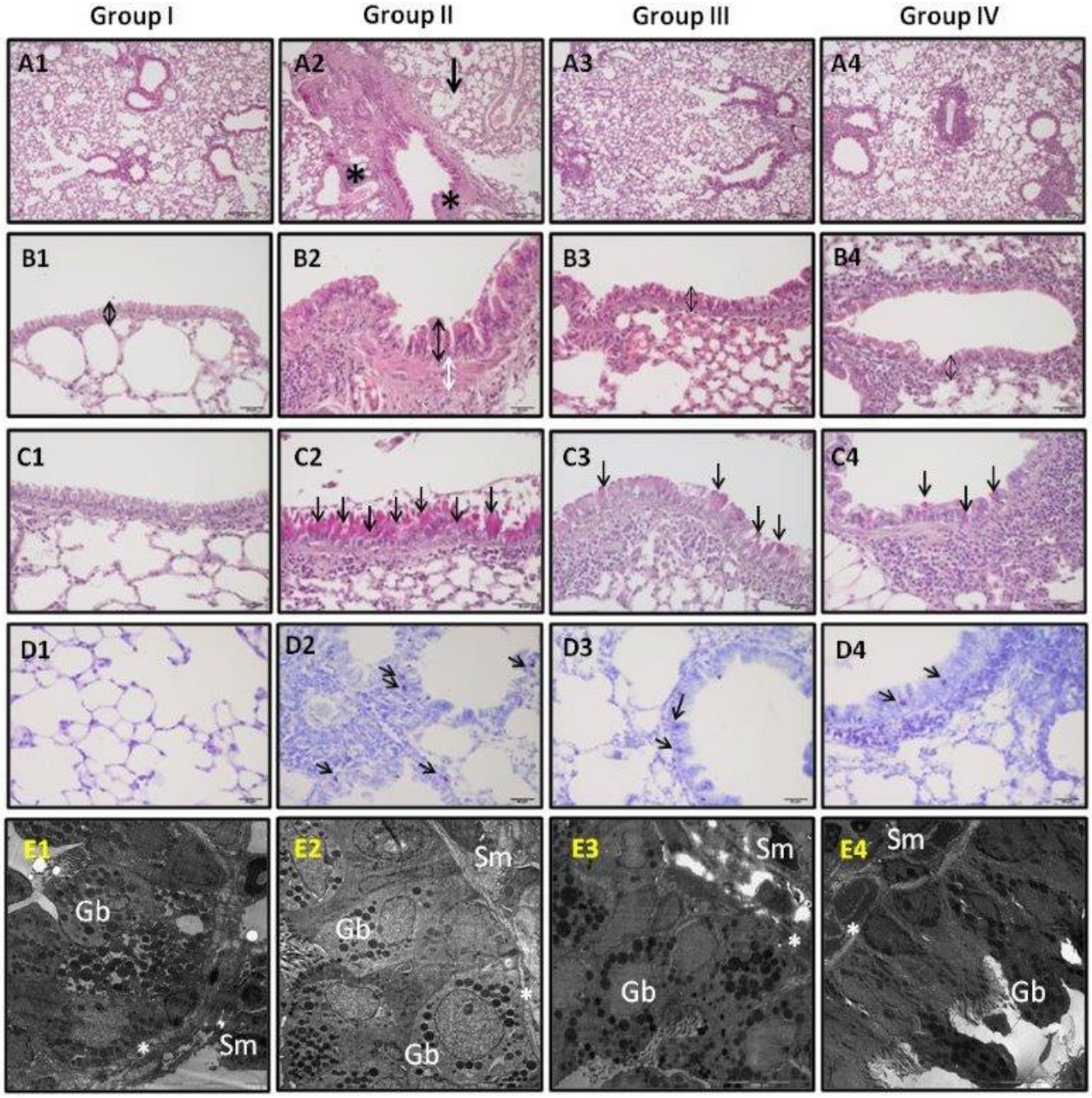

Figure 2. Light and electron microscopic findings of lung tissues in the murine model of allergic airway inflammation. I; control (n= 7), II; allergic airway inflammation (n= 7), III; quercetin (n= 7), IV; dexamethasone (n= 6) A,B; H&E, C1; Periodic acid –Schiff stain D1; toluidine staining. (A) (*) peribronchial mononuclear cell infiltration (↓) irregular pattern of alveoli. (B) Arrow with two heads; epithelial thickness (black arrow), subepithelial smooth muscle thickening (white arrow), (C) arrows; goblet cells, (D) arrows; mast cells. (E) electron microscopic views. (Gb); goblet cells, white star; basement meembrane, (Sm); smooth muscle cell.

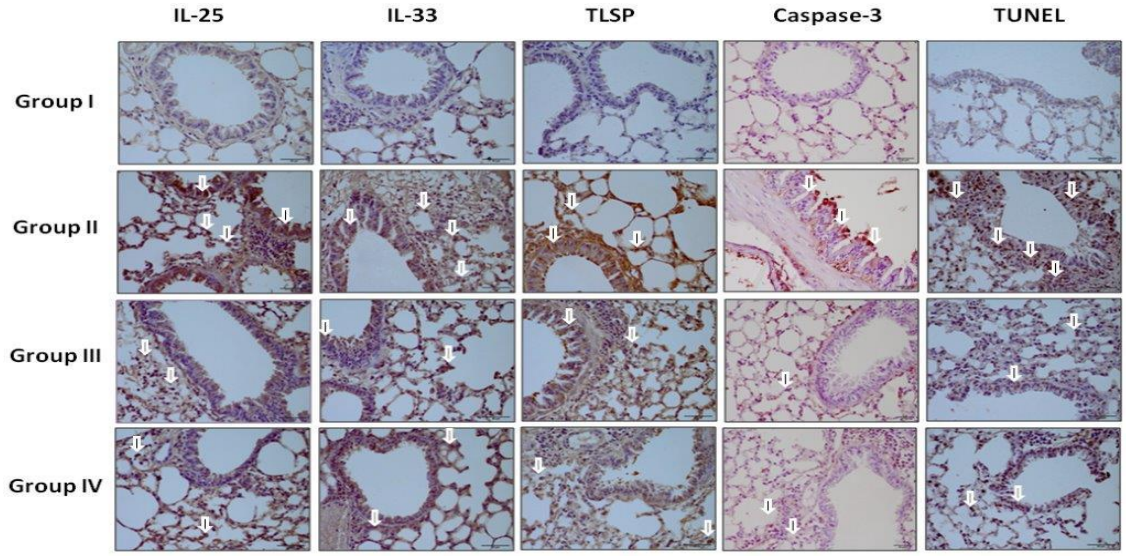

Figure 3.Presentation of immunohistochemical findings of lung tissue in murine model of allergic airway inflammation. I; control (n=7), II; Allergic airway inflammation (n=7), III; quercetin (n=7), 2. IV; dexamethasone (n=6). White arrows show positive staining with IL25, IL-33, TSLP and caspase.

IL: interleukin; TSLP: thymic stromal lymphopoietin; Caspase: cysteine dependent aspartate specific proteases; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling

Table 3. Comparison of the serum cytokine levels in the murine model of allergic airway inflammation between groups

	Group 1 Mean±SD Median(IQR) Control	**Group 2 Mean±SD Median(IQR) Allergic airway inflammation**	**Group 3 Mean±SD Median(IQR) Quercetin**	**Group 4 Mean±SD Median(IQR) Dexamethasone**	***p*[a] value**
IL-4(pg/ml)	5.56±3.95	48.83±2.78[b,c,d]	5.05±0.90	5.86±3.40	0.001
(BALF)	3.62	48.1	4.76	4.3	
	(3.34-7.24)	(46.42-51.39)	(4.4-5.68)	(3.89-7.84)	
IL-33(pg/ml)	140.41±153.08	1554.62±178.22[b,c,d]	130.75±97.07	180.10±162.76	0.001
(BALF)	106.3	1547.95	120.064	139.575	
	(46.23-135.69)	(1416.39-1645.08)	(48.24-251.38)	(34.42-365.19)	
IL-25(pg/ml)	8.14±5.03	159.73±20.58[b,c,d]	15.12±14.51	23.11±28.79	0.001
(BALF)	6.63	149.62	7.06	14.855	
	(4.03-14.98)	(144.06-180.52)	(2.9-32.28)	(2.77-38.95)	
TSLP(pg/ml)	4.95±0.398	151.90±14.52[b,c,d]	5.56±1.09	5.66±0.57	0.001
(BALF)	4.76(4.76-5.41)	140.65	5.2(4.81-5.93)	5.675	
		(140.27-165.26)		(5.14-6.15)	
OVA specific IGE(ng/ml)	268.45±140.21	1281.14±163.60[b,c,d]	305.54±192.85	318.47±84.33	0.001
(serum)	266.28	1239.16(1165-1355)	268.33	331.25	
	(124.16-456.66)		(166.66-406.66)	(246.87-393.54)	

BALF: bronchoalveolar lavage fluid; OVA: ovalbumin; IL: interleukin; TSLP: thymic stromal lymphopoietin

[a]Kruskal-Wallis statistical analysis [b]Significantly higher than Group I

[c]Significantly higher than Group III [d]Significantly higher than Group IV

DISCUSSION

Quercetin has been investigated as a potential therapeutic agent in experimental models of asthma. However, to the best of our knowledge, quercetin effects on airway epithelium-derived cytokines and epithelial apoptosis have been assessed for the first time in our study. In this study, we demonstrated that, quercetin was effective in ameliorating airway remodeling including epithelial thickness, subepithelial smooth muscle thickness and goblet cell hyperplasia except basement membrane thickness. Quercetin regulates epithelial derived cytokines, leads to a prominent anti-inflammatory response and it represents an anti-apoptotic effect in airway epithelial cells. These effects of quercetin were successfully shown using allergic airway inflammation model in the study.

Experimental models of allergic airway inflammation which resemble remodeling processes in humans, are well established and the procedure implemented in our study is a widely used model.[5] Main features of remodeling including epithelial thickness, subepithelial smooth muscle thickness, the number of goblet and mast cells were found significantly higher in untreated mice with allergic airway inflammation group upon OVA exposure compared to control group. These findings indicate that chronic changes associated with the features of remodeling have been successfully developed. Despite conflicting results in the literature, treatments with inhaled and systemic steroids are generally accepted to decrease both basement membrane thickness and smooth muscle mass.[10,13-15] In concordance with these results, our study demonstrated beneficial effects of dexamethasone in all aspects of remodeling. Systemic quercetin treatment was shown to improve indicators of remodeling except basement membrane thickness. Relatively short administration of treatment might have negative impact on this result in our study. Moreover, quercetin and dexamethasone treatments resulted in lower number of mast cells which promotes inflammation by releasing potent inflammatory mediators such as histamine, cytokines and proteases. Although we showed the beneficial effects of quercetin on remodeling, we did not find an advantage of quercetin treatment over steroid treatment.

Previous studies yielded beneficial effects of quercetin on chronic inflammation in experimental models of asthma. Oliveira et al. demonstrated that per oral (po) quercetin treatment decreased the level of inflammatory cytokines (IL-4,IL-5,IL-13) in BAL fluid, eosinophil peroxidase in lung tissue and Blomia tropicalis (Bt)-specific IgE in a murine model of Bt-induced asthma.[16] Park et al. studied the effect of quercetin on Th1/Th2 balance in a mouse model of OVA-induced asthma. They observed that IP quercetin treatment increased the expression of T-box expressed in T cells (T-bet) (Th1 transcription factor) whereas it decreased the expression of GATA which regulates Th2 differentiation..[3] In another study, Rogeria et al. found that the treatment with quercetin-loaded microemulsion (QU-ME) was more effective than the treatment performed with quercetin suspension. They found that QU-ME treatment decreased the number of eosinophils, the expression of IL-4, IL-5 in BAL fluid and inhibited p-selectin expression by blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) signaling in lung tissue.[17] Various researchers demonstrated that airway epithelium acts as innate immune cells in addition to its barrier function. Epithelium-derived cytokines (IL-25, IL-33 and TSLP) promote both innate and adaptive immune response and this feature has attracted considerable interest as a possible therapeutic target.[18] Therefore, further research delineating the treatment modalities and targeting the initiation of inflammation may provide new insights into the development of more effective therapies. Our results showed that the expression of IL-25, IL-33 and TSLP was significantly lower in quercetin-treated group compared to untreated mice with allergic airway inflammation group both in lung tissue and BAL. The expression of these epithelium-derived cytokines was not significantly different between quercetin and dexamethasone groups except for IL-33. Quercetin treatment led to lower expression of this cytokine than dexamethasone treatment in lung tissue. In asthmatic airways, IL-33 is the major cytokine that activates innate lymphoid cells with its receptor T1/ST2. In addition, IL-25 and TSLP also have additive or synergistic effects on innate lymphoid cells.[19] Barlow et al. investigated the role of IL-33 and IL-25 in IL-25 deficient, IL-33 deficient and double deficient BALB/c mice. They found that IL-33 plays an important role in terms of airway hyperreactivity and contraction, and it activates IL-13 producing innate lymphoid cells more potently. Moreover, they observed that, IL-33 was expressed and released faster than IL-25.[20] Another study demonstrated that the administration of IL-33 can

lead to an increase in TSLP expression.[21] In accordance with these findings, quarcetin suppressed the expression of IL-33 more potently compared to other cytokines. Thus, quarcetin might have inhibitor effects on the initiation of the asthmatic inflammation. In our study, quercetin treatment inhibited Th2 immune response, which led to lower levels of OVA-specific IgE in serum and IL-4 in BAL; these findings support its anti-allergic effects.

Apoptosis is a physiological process of cell death in order to maintain the healthy state of the lung. However, excessive epithelial cell apoptosis causes epithelial shedding, which further increases the epithelial barrier disruption. Activated T cells and eosinophils induce epithelial cell apoptosis and insufficient clearance of these cells can lead to development of necrosis with loss of membrane integrity.[22] Penberthy et al. observed that epithelial cells were capable of removing their apoptotic neighbors and releasing anti-inflammatory cytokines during the clearance of these cells. Moreover, they found that when the engulfment capacity of bronchial epithelial cells was deteriorated, mice began to overproduce IL-33. They suggested that impaired apoptotic epithelial cell removal increased the inflammatory response.[23] In accordance with these findings; we indicated that quercetin treatment showed beneficial effects on the epithelial cell apoptosis which was demonstrated using TUNEL and caspase-3 staining techniques. Even though quercetin and dexamethasone treatments did not show statistically significant difference in terms of the epithelial cell apoptosis, quercetin treatment resulted in a lower immunostaining than dexamethasone treatment according to the results of TUNEL and caspase-3 assays. Furthermore, quercetin also suppressed IL-33 expression more effectively compared to dexamethasone. This can be explained with the higher number of remaining apoptotic epithelial cells in dexamethasone group which may cause the excess production of IL-33.

There are some limitations in this study. We could not define the molecular mechanisms of quercetin on epithelium, as we used immunohistochemical scoring in lung tissue. We used an animal model, thus we cannot generalize our results to humans. However, experimental mouse models of allergic airway inflammation are well known for their well correlation to humans.[24] we did not use any method for the evaluation of airway hyper-reactivity, but we assessed the effects of quercetin and dexamethasone histopathologically. Lastly, although we studied the level of IL-4, one of the major indicators of eosinophilic inflammation in BAL, we did not measure inflammatory cells such as eosinophils both in BAL and lung tissue.[25] In conclusion, the present study states that the administration of systemic quercetin is effective in ameliorating histological changes except the basal membrane thickness. Moreover, systemic quercetin treatment has beneficial effects on the regulation of key cytokines and epithelial cell apoptosis in a murine model of allergic airway inflammation Thus, this treatment might be an alternative for patients suffering from steroid-resistant asthma or side effects of steroids. However, clinical investigations of the in vivo effectiveness should be conducted and possible side effects of quercetin should be determined in patients with asthma.

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