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Effects of the Flavanone combination Hesperetin-Naringenin, and Orange and Grapefruit Juices, on Airway inflammation and Remodeling in a murine asthma model

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We investigated whether flavanones, hesperetin–naringenin, orange, and grapefruit juices reduce airway inflammation and remodeling in murine chronic asthma model. To establish chronic asthma, mice received house dust mite (HDM) for 3 days in 2 weeks, followed by twice per week for 4 weeks. Concurrently, during the last 4 weeks, mice received hesperetin plus naringenin (HN), orange plus grapefruit juice (OGJ), orange juice (OJ), or grapefruit juice (GJ); whereas the asthmatic control (AC) group and non-asthmatic control (NC) group consumed water *ad libitum*. In histopathological examination, no goblet cells metaplasia was observed in the HN, OJ, and GJ groups; also, intra-alveolar macrophages decreased compared with those of the AC group. Hesperetin plus naringenin significantly decreased subepithelial fibrosis, smooth muscle hypertrophy in airways, and lung atelectasis compared with the AC group. Also, there was a reduction of subepithelial fibrosis in airways in OJ and GJ groups compared with AC group, but it was not noticed in OGJ group. In bronchoalveolar lavage fluid, macrophages numbers decreased in OJ and OGJ groups, whereas eosinophil numbers were increased in OJ group compared with NC group. Our finding revealed that hesperetin plus naringenin ameliorate airway structural remodeling more than orange juice and grapefruit juice in murine model of HDM-induced asthma.

Keywords: asthma; remodeling; hesperetin; naringenin; orange juice; grapefruit juice.

INTRODUCTION

Despite the different etiologies among asthmatic patients, an abnormal response of the epithelial–mesenchymal trophic unit to environmental challenges has been proposed to play a central role in the airway pathology and physiology of asthma (Holgate *et al.*, 2000). The persistent activation of the epithelial–mesenchymal transforming unit promotes airway remodeling that is characterized by structural changes, including epithelial cell impairment, mucous cell metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, thickening of several airway wall layers, epithelial goblet cell metaplasia, and other remodeling features (Hackett *et al.*, 2009). Pharmacological intervention in asthma focuses on a stepwise approach depending on the clinical control status. Therefore, many patients with severe asthma remain uncontrolled despite intensive multidrug treatment (Doherty *et al.*, 2011). In comparison to the suppression of an inflammatory response, placing epithelium remodeling at the center of asthma pathogenesis helps to sufficiently identify novel therapeutic targets to protect airways from environmental triggers.

Observational studies have established a beneficial association between fruit and vegetable intake and asthma (Seyedrezazadeh *et al.*, 2014). In addition, previous studies that focused on subgroups of fruits have reported that citrus fruits and apples tend to decrease asthma symptoms and incidence (Chatzi *et al.*, 2007). This beneficial effect has been related to their high content of bioactive substances of polyphenols, including flavonoids. Potential therapeutic application of flavonoids is exerted antioxidant/radical scavenging properties, anti-bacterial and anti-viral activity, antiinflammatory/immunemodulatory actions, down-regulation of the expression of pro-inflammatory markers, apoptotic, as well as antiallergic properties in various conditions, and individual type of cells, such as airway epithelial, macrophages, dendritic, lymphocytes, mast cells, and natural killer cells (Gonzalez *et al.*, 2011; Romano *et al.*, 2013). They have a

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well-established capacity to modulate Th2-driven inflammation, reducing total IgE, eosinophil count, and cytokine production (IL-4 and IL-13) (Gonzalez *et al.*, 2011). Flavanones, including hesperetin (predominant in oranges) and naringenin (predominant in grapefruits) are a subclass of flavonoids, which can be found abundantly in citrus fruits. Globally, there has been a steady increase in the production and consumption of citrus fruits, mostly oranges. In addition, there has been growing evidence in *in vitro* and animal model studies that flavanones decrease endothelial dysfunction and modulate expression of genes involved in adhesion and transendothelial migration (Buscemi *et al.*, 2012). Citrus flavanones, hesperetin, and naringenin by reducing the mitochondrial NADH/NAD^+ ratio, and stimulation of the citric acid cycle, also reveal prooxidant effects on hepatic fatty acid oxidation parameters (Constantin *et al.*, 2013).

In recent years, a limited number of studies have investigated the effects of a variety of flavonoids on inflammatory biomarkers that are responsible for asthma and allergic diseases (Park *et al.*, 2009). However, to our knowledge, none of the previous studies have evaluated the effect of orange and grapefruit juice on inflammation and airway remodeling in asthma, although the effects of their major flavonoids source, hesperetin and naringenin, on inflammation and airway remodeling in asthma have been considered in some studies (Iwamura *et al.*, 2010; Wei *et al.*, 2012).

Among many allergens, house dust mite-exposed animals have exhibited severe structural changes, including goblet cell hyperplasia and an increase in subepithelial collagen deposition. On the other hand, even though respiratory exposure to ovalbumin (OVA) does not lead to chronic airway inflammation, because of increased airway resistance. This may be due to the development of tolerance to inhaled protein antigens without systemic sensitization. Therefore, the inhaled delivery of house dust mite (HDM) is more successful than OVA models because of the intrinsic enzymatic activity of this allergen (Johnson *et al.*, 2007).

Hence, in this study, we investigated whether the *in vivo* administration of red orange juice, grapefruit juice, and their flavanones, hesperetin and naringenin, modulate airway inflammation and remodeling by using mouse models with HDM-induced chronic asthma.

MATERIALS AND METHODS

Test compound preparation. Red oranges (*Citrus sinensis* Var. Moro) and white grapefruit (*Citrus paradis*) were purchased from a wholesale fruit market. Hesperetin and naringenin were obtained from Sigma Chemical Co. (Cat. No. W431300, USA and N5893, UK respectively).

Fruits were squeezed, and the resultant juice was filtered and stored at −20 °C in 250-mL dark glass bottles until further use. Hesperetin plus naringenin supplement was prepared daily. We used the US Department of Agriculture database for calculation of hesperetin and naringenin approximate beverage values (Department of Agriculture, January 2007). Therefore, each 100 mL of the prepared fluid contained 7 mg of hesperetin and 9 mg of naringenin in water.

Experimental groups. Six-week-old to 8-week-old male BALB/c mice (purchased from Pasteur Institute of Iran) were used in this study. Mice were randomly divided into six groups, eight to nine in each group given as follows: (1) tap water for the non-asthmatic control (NC) and asthma control (AC) groups; (2) hesperetin plus naringenin for hesperetin–naringenin (HN) group; (3) mixture of orange and grapefruit juice for the orange-grapefruit (OGJ) group; (4) orange juice for the orange (OJ) group; (5) and grapefruit juice for the grapefruit (GJ) group. Animals in each group were housed separately in single cages placed in a room with a 12/12-h light/dark cycle and an ambient temperature of 22 ± 2° C. They were fed a commercially available food pellet diet (normal diet) and water *ad libitum*.

Asthma induction. For chronic asthma-induced experiments, after acclimatization, mice were given an intranasal sensitized on days 0, 7, and 14 with 200, 100, and 100 μg of HDM (Greer Laboratories, Lenoir, North Carolina, USA) adsorbed to 40 μL of phosphate-buffered solution (PBS), respectively. Further intranasal challenges using a dose of 50 μg HDM/40 μL PBS was performed twice a week for 4 weeks to allow progressive airway remodeling (Doherty *et al.*, 2011). The control mice were given PBS in place of HDM in the challenge stages of the protocol.

Design of beverage interventions. Nutritional supplementation began simultaneously with HDM-induced chronic asthma protocol during the last 4 weeks.

All the procedures in this study were performed in accordance with the guidelines for the Care and Use of Laboratory Animals as adopted by the Ethics Committee of the Faculty of Veterinary Medicine of University of Tabriz (140/9970, March, 2012/ Faculty of Veterinary Medicine of University of Tabriz).

Collection of bronchoalveolar lavage fluid. Animals in all groups were killed by exsanguination at the end of each experimental protocol (24 h after the last HDM challenge and at the end of nutritional supplementation). The lungs were lavaged with three separate 1-mL aliquots of phosphate-buffered saline. The recovered bronchoalveolar lavage (BAL) fluid was centrifuged (400 g, 10 min), and the pellet was resuspended in 200 μL phosphate-buffered saline. Cells were counted on a hemocytometer. Differential cell counts were done by light microscopy from Cytospin® preparations stained with Giemsa stain (Merck, Darmstadt, Germany). A total of 200 cells was counted and classified as neutrophils, eosinophils, or mononuclear cells based on normal morphological criteria.

Histopathological examination. Left diaphragmatic lobe of lung and trachea were removed and placed into 10% neutral phosphate-buffered formalin. For histopathological examination, 4 μm sections of fixed embedded tissues were cut on a Leica rotary microtome, mode l2165 (Leica, Nussloch, Germany), placed on glass slides, and deparaffinized. To determine the structural changes in airways, including goblet cells metaplasia, smooth muscle hypertrophy/hyperplasia, and accumulation of

extracellular matrix proteins such as abnormal subepithelial collagen deposition (fibrosis), we used hematoxylin and eosin stain, periodic acid-Schiff (PAS) stain, and Masson's trichrome stain on sections of the lung tissue (Doherty *et al*., 2011). The tissues were then evaluated under a light microscope. The grading system of histologic severity for inflammation was assessed by two investigators using a semi-quantitative method from grade 0 to grade 3 as follows: 0, normal lung; 1, scattered infiltration of inflammatory cells to average four layers around the vessels or airways; 2, average four to seven layers of inflammatory cells around the vessels or airways; and 3, more than seven layers of inflammatory cells around the vessels or airways; on average, 20 fields were analyzed per section for these measurements. The tissues' qualitative changes were also studied using light microscopy.

Statistics. Data were analyzed using IBM SPSS, Version 20.0 (SPSS, Inc., Chicago, IL, USA). Data were log-transformed to achieve normal distribution before statistical analysis. ANOVA and Tukey tests were applied to assess significant differences between the groups. All the data in figures were expressed as mean ± standard error. p values of <0.05 were considered statistically significant.

RESULTS

To determine whether flavanones, hesperetin and naringenin, and their rich sources, including orange and grapefruit juices, improve airway inflammation and structural remodeling due to chronic asthma, we used HDM in mouse model according to the aforementioned protocol. The mice underwent a fibrotic response and other structural changes similar to those observed in human chronic asthma according to a previously published method by Doherty *et al*. (2011). Briefly, after development of acute airway inflammation with three sensitize of HDM extract, mice undergo a fibrotic response with eight challenges during 4 weeks. Simultaneously with fibrotic stage, each interventional group received interventional diet.

Thereafter, chronically exposed to HDM extracted as shown in Fig. 1, the numbers of inflammatory cells increased in asthmatic group (AC) compared with non-asthmatic group (NC). No significant differences were shown between groups in total cell numbers, macrophages, lymphocytes, and neutrophils in the BAL fluids (Fig. 1A–D). However, oral administration of OJ and OGJ reduced macrophage cells in BAL fluid. Reduction in eosinophils numbers were observed with GJ treatment, whereas their infiltration increased significantly in OJ group compared with NC group ($p \leq 0.05$) (Fig. 1E).

Histopathological evaluation and semi-quantitative analysis revealed peribronchial and perivascular cell layers of inflammatory cells in AC group (Figs 2A, 3, and 4). In addition, in the area of the PAS staining, airway goblet cells metaplasia was obvious, and severe respiratory epithelial hyperplasia, but rarely squamous metaplasia, was seen in AC group (Fig. 2B). In this respect, increase in alveolar macrophage counts, with some scattered eosinophilia in the alveoli, airways, or interstitium, and severe infiltration of lymphocytes were

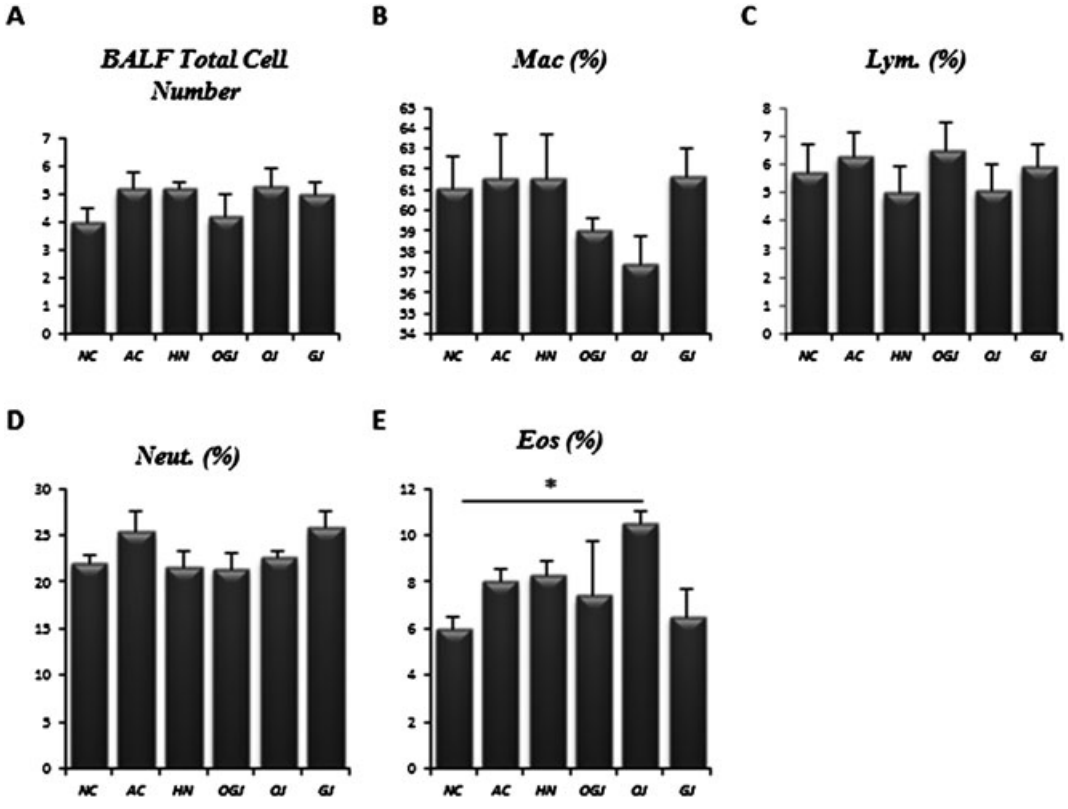

Figure 1. Characterization of bronchoalveolar lavage fluid inflammatory cell populations in chronic house dust mite-challenged male mice. BALF, bronchoalveolar lavage fluid; NC, (non-challenged control) group; AC, (asthma control) group; HN, (mixture of hesperetin and naringenin) group; OGJ, (mixture of orange and grapefruit juice) group; OJ, (orange juice) group; GJ, (grapefruit juice) group. $^{*}p \leq 0.05$.

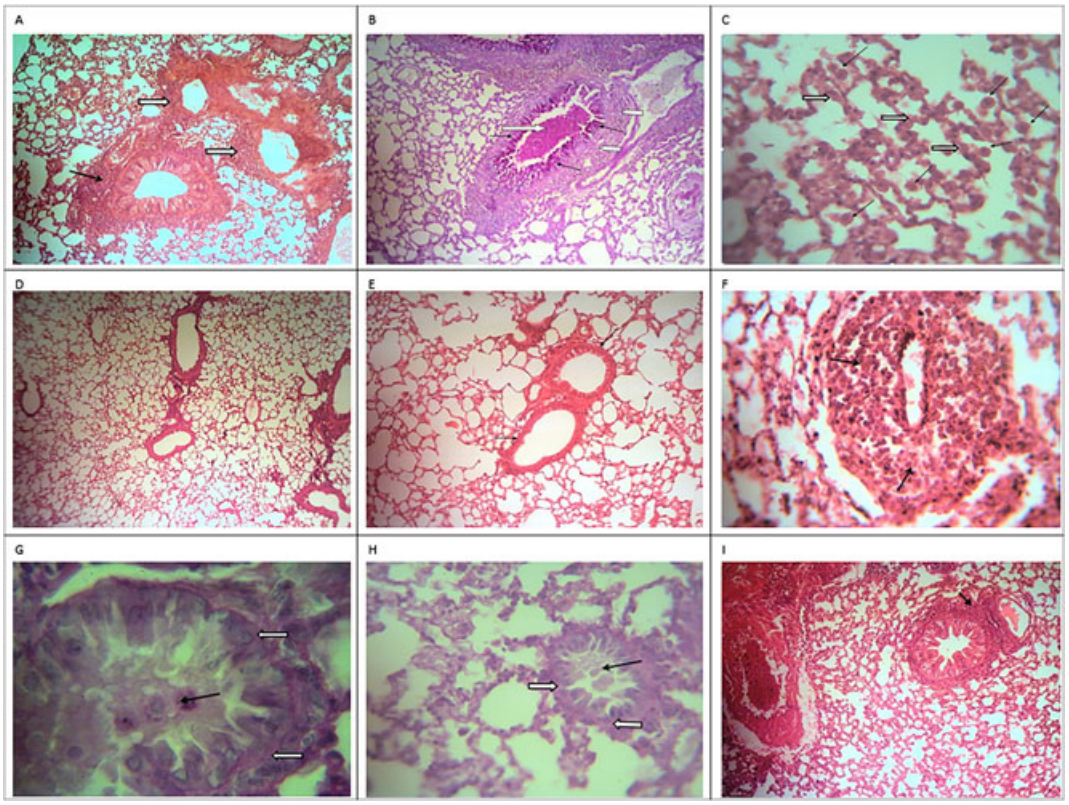

Figure 2. Histological analysis on airway inflammation. (A) Perivascular (white arrows) and peribronchial (black arrows) infiltration of inflammatory cells. Asthma control (AC) group; hematoxylin and eosin (H&E) staining, ×200. (B) Mucus plug (white arrow), goblet cell metaplasia (black arrows) and peribronchial infiltration of inflammatory cells (white pentagons) in a secondary bronchiole. AC group; periodic acid-Schiff (PAS) staining, ×80. (C) Enhancement in alveolar macrophages number (black arrow) and epithelialization (white arrows) in lung parenchyma. AC Group; H&E staining, ×200. (D) Alveolar macrophages and inflammatory cells decreased in number about the same as non-challenged control (NC) group level. Mixture of hesperetin and naringenin (HN) group; H&E staining, ×200. (E) Airways and parenchyma in NC group does not show any marked aggregation of inflammatory cells or any vast presence of alveolar macrophages. H&E staining, ×200. (F) Vasculitis, severe infiltration of inflammatory cells in a venule wall (arrows). Mixture of orange and grapefruit juice (OGJ) group; H&E staining, ×200. (G) Mucus plug formation (black arrow). Thickening and roughening of basement membrane in secondary bronchiole (white arrows). Orange juice (OJ) group; PAS staining, ×200. (H) Mucus plug formation (black arrow) and slight thickening of basement membrane in secondary bronchiole (white arrows). OJ group; PAS staining, ×200. (I) Perivascular and peribronchial infiltration of inflammatory cells (black arrow). Grapefruit juice (GJ) group. Severity of infiltration is markedly less than that in groups 5 and 1. H&E staining, ×80. This figure is available in color online at http://wileyonlinelibrary.com/journal/ptr.

observed in the interstitium of the AC group (Fig. 2C). No goblet cells metaplasia was observed in the HN group. However, in lung sections by morphologic analyses, hesperetin–naringenin-treated mice demonstrated a significant decrease in peribronchial and perivascular inflammatory cell layers ($p < 0.0001$) (Figs 3

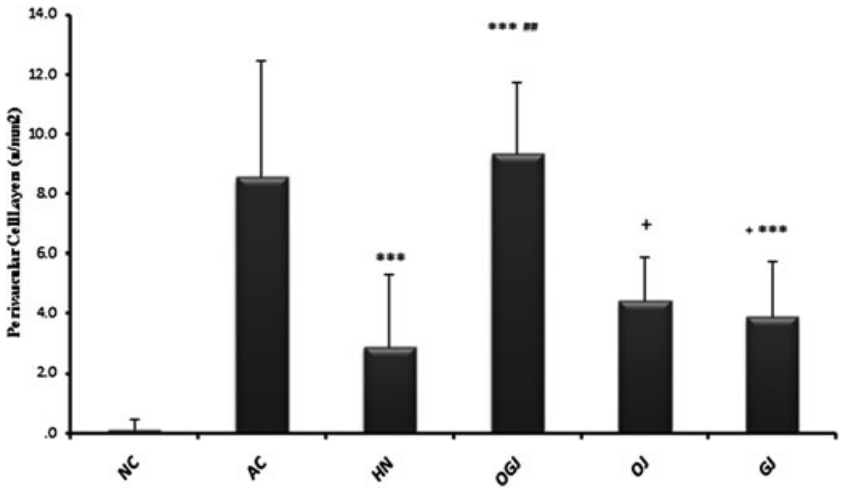

Figure 3. *In vivo* treatment with hesperetin, naringenin, orange juice, and grapefruit juice affected perivascular cell layers in chronic HDM-challenged male mice. Hesperetin and naringenin induced a significant decrease in perivascular cell layers compared with AC and OGJ (***$p < 0.0001$). In OGJ, perivascular cell layers significantly increased compared with NC and HN (***$p < 0.0001$) and GJ ($^{\#\#}p < 0.001$). Perivascular cell layers significantly ($^{+}p < 0.05$) decreased in OJ and GJ (***$p < 0.0001$) compared with AC and OGJ. All the values are presented as Mean ± SE. HDM, house dust mite; NC, (non-challenged control) group; AC, (asthma control) group; HN, (mixture of hesperetin and naringenin) group; OGJ, (mixture of orange and grapefruit juice) group; OJ, (orange juice) group; GJ, (grapefruit juice) group.

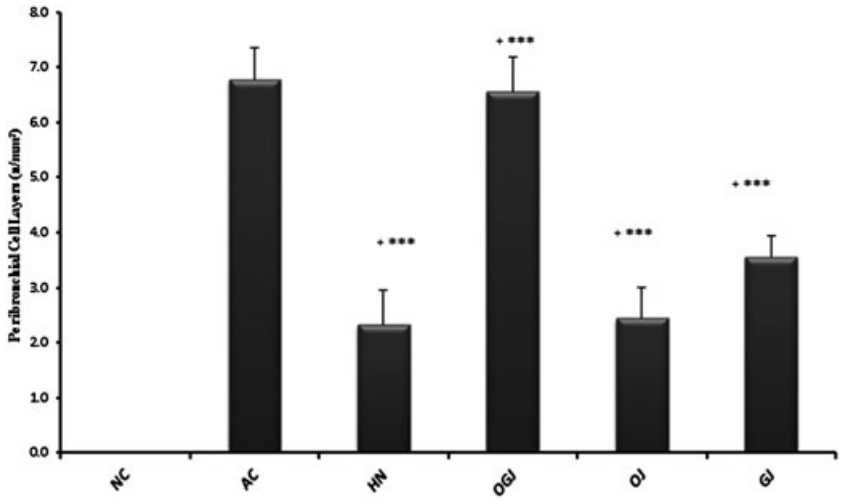

Figure 4. *In vivo* treatment with hesperetin, naringenin, orange juice, and grapefruit juice affected perivascular cell layers in chronic HDM-challenged male mice. In HN, peribronchial cell layers decreased significantly compared with AC and OGJ (***$p < 0.0001$). Mixture of orange and grapefruit juice significantly increased peribronchial cell layers compared with AC and OJ (***$p < 0.0001$) and GJ ($^{+}p < 0.05$). Peribronchial cell layers significantly decreased in OJ and GJ compared with AC and OGJ (***$p < 0.0001$ and $^{+}p < 0.05$, respectively). Significant increases in peribronchial cell layers were observed in HN and OJ ($^{+}p < 0.05$) and in OGJ and GJ (***$p < 0.0001$) compared with NC. HDM, house dust mite; NC, (non-challenged control) group; AC, (asthma control) group; HN, (mixture of hesperetin and naringenin) group; OGJ, (mixture of orange and grapefruit juice) group; OJ, (orange juice) group; GJ, (grapefruit juice) group.

and 4). Mucus plug formation, which is one of the pathological features in asthma, and stromal inflammatory cell infiltration significantly decreased in the NH group compared with the AC group; however, this was still higher compared with the NC group (Fig. 2D, B, and E, respectively) (Table 1). Mice treated with a mixture of orange and grapefruit juice showed mucus plugs and increase of hyperemia and alveolar macrophage numbers (Fig. 2F). Interstitial infiltration of leukocytes, mostly lymphocytes, was severe, with no difference from the AC group. In PAS staining area, goblet cells metaplasia was not seen, whereas the mucus plug formation was found in the orange-intervention group (OJ group) (Fig. 2G and H), which was significantly lower compared with AC group ($p = 0.04$) and higher compared with HN and NC groups (Table 1). Grapefruit group, similar to the OJ group, showed differences in mucus plug formation from the NC and AC groups (Fig. 2I). Stromal cell infiltration in the OJ and GJ group decreased significantly compared with the AC and OGJ groups ($p < 0.0001$).

Masson's trichrome staining of lung sections revealed repetitive challenges of the HDM in the AC group and induced severe remodeling, including abnormal subepithelial collagen deposition (fibrosis) of airway wall, smooth muscle hypertrophy, basement membrane thickening, and severe respiratory epithelium hyperplasia (Fig. 5A) compared with NC group (Fig. 5B and C). The mixture of hesperetin and naringenin treatment (HN group) significantly decreased airway remodeling. Basement membrane thickening was not observed, and smooth muscle hypertrophy was rare in HN group (Fig. 5D and E). Abnormal subepithelial collagen deposition around airways was obvious in the OGJ group (Fig. 5F and G), but it was decreased compared with AC group. In OGJ group, airways showed hyperplastic wall of vessels and hypertrophic muscles, with basement membrane thickening and roughening of the airways and alveoli sections. These findings of the OJ and GJ groups revealed similar histopathological effects on airway tissues; interestingly, fibrosis and remodeling of airways decreased compared with AC group and somewhat less than which was observed in the OGJ group. However, vessel wall hypertrophy was rare or absent (Fig. 5H and I).

Furthermore, histologic assessment showed asthma induction resulted in lung atelectasis and denudation in the AC and OGJ groups, but these changes were

Table 1. Pathological finding score in the intervention groups and controls groups

	Score					
Pathological finding	NC	AC	HN	OGJ	OJ	GJ
Mucus plug	0	2.0 ± 0.23	0.22 ± 0.15[2,4,5]	1.11 ± 0.2[1,2,3]	1.11 ± 0.26[1,2,3]	0.89 ± 0.26[1,2]
Stroma cell infiltration	0	2.89 ± 0.11	0.89 ± 0.2[1,2,4]	2.44 ± 0.18[1,3,5]	1.22 ± 0.15[1,2,4]	1.33 ± 0.17[1,2,4]
Denudation	0.11 ± 0.11	2.0 ± 0.23	0.44 ± 0.2[2]	1.33 ± 0.23[1]	0.89 ± 0.26[2]	1.0 ± 0.24[2]

NC, (non-challenged control) group; AC, (asthma control) group; HN, (mixture of hesperetin and naringenin) group; OGJ, (mixture of orange and grapefruit juice) group; OJ, (orange juice) group; GJ, (grapefruit juice) group ($p < 0.05$).

[1]Significant differences compared with NC.

[2]Significant differences compared with AC.

[3]Significant differences compared with HN.

[4]Significant differences compared with OGJ.

[5]Significant differences compared with OJ or GJ.

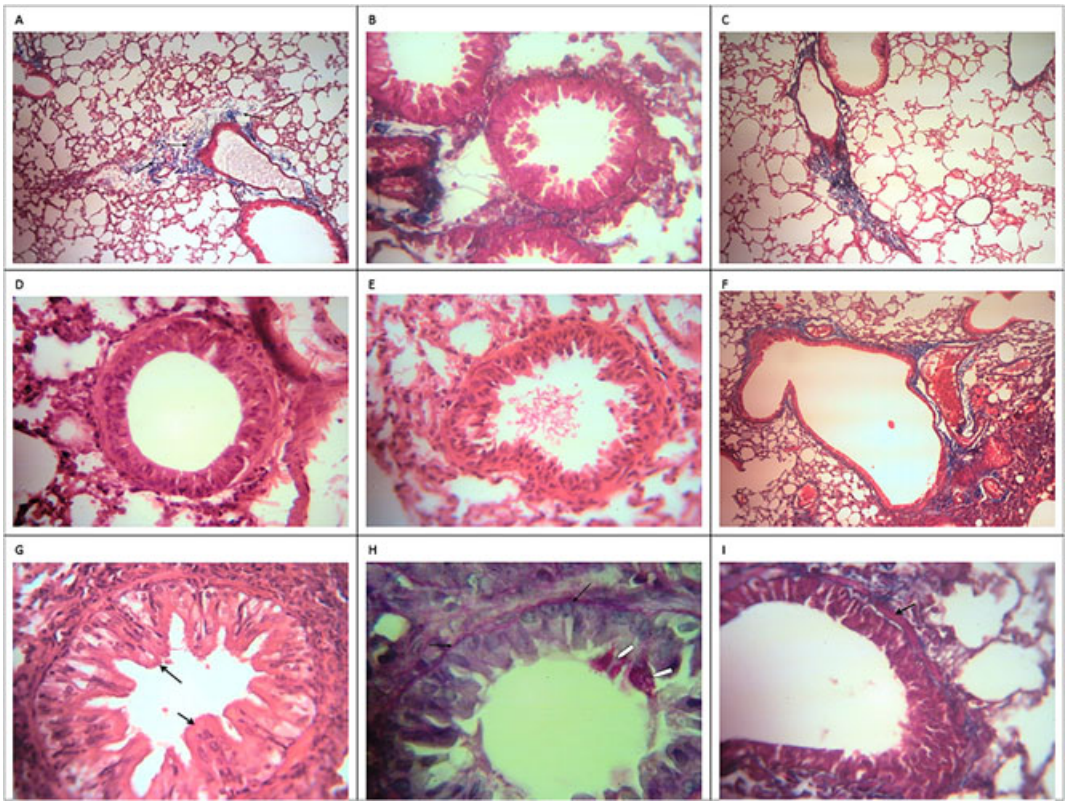

Figure 5. Histological analysis of airway structure changes: (A) Fibrosis around vessels and a bronchiole. Asthma control (AC) group; Masson's trichrome (MT) staining, ×80. (B) Normal bronchioles. non-challenged control (NC) group; MT staining, ×200. (C) Normal bronchioles. NC group; MT staining, ×200. (D) A secondary bronchiole with smooth muscle hypertrophy. Mixture of hesperetin and naringenin (HN) group; hematoxylin and eosin (H&E) staining, ×800. (E) A secondary bronchiole with hyperplastic respiratory epithelium. HN group; H&E staining, ×200. (F) Fibrosis in lung parenchyma mixture of orange and grapefruit juice (OGJ) group: MT staining, ×200. (G) Hyperplasia in respiratory epithelium of a secondary bronchiole (arrows) that caused papillary invagination of the epithelium to the lumen. OGJ group; H&E staining, ×800. (H) A secondary bronchiole with very slight thickening and roughening of basement membrane (black arrows) and slight goblet metaplasia (white pentagons). Grapefruit juice (GJ) group; periodic acid-Schiff (PAS), ×800. (I) A secondary bronchiole with smooth muscle hypertrophy (arrow). GJ group; MT staining, ×800. This figure is available in color online at http://wileyonlinelibrary.com/journal/ptr.

significantly decreased in the HN group compared with AC group. These mentioned histopathological conditions were similar to OJ and GJ groups compared with AC (Table 1).

DISCUSSION

Asthma is a persistent inflammatory airway disease mostly due to intermittent or continuous aeroallergen exposure. The eventual prolonged chronic inflammatory state leads to some structural abnormality both in the epithelial and subepithelial alveolar cell layers, which is considered as airway remodeling. Any inflammatory condition inevitably drives oxidative stress. Imbalances in antioxidant defense mechanisms increase oxidative and inflammatory responses. There has been an increasing consideration towards the use of natural sources of antioxidant to suppress the inflammatory condition and modulate tissue homeostasis. Flavanones and their natural sources, citrus fruits, are very active as reactive oxygen species scavengers (Ghanim *et al.*, 2007).

Our strategy involved observation of the biological effects of flavanones and their rich diet on lung tissue remodeling and repair. In this context, hesperetin plus naringenin, orange plus grapefruit juice, orange juice, and grapefruit juice were separately administrated while simultaneously inducing chronic asthma with exposure to an aeroallergen, HDM, for certain period.

These diets affected airway inflammation and structure. In this respect, non-goblet cells metaplasia was observed, with a significant reduction in mucus plug formation in the HN and GJ groups and to some extent in the OJ and OGJ groups. Moreover, perivascular and peribronchial cells markedly decreased in HN, OJ, and GJ groups. Also, significant reduction of inflammatory cells was seen with HN; however, this difference was only moderate with OJ and GJ groups, and non-significant with OGJ group compared with AC group.

In addition, compared with the AC group, hesperetin–naringenin markedly improved basement membrane thickness and smooth muscle hypertrophy. Each of orange and grapefruit juice also suppressed inflammation and fibrosis moderately, resulting in airway remodeling. However, mixture of orange-grapefruit juice showed a narrow range of properties to attenuate airway structural changes.

To our knowledge, the effects of orange and grapefruit juice on airway inflammation and remodeling have not yet been investigated. Some recent studies have evaluated the potential cellular–molecular mechanisms of orange juice on cardiovascular, metabolic diseases, and inflammation (Cesar *et al.*, 2010; Coelho *et al.*, 2013). Also, one study evaluated the effect of grapefruit intake on DNA damage (Alvarez-Gonzalez *et al.*, 2010). After

1 week of consumption of red orange juice (250 mL, twice daily) by non-diabetic subjects with high risk of cardiovascular disease, endothelial function improved and inflammatory factors including IL-6, TNF-α, and hypersensitivity C-reactive protein decreased (Buscemi *et al*., 2012). In a mouse model of OVA-induced asthma, it was shown that 30 mg/kg hesperidin (glycoside form of hesperetin) reduced the degree of inflammatory cell hyperplasia, mucus hyper secretion, and cytokines concentration such as IL-4, IL-5, and IL-13 in BAL fluid (Wei *et al*., 2012). In addition, the treatment of the asthmatic mice with naringenin-chalcone extract ion from tomato moderately reduced the level of mucus hyperproduction, and IL-4, IL-5, and IL-13 levels in their splenic CD4 T cells *in vitro* (Iwamura *et al*., 2010). Administration of hesperetin (10–30 μmol/kg i.p. or 3–30 μmol/kg o.p.) reduced all types of inflammatory cells and cytokines, including IL-5 and tumor necrosis factor-α (TNF-α) levels in BAL fluid of methacholine-sensitized mice (Yang *et al*., 2011; Shih *et al*., 2012). Our study revealed that hesperetin–naringenin, orange, and grapefruit juices each could significantly reduce infiltration of inflammatory cells to airway and mucus plug formation. Fruit juices and hesperetin–naringenin supplementation also influenced expression of TNF-α and transforming growth factor-β (TGF-β) in lung tissue (data not shown). These findings can be attributed largely to the antiinflammatory properties of flavanones, hesperetin and naringenin. In addition, these substances may potentially interfere with pro-inflammatory cytokines such as IL-5, TNF-α and probably TGF-β, which play a critical role in the pathogenesis of chronic asthma (Durrani *et al*., 2011).

House dust mite was used in our study for chronic asthma induction. Unlike OVA, which has been used in most previous dietary intervention studies, long-term exposure to HDM, the most common environmental allergen, in mice mimics many features of human chronic asthma, including airway remodeling and inducing mesenchymal transition in bronchial epithelial cells (Johnson *et al*., 2007; Heijink *et al*., 2010). Indeed, passive respiratory exposure to OVA leads to immunological tolerance. Also, continuous exposure does not result in chronic airway inflammation (Cates *et al*., 2004). Therefore, the effects of diets were evaluated on chronic asthma model instead of acute asthma model.

Previous studies were shown intranasal delivery of HDM resulted in an increase in total cells that consisted of 76% mononuclear cells and 22% eosinophils, but infiltration of neutrophils was negligible (Cates *et al*., 2004). Fattouh and colleagues demonstrated that eosinophils play negligible roles in the generation of HDM-induced allergic immunity and airway remodeling, including subepithelial collagen deposition and smooth muscle thickening (Fattouh *et al*., 2011). In line with previous studies, our study showed that exposure to HDM increases the levels of eosinophils. However, non-significant reduction was found between beverage intervention groups compared with asthmatic group.

It was shown that the effects of hesperetin and naringenin on inflammation and airway remodeling are somewhat greater than orange juice and grapefruit intake. By using USDA database for the flavonoid content of food, a concentration of 7 mg/100 mL fluid for hesperetin and 9 mg/100 mL fluid for naringenin was selected as supplementation (Department of Agriculture, January 2007). Moreover, it is important to know how much of these nutrients are bioavailable. In food, flavanones can be naturally found in glycosylated forms, which are not absorbable (Kawai *et al*., 2007). These substances must be hydrolyzed by intestinal enzymes or by colonic microflora to aglycone forms before absorption. The concept of bioavailability is under the influence of several factors, including intestinal absorption, metabolism by microflora, cells uptake, and accumulation in tissues (Vallejo *et al*., 2010). The moderate effects of orange and grapefruit juices could be dependent to the compounds of juices and their bioavailability from the fruits. Evaluation of pharmacokinetics indexes of hesperetin and naringenin content of ingested orange juice or grapefruit juice in healthy volunteers individuals were shown remarkable inter-individual variation in AUC_{0-24} (the area under the plasma concentration–time curve) and C_{max} (the peak plasma concentrations) values. So, C_{max} was 0.6 ± 0.4 mmol/L for naringenin from orange juice, 6.0 ± 5.4 mmol/L for naringenin from grapefruit juice, and 2.2 ± 1.6 mmol/L for hesperetin from orange juice. Also, the C_{max}-to-ingested dose ratio was almost equal for naringenin from grapefruit juice and orange juice, suggesting that absorption was not saturated at these doses. Therefore, there may be differences in absorption efficiency and urinary clearance of fraction of flavanones (Erlund *et al*., 2001).

Additionally, it was also observed that a mixture of orange and grapefruit juice intake (OGJ group) has no effect on reducing airway inflammation. Citrus fruits, including orange and grapefruit, contain large amounts of vitamin C and substantial amounts of carotenoid, folate, and fiber (Turner and Burri, 2013). Naringenin and hesperetin are the predominant flavanones in grapefruit and oranges, respectively (Erlund *et al*., 2001). In general, they are considered as major fruit allergen in the Mediterranean area. This property is often attributed to it containing the lipid transfer pan-allergen family. Their biological activity is involved in cross-pollen, which plays a role of pollen generation and plant fertilization (Ahrazem *et al*., 2006). Increasing evidence has demonstrated that pharmacological effect of multimedical components combinations could have synergistic activation and amplify the therapeutic efficacies of each agent (Sun *et al*., 2013). However, in this study, it seems consumption of mixed orange and grapefruit juice increases the allergen ingredient of these fruits, which results in an overlap to their effect on airway's constructional changes.

In summary, the results of this study add new information regarding HDM-induced airway inflammation and remodeling. Also, consumption of hesperetin–naringenin was associated with substantial amelioration of airway structural changes. In addition, this study may provide important insight into the role of orange juice or grapefruit juice in decreasing airway fibrosis and mucus plug formation in asthma model. Taking all together, our results may show that oranges and grapefruits and their main flavanone compounds could improve histopathological changes of HDM-induced chronic asthma in mice. Further interventional studies are required to clarify the exact mechanism of citrus fruits and flavanones' effects on inflammatory events in asthmatic subjects.

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Conflict of Interest

The authors have no relevant interests to declare.

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