

AIRWAY REMODELING

The Effect of Rupatadine on Lung Histopathology in a Murine Model of Chronic Asthma

TUBA TUNCEL, M.D.,^{1,*} MERAL KARAMAN, M.D.,² FATİH FIRINCI, M.D.,¹ PINAR UYSAL, M.D.,¹
MUGE KIRAY, ASSOC. PROF.,³ ALPER H BAGRIYANIK, ASSOC. PROF.,³ OSMAN YILMAZ, PROF.,²
OZKAN KARAMAN, PROF.,¹ AND NEVIN UZUNER PROF.¹

¹Division of Allergy, Department of Pediatrics, Dokuz Eylul University Hospital, Izmir, Turkey.

²Department of Multidisciplinary Laboratory, Dokuz Eylul University Hospital, Izmir, Turkey.

³Department of Histology and Embriology, Dokuz Eylul University Hospital, Izmir, Turkey.

Introduction. Rupatadine is a new second-generation antihistamine with H₁ receptor antagonist activity and platelet-activating factor antagonist properties. This study aimed to investigate the effect of rupatadine on histologic changes in the lungs in a murine model of chronic asthma. **Materials and Methods.** Thirty-five BALB/c mice were divided into five groups of seven mice each: group I (control), group II (placebo [saline]), group III (dexamethasone 1 mg · kg⁻¹ · d⁻¹), group IV (rupatadine 3 mg · kg⁻¹ · d⁻¹), and group V (rupatadine 30 mg · kg⁻¹ · d⁻¹). Groups II through V were sensitized and challenged with ovalbumin and treated once per day via the oral route (gavage). Animals were sacrificed 24 h after the last treatment was administered. Airway histopathology was evaluated using light and electron microscopy in all groups. **Results.** There were no significant differences observed in any of the histologic parameters between groups II and IV. There were significantly thinner basement membrane, subepithelial smooth muscle layer, and epithelia were significantly thinner in group V than in group II ($p < .05$). There were no statistically significant differences in the thicknesses of the basement membrane, subepithelial smooth muscle layer and epithelia between groups III and V. **Conclusion.** Rupatadine had a beneficial effect on histologic changes in a chronic murine model of asthma.

Keywords antihistamines, asthma, lung histology, mice, rupatadine

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by airway hyperresponsiveness to various stimuli, reversible bronchoconstriction, and partially irreversible structural changes referred to as airway remodeling. Asthma is a problem worldwide, with an estimated 300 million affected individuals (1). The prevalence of asthma is increasing, especially in children, and is the most common chronic disease of children in industrialized countries (2).

Inflammation is essential to the pathogenesis of asthma; therefore, treatment methods are directed towards suppressing inflammation (3). Steroids are the most effective drugs for the treatment of asthma, but due to their side effects inhaled forms are preferred. Inhaled steroids may also have side effects, such as suppression of the hypothalamic–pituitary–adrenal axis, and growth failure, especially in children and after long-term treatment (2). Furthermore, although current asthma treatment strategies effectively suppress inflammation, their effects are temporary. However, airway remodeling is not very responsive to these agents (4). New treatment methods and drugs that are effective in treating airway inflammation and remodeling with fewer side effects are currently under investigation.

Histamine is an essential mediator released during mast cell degranulation in asthma which can cause vasodilatation in post-capillary venules, increased vascular permeability, mucosal edema, smooth muscle contraction, bronchospasm, cough, and mucus hypersecretion (5). As the role of histamine in the pathogenesis of allergic disorders is well established, antihistamines are commonly used to treat such allergic diseases as allergic rhinitis and urticaria (6). Although there is some evidence that antihistamines are effective for the treatment of asthma, there is no recommendation for their use in current asthma guidelines (7). However, histamine is not the only mediator involved in the allergic inflammatory process.

Drugs that can inhibit inflammatory mediators besides histamine might be more effective in the treatment of asthma (8). Rupatadine is a new second-generation antihistamine with anti-inflammatory properties and platelet-activating factor (PAF) antagonist properties that are different from the other antihistamines (9). PAF is a proinflammatory mediator involved in the pathogenesis of asthma (10). Because of its anti-inflammatory and anti-PAF properties, as well as antihistaminic activity, rupatadine might have beneficial effect on patients suffering with asthma, although effect of rupatadine in the treatment of asthma has not been studied. The present study was aimed to investigate the effect of rupatadine on histologic changes in the lungs in a murine model of chronic asthma.

*Corresponding author: Tuba Tuncel, Division of Allergy, Department of Pediatrics, Dokuz Eylul University Hospital, 35340, Balcova, Izmir, Turkey; E-mail: tuba.tuncel@deu.edu.tr

MATERIALS AND METHODS

Experimental Animals

The study included 35 specific pathogen-free 6- to 8-week-old male BALB/c mice weighing 20–25 g. The animals were purchased from Dokuz Eylul University, Department of Multidisciplinary Animal Laboratory (Izmir, Turkey) and maintained in a pathogen-free laboratory in the same department. They were kept in hygienic macrolene cages in air-conditioned rooms with a 12-h light/12-h dark cycle and were provided ad libitum food and water. All experimental procedures complied with the requirements of the Dokuz Eylul University Animal Care and Ethics Committee.

Study Groups

The 35 BALB/c mice were divided into five groups of seven mice each: group I (control), group II (placebo), group III (dexamethasone $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), group IV (rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), and group V (rupatadine $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$).

Sensitization and Inhalational Exposure

BALB/c mice were selected for the study because of their high responsiveness to ovalbumin (11). The study was conducted according to a previously described murine model of chronic asthma (12). Mice in groups II–V were sensitized via 2 intraperitoneal injections of ovalbumin (grade V, $\geq 98\%$ pure; Sigma, St. Louis, MO, USA) $10 \mu\text{g}$ 0.1 mL^{-1} with alum as an adjuvant on experimental day 0 and day 14. The mice in groups II–V were then exposed to aerosolized ovalbumin solution 30 min d^{-1} thrice in a week for 8 weeks, starting on the 21st day of the study. Exposure was carried out in a whole-body inhalation exposure chamber. A solution of 2.5% ovalbumin in normal saline was delivered into the chamber via aerosolization of compressed air using a SideStream jet nebulizer. The aerosol generated by this nebulizer was comprised of $>80\%$ particles with a diameter $<4 \mu\text{m}$. Particle concentration was maintained in the range of $10\text{--}20 \text{ mg mm}^{-3}$. A schematic presentation of sensitization and inhalation exposure is shown in Figure 1.

Mice in group I were administered NaCl 0.9% with alum via the intraperitoneal route on experimental day 0 and day 14 and then were exposed to aerosolized NaCl 0.9% for 30 min d^{-1} thrice in a week for 8 weeks, starting on the 21st day of the study. A solution of normal saline was delivered to the whole-body inhalation exposure chamber via aerosolization of compressed

air using a SideStream jet nebulizer. Temperature and relative humidity were maintained at $20\text{--}25^\circ\text{C}$ and $40\text{--}60\%$, respectively.

Study Drugs

Group II (placebo) received saline, group III received dexamethasone $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, group IV received rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, group V received rupatadine $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, all via the oral route (gavage) once daily during the last 5 days of the challenge period. The rupatadine used was a preservative-free powder and was diluted with normal saline. Rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ and dexamethasone $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ were given orally in other studies conducted with BALB/c mice (9,13). Rupatadine was provided by Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey).

Histopathological Analysis

Animals were sacrificed via an overdose of ketamine 24 h after the last treatment was administered. The histopathology findings were evaluated by two investigators that were blinded to the study. Tissue specimens were obtained from the mid zone of the left lung of each mouse. Samples were fixed in 10% formalin for light microscopic evaluation. Some $1\text{--}2 \text{ mm}^{-3}$ tissue samples were obtained from adjacent regions and fixed in 2% glutaraldehyde for electron microscopic evaluation. After fixation, samples were embedded in paraffin for light microscopic evaluation and $5 \mu\text{m}$ serial sections were prepared. After choosing the first section randomly, 10 sections from each mouse were selected by skipping over 10 sections, followed by staining. Consecutively, 30 sections were selected from each mouse and then stained. For light microscopic evaluation, three different staining processes were used. The first 10 samples were stained with hematoxylin and eosin. In these samples, general tissue features were examined, and the 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, measurements were obtained from four points in each airway at levels of 3, 6, 9, and 12 o'clock. Considering that each section contained approximately 2–3 airways, nearly 20 or more airways were evaluated in each mouse (14).

Photomicrographs were obtained using a high-resolution video camera (Olympus DP71, Japan) attached to an Olympus BX-51 microscope (Olympus Optical, Tokyo, Japan). Histological analysis was performed using UTHSCSA Image Tool for Windows v.3.00 software. The 10 consecutive

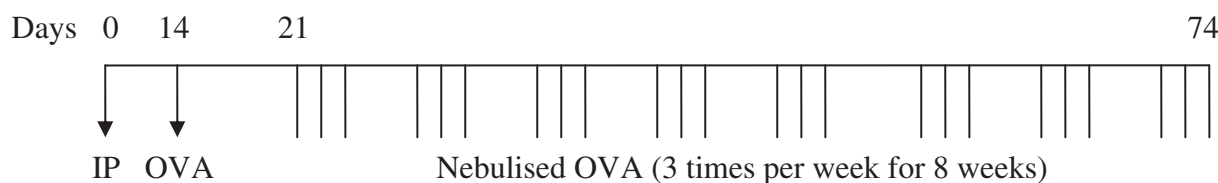


FIGURE 1.—Schematic presentation of sensitization and inhalation exposure in the murine model of chronic asthma.

sections were stained with Toluidine blue and the other 10 sections were stained with periodic acid-Schiff (PAS). Photomicrographs were obtained from five random fields of each section that were stained with Toluidine blue. For mast cell enumeration, a standard transparent counting frame representing an area of $20,000 \mu\text{m}^{-2}$ was manually used and eight fields in each photograph were examined for each mouse. Goblet cells stained with PAS were enumerated in 10 sections for each mouse. In each section, 3–5 randomly selected airways were photographed. The circumferences of all airways were measured and the number of goblet cells in these areas was recorded. The number of goblet cells in $100 \mu\text{m}^2$ was analyzed by dividing the total number of goblet cells by the total airway circumference and multiplying the result by 100 for standardization.

For ultrastructural investigation lung pieces were fixed in 2.5% glutaraldehyde for 24 h. Tissue was postfixed with osmium tetroxide, dehydrated in a graded series of alcohol, and then embedded in Araldite® CY212. For each mouse 5–7 thin (60–90 nm) sections were obtained using an ultra-microtome (Leica), stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (Carl Zeiss Libra 120). After that 8–10 areas were photographed using a Trondle (2048 × 2048 pixels) digital camera attached to the electron microscope. Basement membrane thickness of the respiratory epithelium was measured at 20 points of equal distance and the data were recorded in sequence.

Statistical Analysis

SPSS v.15 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Data are presented as the mean ± standard

error of the seven animals in each group. Comparisons between all the groups were conducted using the one-way ANOVA post hoc Bonferroni method. $p < .0125$ was considered statistically significant.

RESULTS

All histologic parameters, including basement membrane, epithelium, and subepithelial smooth muscle layer thickness, and goblet cell and mast cell counts were significantly high in group II (placebo group) than in group I (control). These results show that the asthma model was successfully established (Table 1). In group III (dexamethasone $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), the basement membrane, epithelium, and subepithelial smooth muscle layer thickness, goblet cell count, and mast cell count were significantly lower than in group II (Table 2).

All histologic parameters were lower in group IV (rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) than in group II, but the differences were not statistically significant (Table 2). Basement membrane, subepithelial smooth muscle layer, and epithelium were significantly thinner in group V (rupatadine, $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) than in group II. Although goblet and mast cell counts were lower in group V than in group II, the differences were not statistically significant. There were not any statistically significant differences in basement membrane, subepithelial smooth muscle layer, and epithelium thickness between groups III and V ($p = 1.000$).

Although all histologic parameters were lower in group V (rupatadine, $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) than in group IV (rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), the differences in the thicknesses of

TABLE 1.—Comparison of groups I and II.

	Group I (control) mean ± SE ($n = 7$)	Group II (placebo) mean ± SE ($n = 7$)	<i>P</i>
Basement membrane thickness (nm)	275.21 ± 15.24	654.17 ± 14.51	<.001
Smooth muscle layer thickness (μm)	3.23 ± 0.43	8.06 ± 0.98	<.001
Epithelium thickness (μm)	10.16 ± 0.61	23.01 ± 2.91	<.001
Number of goblet cells $20,000 \mu\text{m}^{-2}$	0.41 ± 0.07	2.21 ± 0.27	.004
Number of mast cells $100 \mu\text{m}^{-1}$	0.74 ± 0.23	2.39 ± 0.45	.003

TABLE 2.—Comparison of groups II, III, and IV.

	Group II (placebo) mean ± SE ($n = 7$)	Group III (dexamethasone $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) mean ± SE ($n = 7$)	Group IV (rupatadine $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) mean ± SE ($n = 7$)	Group V (rupatadine $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) mean ± SE ($n = 7$)
Basement membrane thickness (nm)	654.17 ± 14.51	479.90 ± 24.75^a	595.43 ± 11.48	443.84 ± 30.07^{ab}
Smooth muscle layer thickness (μm)	8.06 ± 0.98	3.28 ± 0.29^a	7.89 ± 0.58	4.40 ± 0.50^{ab}
Epithelium thickness (μm)	23.01 ± 2.91	11.82 ± 0.99^a	16.94 ± 1.59	12.33 ± 0.54^a
Number of goblet cells $20,000 \mu\text{m}^{-2}$	2.21 ± 0.27	0.81 ± 0.03^a	2.06 ± 0.52	1.31 ± 0.38
Number of mast cells $100 \mu\text{m}^{-1}$	2.39 ± 0.45	1.11 ± 0.24^a	1.59 ± 0.22	1.43 ± 0.20

Note: ^a $p < .0125$ versus placebo.

^b $p < .0125$ versus Group IV.

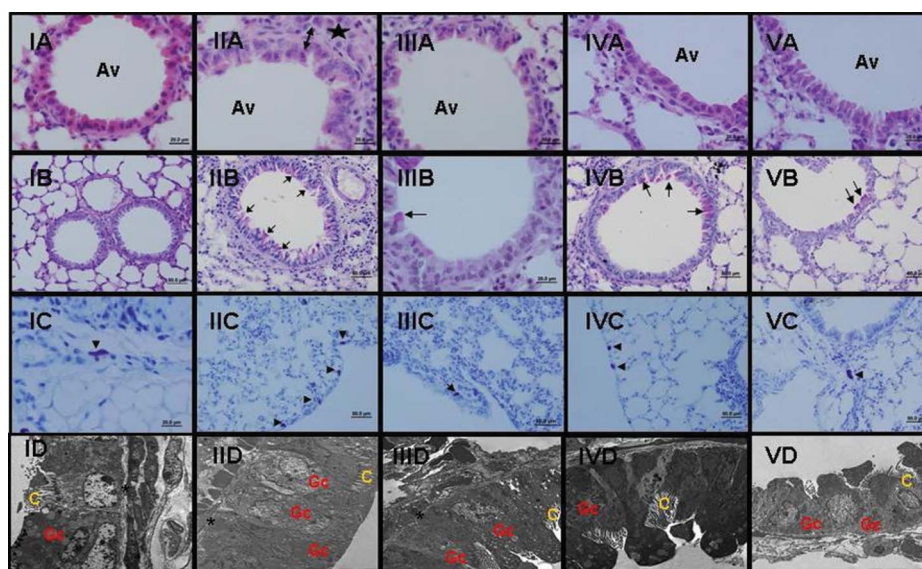


FIGURE 2.—Light and electron microscopy findings in groups I, II, III, IV, V. Lung tissues were stained with H&E (A), PAS (B), and Toluidine blue (C). Electron microscopy images are also shown (D).

H&E; *, peribronchiolar mononuclear infiltration, arrow with two heads; subepithelial smooth muscle layer, PAS; arrows indicated goblet cells, Toluidine blue; arrowhead indicated mast cells, Electron microscopy; *, the basement membrane, C; epithelial cells with cilia.

Av; airways.

the basement membrane and subepithelial smooth muscle layer were statistically significant (Table 2).

Histological views of the airways are shown in Figure 2. Light and electron microscopic examinations demonstrate that the histology of the control group samples was normal. Light and electron microscopic histologic views of the asthmatic mice (placebo group) show epithelium, basement membrane, and subepithelial smooth muscle layer thicknesses, and increased goblet cell and mast cell counts (Figure 2 [IIA–D]). Histopathological parameters in groups III and V improved after the treatment (Figure 2 [IIIA–D and VA–D]).

DISCUSSION

The present study investigated the effect of rupatadine on histologic findings of inflammation and remodeling in the chronic murine model of asthma and showed amelioration on the thicknesses of the basement membrane, subepithelial smooth muscle layer, and epithelium.

Asthma is a chronic disease characterized by reversible airway obstruction, airway inflammation and remodeling (1). Inflammation is essential in asthma. Important consequences of inflammation are remodeling, structural changes in cells, and extracellular matrix in the airways (15,16). Current treatment strategies aim to suppress inflammation, thus preventing remodeling and ameliorating symptoms; however, current asthma treatments are ineffective against remodeling (17). The present study aimed to investigate the effect of rupatadine—a new second-generation antihistamine—on lung histology in a murine model of chronic asthma.

Histamine is the essential mediator released during mast cell degranulation in asthma. The efficacy of antihistamines to treat asthma has been studied extensively, although most

studies were not placebo-controlled and used high doses of first-generation antihistamines. These studies showed that antihistamines cause bronchodilatation and reduce the need for rescue medication (7). More recent studies investigated the efficacy of second-generation antihistamines (cetirizine, azelastine, and desloratadine) at standard doses in patients with both asthma and allergic rhinitis. According to these studies, second-generation antihistamines are effective in reducing the symptoms of asthma, the need for bronchodilators, and the dose of inhaled steroids; the effects of desloratadine did not differ from those of montelukast (18–20). One study on desloratadine reported that it reduced neutrophil, eosinophil, and lymphocyte counts, as well as T-helper cell type 2 (Th 2)-derived cytokine levels, such as interleukin-5 (IL-5) and IL-13, in bronchoalveolar lavage in a murine model of asthma (21). When the studies about allergic rhinitis and comorbid asthma are reviewed, antihistamines are thought to have effect on symptoms of asthma and the quality of life (22). However, antihistamines are not recommended in current asthma treatment guidelines (1,23). The literature does not include any studies on the effects of antihistamines on airway remodeling.

Rupatadine is an N-alkyl pyridine derivate classified as a new second-generation antihistamine. The efficacy of rupatadine in patients with seasonal, perennial, and persistent allergic rhinitis, and chronic urticaria was shown in clinical trials (7). It was reported that rupatadine is a selective and potent histamine receptor antagonist (9). In vitro studies reported that rupatadine-inhibited mast cell degranulation elicited by both immunological and non-immunological stimuli (24). Furthermore, rupatadine inhibited not only pre-formed mediators such as histamine, but also newly produced mediators, including leukotriene C4 (LTC4) and tumor necrosis factor- α (TNF- α) (25). Rupatadine also had

an inhibitory effect on eosinophil chemotaxis, which plays a role in late phase allergic reaction and the pathogenesis of asthma, and decreased eosinophil recruitment after allergen challenge (26). Moreover, rupatadine inhibited the production of IL-5, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In comparison with desloratadine, rupatadine has a significantly stronger effect on the suppression of IL-5, IL-6, and TNF- α (27).

Compared with other antihistamines, such as loratadine, ketotifen, mepyramine, and terfenadine, rupatadine's anti-PAF activity is unique (9). PAF is an important mediator in the pathogenesis of asthma by inducing bronchoconstriction, bronchial hyperresponsiveness, mucus secretion, and allergic rhinitis. In summary, it is thought that PAF is responsible for the persistence of allergic and non-allergic inflammation in the airways, and bronchial hyperresponsiveness (28,29).

Treatment with anti-PAF agents has been investigated in asthma, but drugs that only have anti-PAF activity were not effective in asthma patients in controlled clinical studies (30,31). The administration of PAF-acetylhydrolase (AH) was not found effective in reducing dual-phase asthmatic response induced by an allergen (32). Rupatadine is an antihistamine that inhibits important processes and secretion of mediators in the pathogenesis of asthma, such as mast cell degranulation, recruitment of eosinophils, and production of IL-5, IL-6, TNF- α , and PAF. Rupatadine may be more effective than other antihistamines and PAF antagonists, but until now there has not been a study that examined the effect of rupatadine in asthma.

In the present study, the murine model of chronic asthma reported by Temelkovski et al. was established for examining histologic changes that simulate airway remodeling in humans. Histologic parameters in the placebo group were higher than those in control group, and histologic changes associated with asthma and remodeling were successfully established ($p < .0125$).

Because steroids are currently the most effective drugs for treating asthma, we used dexamethasone in the asthma treatment group in order to compare it to rupatadine. All the histologic parameters improved in the dexamethasone group, when compared with the placebo group ($p < .0125$).

Rupatadine was given by intravenous and peroral route in the previous animal studies. Although there may be other potential ways for drug application, in our study, we preferred peroral use which is the usual route of rupatadine for humans. It was shown that peroral dose of $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ prevents PAF induced mortality in mice. In addition, $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of dose is not related to toxicity in mice (13). There is no data about the dose of rupatadine for the antiinflammatory effect in mice. Therefore we planned to use the highest tolerated and the lowest effective dose in order to compare the effectiveness of the doses on the change of chronic asthmatic histological parameters and we chose $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (low dose) and $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (high dose) doses of rupatadine.

We observed that rupatadine ($30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) reduced basement membrane ($p < .000$), subepithelial smooth

muscle layer ($p = .001$), and epithelial ($p < .000$) thicknesses, and that these effects were not different than in the dexamethasone group. Mast cell and goblet cell counts were lower in the rupatadine group when compared with placebo group, but the difference was not statistically significant. These results indicate that rupatadine had an effect on suppression of inflammation and amelioration of the remodeling process in asthma in high doses. This difference might be caused by the need of higher dose of rupatadine for anti-inflammatory effect than the dose for the anti-PAF effect.

The present study has some limitations. The obtained results may not be translated to positive findings in human clinical trials. In the present study we found that the higher doses of rupatadine were effective on the histologic findings of asthma but it is inappropriate to propose the use of high doses in clinical settings because human doses are different than mice. Additionally, we performed only histological assessment. We could not measure cytokine levels or eosinophil counts and bronchial hyperresponsiveness. Nonetheless, previous studies reported that rupatadine had an inhibitory effect on cytokine production, such as IL-5, IL-6, IL-8, and GM-CSF, TNF- α , and LTC₄, and inhibited the recruitment of eosinophils (9,24).

In conclusion, the present study shows that rupatadine was effective in amelioration of the established chronic histopathologic changes in the lungs in a murine model of chronic asthma especially in higher doses. As the concomitant occurrence of asthma and allergic rhinitis is frequent, and both of the diseases influence each other, we suggest that rupatadine may have a therapeutic role in patients with asthma, particularly in cases with allergic rhinitis. Additional clinical research is needed in order to evaluate the efficacy of rupatadine monotherapy in the treatment of asthma.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The authors have confirmed that the study drug used was provided by Abdi Ibrahim Pharmaceuticals, Istanbul, Turkey.

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