

Impact of Nebulized Sildenafil on Lung Histology in a Murine Model of Chronic Asthma

Nebulize Sildefanilin Bir Astım Fare Modelinde Akciğer Histolojisi Üzerine Etkisi

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ABSTRACT Objective: Sildenafil, which is used for the treatment of erectile dysfunction, has been shown to inhibit inflammation in an animal model of asthma. We aimed to determine the impact of nebulized sildenafil on lung histology in a murine model of chronic asthma. **Material and Methods:** Forty-two BALB/c mice were divided into six groups; A, B, C, D, E, and the control group. All groups except controls were sensitized and challenged with ovalbumin. Then, mice in Group A received nebulized saline. Nebulized sildenafil citrate was administered to Group B, C, and D at concentrations of 0.035 mg/ml, 0.07 mg/ml, and 0.105 mg/ml respectively, and nebulized budesonide was administered at a concentration of 0.25 mg/ml to Group E for 15 minutes once a day, for a week. Animals were sacrificed 24 hours after the last inhalational exposure and the airway samples were evaluated histologically by light and electron microscopy. **Results:** All the histologic parameters of asthma in Group B were significantly ameliorated when compared to the ones in Group A. In Groups C and D, all the parameters except thicknesses of subepithelial smooth muscle layers were significantly improved when compared to Group A. Thicknesses of basement membrane, subepithelial smooth muscle, and epithelium were significantly smaller in Group B than Group E. Thicknesses of basement membrane and subepithelial smooth muscle were significantly smaller in Group C than Group E. Thickness of subepithelial smooth muscle was significantly smaller in Group D than Group E. **Conclusion:** The responses regarding improvement in asthma pathology were found better with all doses of nebulized sildenafil than placebo and even nebulized steroid.

Key Words: Sildenafil; asthma; mice, inbred BALB C

ÖZET Amaç: Erektile disfonksiyonun tedavisinde kullanılan sildenafilin bir astım hayvan modelinde inflamasyonu inhibe ettiği gösterilmiştir. Biz ilk kez bir kronik astım fare modelinde akciğer histolojisi üzerine nebulize sildenafilin etkisini araştırmayı amaçladık. **Gereç ve Yöntemler:** Kırk iki BALB/c fare altı gruba ayrıldı; A, B, C, D, E ve kontrol grubu. Kontroller dışındaki tüm fareler ovalbumin ile duyarlılaştırıldı ve provake edildi. Daha sonra Grup A'daki fareler nebulize salin, Grup B, C ve D'dekiler sırası ile 0.035 mg/ml, 0.07 mg/ml ve 0.105 mg/ml konsantrasyonlarda nebulize sildenafil sitrat, Grup E'deki fareler 0.25 mg/ml konsantrasyonda nebulize budesonidi günde 15 dakika olmak üzere bir hafta boyunca aldı. Hayvanlar son inhalasyon maruziyetinden 24 saat sonra sakrifiye edildi ve havayolu örnekleri histolojik olarak ışık ve elektron mikroskop ile değerlendirildi. **Bulgular:** Grup B'de astıma ait tüm histolojik parametreler Grup A ile karşılaştırıldığında anlamlı olarak düzeldi. Grup C ve D'de subepitelyal düz kas kalınlığı dışındaki tüm parametreler Grup A ile karşılaştırıldığında anlamlı olarak iyileşti. Bazal membran, subepitelyal düz kas ve epitel kalınlıkları Grup B'de Grup E'ye göre anlamlı olarak azdı. Bazal membran ve subepitelyal düz kas kalınlıkları Grup C'de Grup E'ye göre anlamlı olarak azdı. Grup D'de subepitelyal düz kas kalınlığı Grup E'den anlamlı olarak daha azdı. **Sonuç:** Tüm dozlardaki nebulize sildenafil, astımda iyileşmeyi gösterecek şekilde plasebodan ve hatta nebulize steroidden daha iyi sonuç verdi.

Anahtar Kelimeler: Sildenafil; astım; fare, inbred BALB C

Asthma is a chronic disease characterized by reversible airway obstruction, airway inflammation and remodeling.¹ Current strategies for the management of asthma focus on suppressing airway inflammation.² Inhaled glucocorticoids are currently the mainstay of asthma therapy although several side effects may occur when they are used at high doses or for a prolonged time.³ Airway remodeling is progressive structural changes in the composition, content, and organization of the cellular and molecular constituents of the airway wall.⁴ Although current asthma therapies are effective in reducing inflammation, airway remodeling is poorly responsive to current therapies, such as inhaled corticosteroids, antileukotrienes, and theophylline.^{4,5} Thus, new therapeutic options are required.

Phosphodiesterases (PDE) are the major catalyzing enzymes of hydrolysis of the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).⁶ Inhibitors of PDE allow the elevation of cAMP and cGMP which lead to airway smooth muscle relaxation and inhibition of cellular inflammation.⁷ Toward et al.⁸ reported that the PDE5 inhibitor, sildenafil, inhibits inflammation and airway reactivity in an animal model of airway disease. However, there is no evidence that sildenafil affects airway structure and influences airway remodeling. The aim of the study was to investigate the efficacy of nebulized sildenafil on lung histology in a murine model of asthma for the first time.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

Pathogen free, 6- to 8-week-old, male BALB/c mice, weighing 18 to 20 g, were purchased from Bornova Veterinary Control and Research Institute (Izmir, Turkey) and maintained in the pathogen-free animal laboratory of Dokuz Eylül University. They were kept in hygienic macrolene cages with food and water allowed ad libitum in air-conditioned rooms on a 12 hour light/12 hour dark cycle. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the Dokuz Eylül University. Forty two mice were divided into six groups, the study groups

A, B, C, D, E, and the control group each including seven mice.

SENSITIZATION AND INHALATIONAL EXPOSURE

BALB/c mice are high responders to ovalbumin.⁹ The mice in the study Groups A, B, C, D, E were sensitized via two intraperitoneal injections, on days 0 and 14 of the experiment, with 10 µg/0.1 mL chicken egg albumin (ovalbumin, grade V, ≥98% pure; Sigma, St. Louis, MO, USA) that contained alum as an adjuvant. The mice in the study Groups A, B, C, D, E were then exposed to aerosolized ovalbumin for 30 min per day, on three days of the week, for eight weeks, beginning from the 21st day of the study. The mice in control group received normal saline with alum intraperitoneally on days 0 and 14 of the experiment and aerosolized saline without alum for 30 min per day, on three days of the week, for eight weeks, beginning from the 21st day of the study.^{9,10} Exposures were carried out in a whole body inhalation exposure system. Temperature and relative humidity were maintained at 20-25°C and 40-60%, respectively. A solution of 2.5% ovalbumin in normal saline was aerosolized by delivery of compressed air to a sidestream jet nebuliser and injected into a chamber. The aerosol generated by this nebuliser comprised >80% particles with a diameter of <4 µm. Particle concentration was maintained in the range of 10-20 mg/mm.^{3,10,11}

STUDY DRUGS

Mice with experimentally induced chronic asthma in Group A received nebulized saline (placebo), Groups B, C, and D received nebulized sildenafil citrate dissolved in saline at the concentrations of 0.035 mg/ml, 0.07 mg/ml, and 0.105 mg/ml respectively, and Group E was given nebulized budesonide once a day for 15 minutes in the last week of the challenge period by the same system used for administration of aerosolized ovalbumin. Aerosolized sildenafil citrate dose of 0.07 mg/ml was chosen similar to another study conducted with BALB/c mice.¹² Sildenafil citrate was kindly donated by Fako Drug Industry (Istanbul, Turkey). Although nebulized sildenafil citrate was not available in the market, it was prepared as done in

a previous study.¹³ Group E was treated with inhalation of 1 mg budesonide aerosol (0.25 mg/ml).¹⁴

HISTOLOGIC AND MORPHOMETRIC ANALYSIS

Two investigators blinded to the treatment groups interpreted the histology. Animals were sacrificed by an overdose of ketamine 24 hours after the last drug administration, and tissue specimens were taken from the mid zone of the left lung of mice. For light microscopic evaluation, samples were fixed in 10% formalin. Some tissue samples were stocked in 2.5% gluteraldehyde for electron microscopic evaluation.

After samples were embedded in paraffin for light microscopic evaluation, serial sections with 5 micrometer thickness were obtained. The first section was selected randomly, and afterwards ten sections in each mouse were chosen by skipping over ten sections and proceeded to staining process. Three different staining processes were used for light microscopic evaluation. General tissue features were examined and thicknesses of epithelium and subepithelial smooth muscle layers of the medium and small airways were measured in the first ten samples which were stained with hematoxylin and eosin (H&E).

The consecutive ten sections were stained with toluidine blue and the other ten sections with periodic acid Schiff (PAS).

The thicknesses of epithelium and subepithelial smooth muscle layers were measured from four points of each airway at levels of 3, 6, 9 and 12 o'clock. As each section contained two to three airways, nearly twenty or more airways were evaluated in each mouse. Photomicrographs were taken by JVC TK-890-E camera (Japan) which was adapted on Olympus BH-2 RFCA model microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The histological analysis was carried out with UTHSCSA Image Tool for Windows Version 3.00 software.

A standard transparent counting frame representing an area of 16400 square micrometers was manually used for mast cell enumeration. Photomicrographs were taken randomly from five fields of each section which were stained with toluidine blue. For each mouse, eight fields in each photograph were examined.

In ten sections of each mouse, goblet cells stained with PAS were enumerated. Randomly selected three to five airways were photographed in each section. Circumferences of all airways were measured and goblet cell numbers in these areas were recorded. Goblet cell numbers in 100 microns were analyzed by division of total goblet cell number to the total length of airway circumferences and multiplying the result by one hundred.

Tissues were embedded in EPON for electron microscopic evaluation. Airways were marked from the semithin sections by light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate. Libra 120 Carl Zeiss electron microscope (Oberkochen, Germany) was used for this evaluation. Five to seven ultrathin sections were taken from each two blocks in each mouse and epithelium of the airway, the surrounding structures, and the intercellular connections were evaluated. Eight to ten areas were photographed in each mouse by Trondle (2048 x 2048 pixel) digital camera which was attached to the electron microscope. thickness of the basement membrane of the respiratory epithelium was measured at twenty points which had equal distances to each other.¹¹

STATISTICAL ANALYSIS

SPSS 11 package program was used in the statistical analysis. Data were presented as mean \pm standard deviation (SD) (minimum-maximum) of seven animals in each group. The comparisons between all groups were conducted by using Kruskal-Wallis method. When differences were statistically significant, Mann-Whitney U test was used for intergroup comparisons. A $p < 0.05$ was considered statistically significant.

RESULTS

In order to show that the model of asthma was established, we compared control group (non asthmatic) with Group A (asthmatic, placebo). In the chronic asthma group (placebo), the numbers of mast cells as well as the thicknesses of basement membrane, epithelium, and subepithelial smooth muscle layer were significantly higher when compared to the control group ($p < 0.01$, $p < 0.01$, $p <$

0.01, $p < 0.01$ respectively). The numbers of goblet cells were higher in asthma group than the control group, but it was not statistically significant.

These results suggested that the model was successfully established. In Table 1, data of each group were presented as mean \pm SD of seven animals.

In Group B (sildenafil 0.035 mg/ml); the thicknesses of basement membrane, epithelium, and su-

bepithelial smooth muscle layer as well as the number of mast and goblet cells were significantly smaller than the asthma group (Group A) ($p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$).

Group C (sildenafil 0.07 mg/ml) and Group D (sildenafil 0.105 mg/ml) had significantly lower number of mast and goblet cells and decreased thickness of basement membrane and epithelium when compared to the asthma Group (Group A).

TABLE 1: Comparisons between placebo and control groups.

	Placebo	Control	p
Basement membrane thickness (nm)	651.47 \pm 99.72 (485.35-770.23)	299.68 \pm 37.53 (245.19-380.25)	< 0.01
Subepithelial smooth muscle thickness (μ m)	10.65 \pm 2.06 (6.45-13.03)	7.06 \pm 1.42 (5.02-9.65)	< 0.01
Epithelial thickness (μ m)	32.50 \pm 8.70 (20.34-45.12)	19.27 \pm 1.56 (16.34-21.5)	< 0.01
Numbers of mast cells /16400 (μ m ²)	1.15 \pm 0.54 (0-2.2)	0.39 \pm 0.22 (0-0.7)	<0.01
Numbers of goblet cells/100(μ m)	2.22 \pm 0.83 (1-3)	1.33 \pm 0.81 (0-2)	0.08

Data were presented as mean \pm SD (min-max) of 7 animals in each group.

TABLE 2: Comparisons between placebo and nebulized sildenafil citrate groups.

	Nebulized sildenafil citrate			Placebo
	0.035 mg/ml	0.07 mg/ml	0.105 mg/ml	
Basement membrane thickness (nm)	247.45 \pm 65.87 (174.83-447.77) *, #	279.02 \pm 73.60 (161.18-398.66) *	342.37 \pm 95.89 (203.92-563.02) *	651.47 \pm 99.72 (485.35-770.23)
Subepithelial smooth muscle thickness (μ m)	6.25 \pm 1.84 (3.33-9.04) *, †, #	9.71 \pm 2.14 (5.66-13.06)	9.86 \pm 2.34 (6.83-13.68)	10.65 \pm 2.06 (6.45-13.03)
Epithelial thickness (μ m)	14.34 \pm 1.70 (11.49-16.81) *, †, #	22.63 \pm 2.36 (18.85-27.3) **	23.33 \pm 1.87 (19.98-25.93) **	32.50 \pm 8.70 (20.34-45.12)
Numbers of mast cells / 16400 (μ m ²)	0.33 \pm 0.61 (0-2) *	0.2 \pm 0.41 (0-1) *	0.33 \pm 0.61 (0-2) *	1.15 \pm 0.54 (0-2.2)
Numbers of goblet cells / 100 μ m	0.4 \pm 0.54 (0-1) *	0.2 \pm 0.44 (0-1) *	0.8 \pm 0.83 (0-2) **	2.22 \pm 0.83 (1-3)

*: $p < 0.01$ in groups vs. placebo, ** $p < 0.05$ in groups vs. placebo

†: $p < 0.01$ in 0.035 mg/ml vs. 0.07 mg/ml sildenafil groups

#: $p < 0.01$ in 0.035 mg/ml vs 0.105 mg/ml sildenafil groups

Data were presented as mean \pm SD (min-max) of 7 animals in each group.

Thicknesses of subepithelial smooth muscle layers were not different in these groups when compared to the asthma group (Group A) (Table 2).

Thicknesses of basement membrane, subepithelial smooth muscle, and epithelium were significantly smaller in Group B (sildenafil 0.035 mg/ml) than Group E (budesonide) ($p < 0.01$, $p < 0.01$, $p < 0.01$) (Table 3). Thicknesses of basement membrane and subepithelial smooth muscle were significantly smaller in Group C (sildenafil 0.07 mg/ml) than Group E (budesonide) ($p < 0.01$, $p < 0.01$). Thickness of subepithelial smooth muscle was significantly smaller in Group D (sildenafil 0.105 mg/ml) than Group E (budesonide) ($p < 0.01$). In Figure 1A-1E, histological views of the airways taken by light and electron microscopes of Group A - E were shown respectively. When three different doses of nebulized sildenafil citrate were compared to each other, thicknesses of subepithelial smooth muscle and epithelium were found significantly smaller in Group B (sildenafil 0.035 mg/ml) than Group C (sildenafil 0.07 mg/ml) ($p < 0.01$, $p < 0.01$). Thicknesses of basement membrane, subepithelial smooth muscle, and epithelium were smaller in Group B (sildenafil 0.035 mg/ml) than Group D (sildenafil 0.105 mg/ml) ($p < 0.01$, $p < 0.01$, $p < 0.01$). No difference was found between the nebulized sildenafil citrate doses of 0.07 mg/ml and 0.105 mg/ml (Table 2).

DISCUSSION

Airway inflammation in asthma causes subsequent structural changes called as remodeling.¹⁵ The

structural changes in asthmatic airways occur as a result of an injury/repair process. Currently, drugs used for the treatment of asthma have a little effect on airway remodeling.¹⁶ Thus, we aimed to investigate the efficacy of nebulized sildenafil especially on remodeling in a murine model of chronic asthma.

Remodeling includes goblet cell hyperplasia in the epithelium, mucous gland hyperplasia, reticular basement membrane thickening, increased vascularity of mucosa, and thickening of the smooth muscle layer.¹⁷ In our study, the structural changes observed in the asthmatic group revealed that the model was established properly.

Sildenafil, a PDE5 inhibitor, is frequently used for the treatment of erectile dysfunction.¹⁸ PDE5 is expressed in pulmonary vascular smooth muscle, bronchial blood vessels and airway smooth muscle.^{19,20} Airway smooth muscle plays an important role in airway inflammation and remodeling.²¹ The inhibition of PDE5 leads to an increase in cGMP levels and causes protein kinase G dependent smooth muscle relaxation.²² High cAMP levels after PDE4 inhibition also causes bronchodilation dependent on protein kinase C and G,²³ and suppression of inflammation.²⁴ There is evidence that cAMP plays an important regulatory role in the pathophysiology of asthma.²⁵ cAMP also inhibits PDE5 and allows increase in cGMP levels.²⁶ PDE4 and PDE5, which predominate in airway epithelial cells, hydrolyze most of the cAMP and cGMP.²⁷ Similar inflammatory activities of cAMP and

TABLE 3: Comparisons between nebulized sildenafil (0.035 mg/ml) and nebulized budesonide groups.

	Nebulized sildenafil citrate (0.035 mg/ml)	Nebulized budesonide	p
Basement membrane thickness (nm)	247.45 ± 65.87 (174.83-447.77)	366.49 ± 104.62 (71.35-494.48)	< 0.01
Subepithelial smooth muscle thickness (µm)	6.25 ± 1.84 (3.33-9.04)	13.39 ± 2.42 (9.84-17.48)	< 0.01
Epithelial thickness (µm)	14.34 ± 1.70 (11.49-16.81)	27.54 ± 7.22 (13.96-36.54)	< 0.01
Numbers of mast cells /16400 (µm ²)	0.33 ± 0.61 (0-2)	0.4 ± 0.63 (0-2)	0.718
Numbers of goblet cells / 100 (µm)	0.4 ± 0.54 (0-1)	2.8 ± 2.77 (0-6)	0.219

Data were presented as mean ± SD (min-max) of 7 animals in each group.

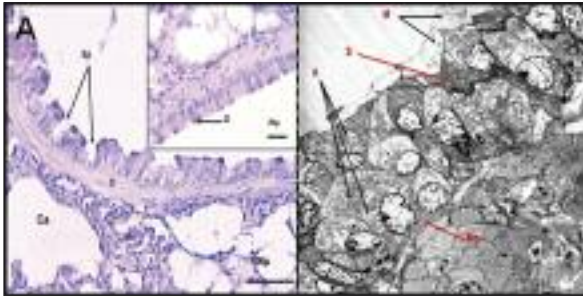


FIGURE 1A: (Placebo group): In asthma group, light microscopic findings revealed disrupted respiratory epithelium (Ep) and pericapillary (Ca) mononuclear cell (Mn) infiltration and increased thickness of smooth muscle layer. In the high-power, PAS (+) goblet cells (G) were seen. In electron microscopy, apical cytoplasm (A) of goblet cells were degranulated, the surface of epithelium was covered with mucus (M), ciliary structure (S) was normal. Basement membrane (Bm).

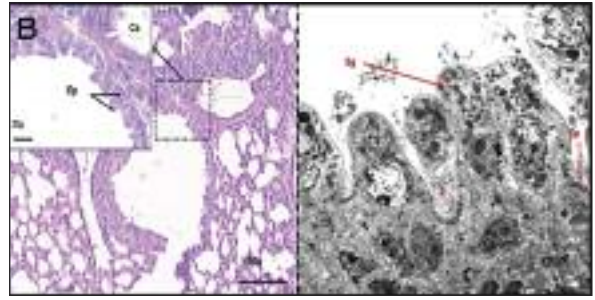


FIGURE 1B: (Nebulized sildenafil 0.035 mg/ml): In light microscopic findings, respiratory epithelium (Ep) was regular. In small figure, the length of epithelium was normal, thickness of smooth muscle was decreased and goblet cells were not detected. Capillary section (Ca). In electron microscopy, apical cytoplasm of secretory granules (Sg), whole epithelial cells (Ep) and ciliary structure (S) were normal.

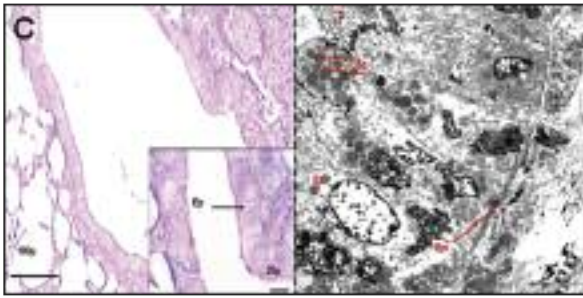


FIGURE 1C: (Nebulized sildenafil 0.07 mg/ml): In light microscopy, respiratory tract epithelium (Ep) and surrounding structures were normal. In electron microscopy, cilia (S) and epithelial cells (Ep) were normal. Integrity of basement membrane (Bm) was not disrupted, apical part of the goblet cells were full of secretory granules (Sg).

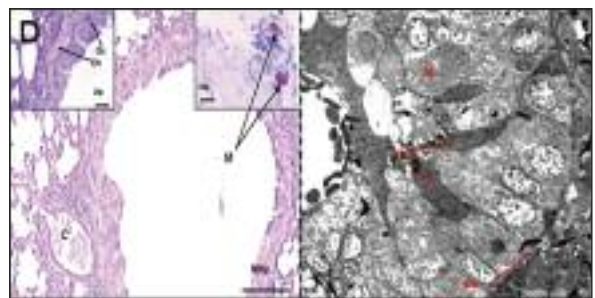


FIGURE 1D: (Nebulized sildenafil 0.105 mg/ml): In light microscopy, general structure was normal. Capillary section (C). In small figures, Goblet cells (Gc), smooth muscle cells (Dh), perivascular mast cells (M) with toluidine blue staining were seen. In electron microscopy, epithelial cells and ciliary structure (S) were normal, apical cytoplasm of some Goblet cells were full of secretory granules (Sg). Basement membrane (Bm) was seen normal.

cGMP create the opportunity of new drug development for treatment of asthma via regulation of cGMP.^{8,19,23}

PDE4 inhibitors have been recently investigated as new treatment options in asthma.²⁸ LASSBio596, a novel PDE4 and 5 inhibitor, ameliorated morphometrical and mechanical changes of lung in a mouse model of asthma.²⁹ PDE4 and PDE5 inhibitors may have similar anti-inflammatory properties. In one study, sildenafil inhibited airway hyperresponsiveness, leucocyte infiltration and exhaled nitric oxide levels after allergen exposure in sensitized guinea-pigs.⁸ It conflicts with another study which indicated that sildenafil produced any significant anti-inflammatory effect in a mouse model of asthma.³⁰ However, in these two studies, different animals, drug doses and routes were used. It is known that inhalation drug therapy has lower

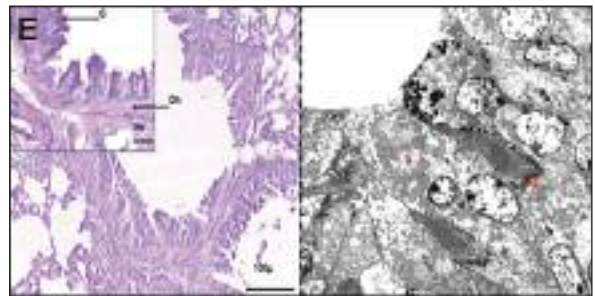


FIGURE 1E: (Nebulized budesonide): Goblet cells (G) and smooth muscle cells (Dh) were seen in light microscopy. Epithelial cells (Ep) with and without cilia and Goblet cells (G) were shown in electron microscopy.

systemic side effects and increased local efficacy in target organ, in our case, the lung.³¹ In our study, nebulized sildenafil, was used in a murine model of asthma for the first time. We evaluated the efficacy of three different doses of nebulized sildenafil and best improvement was achieved with the

smallest dose at a concentration of 0.035 mg/ml. In accordance with our results, Toward et al.⁸ suggested that low dose sildenafil attenuated bronchial hyperreactivity and inflammation. However, in another study, repeated administrations of high doses of sildenafil showed no positive effect on bronchial hyperreactivity and inflammation in sensitized BP2 mice.³² In our study, the improvement of asthma was found with all doses of nebulized sildenafil when compared to placebo and even compared to nebulized steroid.

Nitric oxide inhibits histamine mediated vasodilatation, vasopermeation and leucocyte-endothelial cell attachment which are all mast cell-dependent processes.³³ It also inhibits proliferation of airway smooth muscle, thus may protect against airway remodeling. cGMP, a second messenger in airway smooth muscle, has important relaxant and antiproliferative effects.³⁴ Smooth muscle cells in asthma can produce chemokines and cytokines which may cause airway remodeling.³⁵ We suggest that the favourable effect of nebulized sildenafil on airway remodeling might come along via those inhibitory mechanisms.

There were some limitations of our study such as cytokine levels could not be evaluated and the results found in our study may not translate to positive findings in human clinical trials. In addition, the short treatment period for improvement in airway remodeling is another limitation of the current study. Here, to determine the effect of nebulized sildenafil in the treatment of chronic asthma, we evaluated only histological changes. Most of the asthma models are devoid of the chronic histopathologic changes seen in human asthma because of short term exposure to inhaled antigen. Temelkovski et al.⁹ suggested that this experimental model replicated many features of human asthma. Although we could only evaluate the histological changes of asthma, the validity of our method increases the value of our study.

Our results pointed to the ameliorating effect of nebulized sildenafil on the chronic changes in the airways of asthmatic mice for the first time. Further studies with long term treatments which evaluate the effects of sildenafil on lung inflammation and remodeling are needed.

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