Effects of Nebulized Sildenafil on Lung Histology, Oxygen Saturation and Blood Gas Parameters in Asthma Attack of BALB/c Mice

Astım Atağındaki BALB/c Farelerde Nebulize Sildenafilin Akciğer Histolojisi, Oksijen Saturasyonu ve Kan Gazı Parametreleri Üzerine Etkileri

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ABSTRACT Objective: Sildenafil is a phosphodiesterase 5 inhibitor which is used for the treatment of erectile dysfunction. Inhibition of phosphodiesterase 5 promotes alveolar growth and angiogenesis, and attenuates inflammation and airway reactivity in animal models. We aimed to investigate the effects of nebulized sildenafil in asthma attack. **Material and Methods:** In all mice, except Group 1 (control), chronic asthma was established with repeated intraperitonal and aerosolized ovalbumin administrations. In three groups of chronic asthmatics (Group 3, 4 and 5), asthma attack was developed with increasing doses of methacoline inhalation. Afterwards,s nebulized sildenafil (0.07 mg/ml), nebulized budesonide (0.25mg/ml) and nebulized saline were given to Group 3, 4 and 5, respectively. Group 1 and Group 2 were not treated with either methacholine or nebulized therapies. Heart rates and oxygen saturations were measured. Blood gas analyses were performed and lung histologies were examined by light and electron microscopies. **Results:** Thicknesses of basement membrane, subepithelial smooth muscle layer and epithelium and numbers of mast and goblet cells were significantly lower in control group (Group 1) compared to the chronic asthmatics (Group 2) ($p<0.001$, $p=0.002$, $p=0.001$, $p<0.001$, $p<0.001$, respectively). These results suggested that the model of chronic asthma was successfully established. Numbers of mast cells were significantly lower in sildenafil group compared to the saline group ($p=0.020$). Oxygen saturation, heart rate, and blood gas parameters did not show differences between the treatment groups. **Conclusion:** Nebulized sildenafil significantly decreased the numbers of mast cells compared to placebo. Nevertheless, nebulized sildenafil was nor effective on oxygen saturation, heart rate, and blood gas parameters.

Key Words: Microscopy, electron; phosphodiesterase 5 inhibitors; mice, inbred BALB C; asthma

ÖZET Amaç: Sildenafil erektil disfonksiyonun tedavisi için kullanılan bir fosfodiesteraz 5 inhibitörüdür. Hayvan deneylerinde fosfodiesteraz 5'in baskılanması; alveol büyümesini ve anjiogenezi desteklemekte, inflamasyon ve havayolu reaktivitesini azaltmaktadır. Biz astım atağında nebulize sildenafilin etkilerini araştırmayı amaçladık. **Gereç ve Yöntemler:** Grup 1 (kontrol) dışındaki tüm farelerde tekrarlanan intraperitoneal ve aerolize ovalbumin uygulamaları ile kronik astım oluşturuldu. Kronik astım gruplarından üç tanesinde (Grup 3, Grup 4 ve Grup 5) giderek artan dozlarda metakolin inhalasyonu ile astım atağı geliştirildi. Hemen sonrasında Grup 3, 4 ve 5'e sırası ile nebulize sildenafil (0,07 mg/ml), nebulize budesonid (0,25mg/ml) and nebulize salin verildi. Grup 1 ve Grup 2'ye metakolin veya nebulize tedaviler verilmedi. Kronik astım oluşturulduktan sonra tedavi gruplarında Kalp hızları ve oksijen saturasyonları ölçüldü. Kan gazı analizleri yapıldı ve akciğer histolojileri ışık ve elektron mikroskopileri ile değerlendirildi. **Bulgular:** Bazal membran, subepitelyal düz kas ve epitel kalınlıkları, mast ve goblet hücre sayıları kronik astımlılara (Grup 2) göre kontrol grubunda (Grup 1) anlamlı olarak düşüktü. (sırası ile $p<0,001$, $p=0,002$, $p=0,001$, $p<0,001$, $p<0,001$). Bu sonuçlar kronik astım modelinin başarı ile geliştirildiğini göstermektedir. Mast hücre sayıları sildenafil grubunda salin grubuna göre anlamlı olarak düşüktü ($p=0,020$). Oksijen saturasyonları, kalp hızları ve kan gazı parametreleri tedavi grupları arasında farklılık göstermedi. **Sonuç:** Nebulize sildenafil mast hücre sayılarını plaseboya göre anlamlı olarak azalttı. Bununla birlikte nebulize sildenafilin oksijen saturasyonları, kalp hızları ve kan gazı parametreleri üzerine etkisi gösterilemedi.

Anahtar Kelimeler: Mikroskopi, elektron; fosfodiesteraz 5 inhibitörleri; kendi içinde melezlenmiş BALB C fareler; astım

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Asthma is described as a chronic disease that involves inflammation of the pulmonary airways and airway hyperresponsiveness that results in the clinical expression of a lower airway obstruction.[1] Airway inflammation is a key part of the lower airway response in asthma exacerbation.[2] Microscopically, asthma is characterized by the presence of increased numbers of eosinophils, neutrophils, lymphocytes, and plasma cells in the bronchial tissues, bronchial secretions, and mucus. The repeated cycles of inflammation in the lungs with injury to the pulmonary tissues followed by repair may produce long-term structural changes ("remodeling") of the airways.[1] Airway remodeling constitutes subepitelial fibrosis, increased deposition of extracellular matrix protein, goblet cell hyperplasia and mucus gland hypertrophy, smooth muscle hypertrophy, angiogenesis, and epithelial damage.[3-5]

Phosphodiesterases (PDEs) are a family of enzymes which catalyse the metabolism of the intracellular cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) that are expressed in a variety of cell types.[6] Inhibition of cyclic nucleotide PDEs allow cAMP/cGMP to elevate within cells. Therefore, inhibition of PDE is a useful way of causing a variety of cellular effects and can influence inflammatory cell activation, immune cell activation and smooth muscle contractile responses.[7,8] It is known that PDE inhibitors lead to airway smooth muscle relaxation and inhibition of cellular inflammation.[9] PDE4 and PDE5 hydrolyze most of cAMP and cGMP respectively, and predominate in airway epithelium which plays a key role in airway inflammation.[10] It is recognized that the use of PDE3, PDE4 and mixed PDE3/4 inhibitors can provide clinical benefit to patients with asthma or chronic obstructive pulmonary disease (COPD).[6] Sildenafil is a potent and selective PDE5 inhibitor efficient in the treatment of male erectile dysfunction.[11] There is some evidence that PDE5 inhibitors may share similar anti-inflammatory properties as PDE4 inhibitors. Inhibition of PDE5 promotes alveolar growth and angiogenesis, and attenuates inflammation and airway reactivity in animal models.[12-14] Recently we investigated the efficacy of nebulized sildenafil on remodeling in a murine model of chronic asthma.[15] However, there is no study in the literature that determined the efficiency of nebulized sildenafil in asthma attack. Thus, we aimed to use the nebulized sildenafil in chronic asthmatic mice in which an attack was provoked, and investigate the effects of sildenafil on lung histology, oxygen saturations (SaO_2) and blood gas parameters for the first time in the literature.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

Pathogen-free, 6-to 8-week-old male BALB/c mice, weighing 18 g to 20 g were obtained from Bornova Veterinary Control and Research Institute (Izmir, Turkey). They were maintained in the pathogen-free animal laboratory of Dokuz Eylul University (Izmir, Turkey) and kept in hygienic macrolene cages in air-conditioned rooms on a 12-hour light/12-hour dark cycle. The study was done in accordance with Guide for the Care and Use of the Laboratory Animals. The Animal Care and Ethics Committee in Dokuz Eylul University approved the experimental procedures (No:103/2007).

STUDY GROUPS

Thirty five BALB/c mice were divided into five groups: Group 1 (control group), Group 2 (chronic asthmatics), Group 3 (nebulized sildenafil group), Group 4 (nebulized budesonide group), Group 5 (nebulized saline group).

SENSITIZATION AND INHALATIONAL EXPOSURE

BALB/c mice are high responders to ovalbumin.[16] The mice in study groups except Group 1 (control group) were sensitized via two intraperitoneal injections, on days 0 and 14 of the experiment, of 10 μg/0.1 ml chicken egg albumin (OVA, grade V, ≥98% pure; Sigma, St. Louis, MO, USA) with alum as an adjuvant. The sensitized mice were then exposed to aerosolized OVA for 30 minutes per day on 3 days of the week for 8 weeks beginning from the 21st day of the study, so the model of chronic asthma was established with OVA administrations

during 74 days. 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 minutes per day on 3 days of the week for 8 weeks beginning from the 21st day of the study.[16,17] Exposures were carried out in a whole body inhalation exposure system. Temperature and relative humidity were maintained at 20-25^{0}C and 40% to 60%, respectively. A solution of 2.5% OVA in normal saline was aerosolised by delivery of compressed air to a sidestream jet nebulizer 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 to 20 mg/mm^3. [16,18] Mice with experimentally induced chronic asthma in study groups 3, 4 and 5 were given 3 doses of methacholine (at 6.25, 12.5 and 25 mg/ml concentrations) for three minutes by the same system used for administration of aerosolized OVA on day 75. The time interval between the methacholine doses was one hour. Heart rates and SaO_2 were measured by pulse oxymeter (Nellcor N595) just before (0 minute) and five minutes after each dose of methacholine. [18] Ten minutes after the last methacholine dose, nebulized sildenafil (0.07 mg/ml),[15,19] nebulized budesonide (0.25 mg/ml),[20] and nebulized saline were given to Group 3, 4 and 5 respectively for 15 minutes. SaO_2 and heart rates were also obtained 15 minutes after the nebulized therapies. The control group of mice without asthma (Group 1) and the mice with chronic asthma in Group 2 were not treated with either methacholine or nebulized therapies. Thus, the responses to methacholine and nebulized therapies, as determined by SaO_2 and heart rates, were compared among three study groups (Group 3, 4 and 5) of asthmatic mice. One hour after the treatments, mice in group 3, 4 and 5 were sacrificed after anesthesia by blood suction from the hearts, and the blood samples were used for blood gas analysis. In blood gas analysis, pH (acidity), pCO_2 (partial pressure of carbon dioxide) and HCO_3 (bicarbonate) were measured. The mice in Group 1 and 2 were also sacrificed by the same method. Ketamine and xylazine with doses of 35 mg/kg and 5 mg/kg respectively were given for anesthesia.

HISTOLOGICAL EXAMINATION

Two investigators blinded to the groups interpreted the histology. Tissue specimens were taken from the mid zone of the left lung of mice. For light microscopic evaluation, tissue samples were fixed in 10% formalin and embedded in paraffin using routine histologic procedures. Some tissue samples of 1 to 2 mm^3 taken from adjacent regions were stocked in 2% gluteraldehyte for electron microscopic evaluation.

Serial sections of 5 micrometer thickness were then cut from the paraffin blocks and selected for staining. Ten sections were taken from every mouse. (Every 10th section starting from a randomly chosen section was selected). Three different staining processes were used for light microscopic evaluation. The first 10 samples were stained with hematoxylin and eosin (H&E). The slides stained with H&E were analyzed for tissue structure and morphometric features such as the thickness of the epithelium and the subepithelial smooth muscle layers of the medium and small airways. Photomicrographs of 3 fields from each section containing airways were taken using a digital camera (JVC TK-890-E; JVC, Yokohama, Japan) which was adapted to an Olympus BH-2 RFCA microscope (Olympus Optical Co. Ltd, Tokyo, Japan) for these measurements.[17] Morphometric analysis was carried out using version 3 of the UTHSCSA Image Tool for Windows (The University of Texas Health Science Center, San Antonio, Texas, USA).[21] Epithelium and subepithelial smooth muscle layer thicknesses were measured using a calibrated micrometric analyzer at 8 different points on 2 to 3 different airways. The consecutive 10 sections were stained with toluidine blue and the other 10 sections with periodic acid–Shiff (PAS). Photomicrographs were taken randomly from 5 fields of each section which were stained with toluidine blue. A standard transparent counting frame representing an area of 16400 μm^2 was used manually for mast cell enumaration and for each mouse 8 fields in each photograph were examined. Goblet cells stained with PAS were enumerated in 10 sections of each mouse. In each section, randomly selected 3 to 5 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 were analyzed using the formula: total goblet cell numbers /total airway circumference x 100.[17,21]

Tissue samples which were fixed with 2.5% glutaraldehyde and postfixative osmium tetraoxide were evaluated with a Libra 120 electron microscope (Carl Zeiss, Oberkochen, Germany). The tissues were embedded in Epon after routine electron microscopic procedures. Airways were marked from the semi-thin sections by light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs were taken using a TRS Sharp: eye dual speed CCD camera (Troendle, Moorenwies, Germany) fitted to the electron microscope. Five to 7 ultrathin sections were taken from each 2 blocks in each mouse and epithelium of the airway, the surrounding structures, and the intercellular connections were evaluated. Eight to 10 areas were photographed in each mouse. Thicknesses of the basement membrane of the respiratory epithelium were measured from 20 points that were at equal distances to each other using the iTEM software package (version 5.0) (Olympus Soft Imaging Solutions GmbH, Münster, Germany).[17,21]

STATISTICAL ANALYSIS

Statistical analysis was performed using version 11 of the SPSS software package (SPSS Inc, Chicago, Illinois, USA). Data were presented as median and ranges (minimum-maximum) of 7 animals in each group. Differences between two groups were analyzed by Mann-Whitney U test. The comparisons between three groups were conducted by using Kruskal-Wallis method. When differences were statistically significant, Mann-Whitney U test was used for inter group comparisons. Wilcoxon signed-rank test was used for two-sample designs involving repeated measures or "before" and "after" measures. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We compared non-asthmatic control group (Group I) with chronic asthmatic group (Group 2) in order to show that the model of asthma was established. In the chronic asthma group (Group 2), the numbers of mast cells and goblet cells as well as the thicknesses of basement membrane, epithelium, and subepithelial smooth muscle layer were significantly higher when compared to the control group (Group 1) (Table 1). These results considered that the model of chronic asthma was successfully established. Light microscopic findings of Group 1 and Group 2 are shown in Figure 1.

Numbers of mast cells analyzed for Group 3 (nebulized sildenafil), Group 4 (nebulized budesonide) and Group 5 (nebulized saline) were significantly different among the groups (p=0.011). Median and ranges (minimum-maximum) of mast cell numbers for Group 3 (nebulized sildenafil), Group 4 (nebulized budesonide) and Group 5 (nebulized saline) were as follows: 1.00 (0.00-3.00), 0.00

TABLE 1: Comparisons between Group 1 and Group 2.

	Control group	Chronic asthmatics	p
Basement membrane thickness (nm)	286.10 (242.16-378.54)	702.36 (487.23-759.32)	<0.001*
Subepithelial smooth musclethickness (μm)	6.33 (5.25-8.89)	11.22 (7.65-13.44)	0.002*
Epithelium thickness (μm)	19.11 (17.05-20.84)	31.66 (21.11-44.02)	0.001*
Numbers of mast cells/16400 (μm^2)	1.00 (0.00-1.60)	3.20 (1.80-6.00)	<0.001*
Numbers of goblet cells/100 (μm)	0.80 (0.11-3.40)	8.90 (1.72-12.20)	<0.001*

Data were presented as median (minimum-maximum) of 7 animals in each group.

* Statistically significant values.

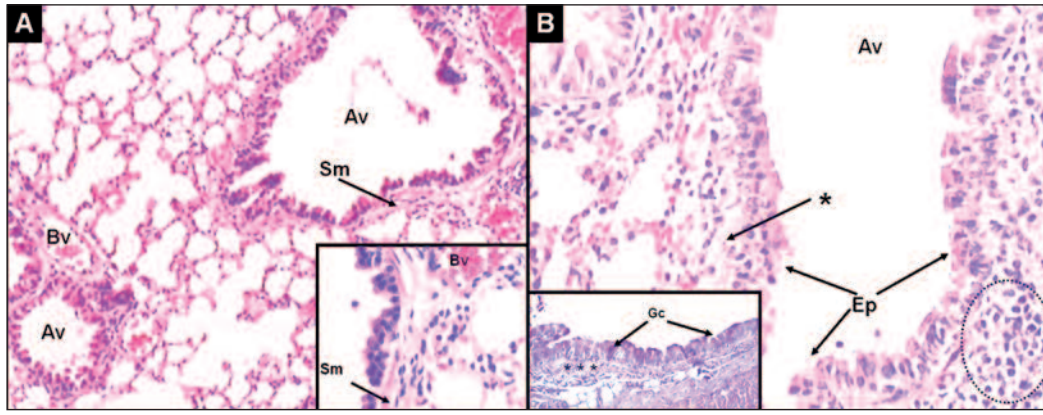

FIGURE 1: A Group 1 (Control group). Epithelium surrounding the airway (Av), subjacent smooth muscle (Sm), blood vessel (Bv) and peripheral parenchyma seemed normal. Staining H&E, magnification x 13.2, small figure x 33. **B:** Group 2 (Asthma group). The increased length of the epithelium (Ep) surrounding the airway (Av), thickened smooth muscle layer (*). In the small figure with PAS staining, increased number of PAS positive Goblet cells (Gc) and peribronchial and perivascular parenchymal mononuclear infiltration are seen (spotted area). Staining H&E, magnification x 33, small figure staining PAS x 33.
(See color figure at http://tipbilimleri.turkiyeklinikleri.com/)

(0.00-3.00), 3.00 (0.00-10.00), respectively (Figure 2). When we compared the groups receiving nebulized therapies, the number of mast cells were significantly smaller in Group 3 (nebulized sildenafil) compared to in Group 5 (nebulized saline) (p=0.020). The numbers of mast cells were significantly smaller in Group 4 (nebulized budesonide) compared to in Group 5 (nebulized saline) (p=0.007). There was no significant difference between Group 3 (nebulized sildenafil) and Group 4 (nebulized budesonide) for mast cell numbers (p=0.404). Light and electron microscopic findings of Group 3, Group 4 and Group 5 are shown in Figure 3.

SaO_2 significantly decreased after second methacholine administration in Group 4 (nebulized budesonide) (p=0.043). Nebulized budesonide significantly decreased SaO_2 in Group 4 (p=0.043). In Group 3 (nebulized sildenafil), SaO_2 significantly increased after third methacholine administration (p=0.027). SaO_2 and heart rates did not show any difference among the groups according to treatments. Analysis of percent changes of SaO_2 and heart rates according to initial parameters inside and between groups were given in table and figures (Table 2) (Figures 4, 5).

Blood gas parameters did not show differences between groups according to the treatments. Blood gas parameters of Group 3, 4 and 5 were given in Table 3.

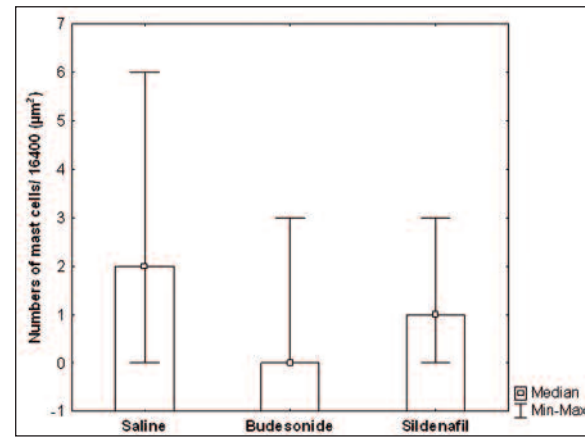

FIGURE 2: Median and ranges (minimum-maximum) of mast cell numbers for Group 3 (nebulized sildenafil), Group 4 (nebulized budesonide) and Group 5 (nebulized saline) (p=0.011).

DISCUSSION

Abnormal structural alterations termed remodeling is an important feature of chronic asthma.[4,5] There is prominent thickening of a fibrillary layer subjacent to the epithelium even in mild or moderate asthma.[22] This is primarily associated with deposition of type III and type V collagens, together with other matrix proteins.[23,24] It is referred to as subepithelial fibrosis or thickening of the reticular basement membrane.[25] The most important feature of asthmatic airway remodeling is thickening of the airway smooth muscle layer.[26] This includes hypertrophy and hyperplasia of the muscle cells, as well as altered deposition of

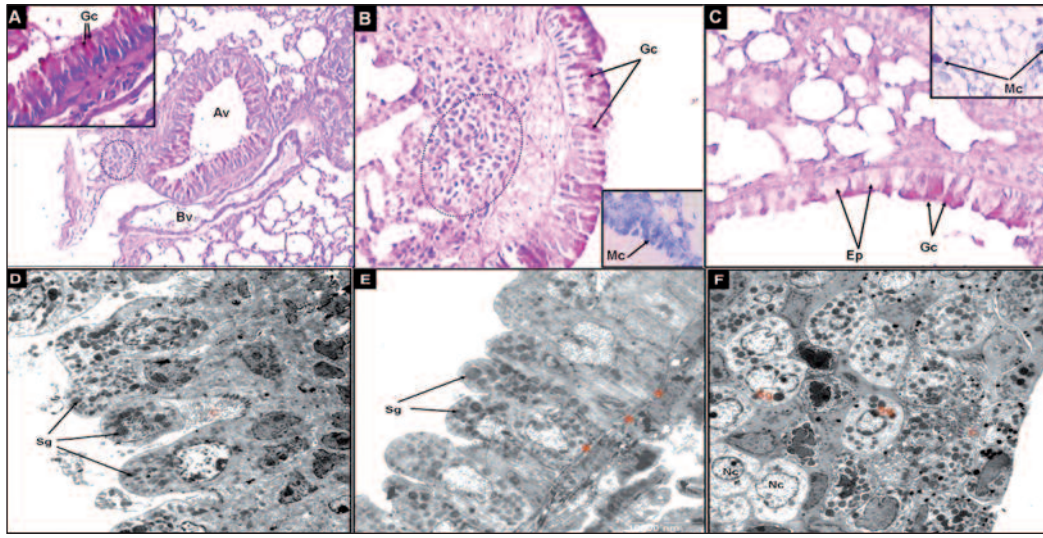

FIGURE 3: A: Light microscopic image of Group 3 (Nebulized sildenafil). Peribronchial infiltration was ongoing in a limited area. PAS positive goblet cells (Gc) were seen around the airway. In the small figure, thickness of smooth muscle was increased. Staining PAS x 13.2, small figure x 33. **B:** Light microscopic image of Group 4 (Nebulized budesonide). Histological view was similar with the previous group. Staining H&E x13.2. In the small figure, intraepithelial Mast cell (Mc) was seen. Staining toluidine blue x 33. **C:** Light microscopic image of Group 5 (Nebulized saline). The length of the epithelium decreased and decreased number of PAS positive goblet cells were seen. Staining PAS x 33. In the small figure, parenchymal mast cell was shown Staining toluidine blue x **66. D:** Electron microscopic figure of Group 3 (Nebulized sildenafil). Respiratory epithelium got full with secretory granules (Sg) and ciliary structure (C) was protected. **E:** Electron microscopic figure of Group 4 (Nebulized budesonide). Respiratory epithelium was full with secretory granules (Sg) which were in secretory phase. Basement membrane (*) was regular. F: Electron microscopic figure of Group 5 (Nebulized saline). Ciliary (C) structure in secretory phase and euchromatic healthy nuclei were seen.
(See color figure at http://tipbilimleri.turkiyeklinikleri.com/)

extracellular matrix.[27] Another characteristic feature of remodeling is an increase in the number of mucin-secreting goblet cells in the surface epithelium, due to both hyperplasia and metaplasia.[28] Airway mucosal vascularity, especially increased density of newly formed small vessels (angiogenesis), is also a feature of remodeling in asthma.[29] The majority of the asthma models involve relatively short-term exposure to aerosolized antigen and are thus devoid of the chronic inflammatory and epithelial changes that typify human asthma. We used BALB/c mice as these are known to be immunoglobulin E (IgE) - high responders to many allergens. Systemic sensitization and repeated allergen challenges were necessary to induce peripheral priming of the immune response and to establish airway inflammation, respectively.[30] In our study, recurrent long term exposure to allergen was provided. The numbers of goblet and mast cells as well as the thicknesses of basement membrane, epithelium, and subepithelial smooth muscle layer were significantly higher in the chronic asthma group when compared to the control group. These results considered that the model of chronic asthma was successfully established. It is known that airway remodeling is poorly responsive to current therapies. Recently, we showed the ameliorating effect of nebulized sildenafil on remodeling parameters in the airways of chronic asthmatic mice for the first time in the literature. In that study, long term treatment with nebulized sildenafil was provided, and the responses regarding improvement in asthma pathology were found better with all three different doses of nebulized sildenafil compared to placebo and even nebulized steroid.[15]

de Visser et al. used sildenafil treatment in experimental bronchopulmonary dysplasia (BPD).[31] Sildenafil treatment, started simultaneously with exposure to hyperoxia after birth, prolonged survival, increased pulmonary cGMP levels, reduced the pulmonary inflammatory response, fibrin deposition and right ventricular hypertrophy, and stimulated alveolarization.[31] Previously it was demonstrated that inhaled nitric oxide (NO) therapy improved lung pathology, reduced fibrin deposition and pulmonary inflammation, and prolonged survival in an animal model of BPD.[32] The authors suggest that the similarity of beneficial effects by

TABLE 2: Percent changes according to initial parameters inside and among the groups.

	Group 3 Percent change Median (Min, Max)	Group 4 Percent change Median (Min, Max)	Group 5 Percent change Median (Min, Max)	p
Oxygen saturations				
0. min-1st methacholine	0.02 (-0.03, 0.25) p*=0.102	-0.07 (-0.32, 0.17) p*=0.273	-0.02 (-0.40, 0.47) p*=0.713	0.244
0.min-2nd methacholine	-0.07 (-0.25, 0.28) p*=0.753	-0.06 (-0.31, 0.19) p*=0.043	-0.22 (-0.36, 0.41) p*=0.715	0.824
0.min-3rd methacholine	0.09 (0.01, 0.39) p*=0.027	0.06 (-0.17, 0.33) p*=0.343	0.20 (-0.28, 0.47) p*=0.588	0.861
0-min-after therapy	-0.24 (-0.53, 0.34)	-0.21 (-0.59, -0.03)	-0.28 (-0.45, 0.32)	0.908
Heart rates				
0.min-1st methacholine	-0.03 (-0.37, 0.55) p*=0.345	-0.06 (-0.30, 0.04) p*=0.144	0.00 (-0.19, 0.25) p*=1.00	0.590
0.min-2nd methacholine	0.08 (-0.30, 0.52) p*=0.173	-0.05 (-0.31, 0.04) p*=0.465	0.18 (0.00, 0.47) p*=0.273	0.088
0.min-3rd methacholine	-0.03 (-0.35, 0.28) p*=0.463	-0.20 (-0.24, 0.22) p*=0.144	0.07 (-0.26, 0.76) p*=0.686	0.565
0-min-after therapy	-0.00 (-0.28, 1.02) p*=0.917	-0.15 (-0.20, 0.21) p*=0.144	0.28 (0.00, 0.95) p*=0.068	0.113

Data are presented as median (minimum, maximum) of 7 animals in each group.

p : Percent changes according to initial parameters between groups (Kruskal-Wallis test),

p**: Percent changes according to initial parameters inside groups (Wilcoxon signed-rank test).

inhaled NO and sildenafil treatment in experimental BPD suggests that the NO-cGMP pathway plays an important role in the pathogenesis of experimental BPD. [31] Inhaled NO can exert its biological effects via the S-nitrosylation or via the NO-cGMP pathway.[31,33] Sildenafil protects cGMP from degradation by inhibiting PDE5 activity whereas NO stimulates the formation of cGMP in the endothelium and smooth muscle cells.[34] However, both modalities result in increased intracellular cGMP levels in these cells.[35] Early treatment with inhaled NO causes acute and sustained improvement in oxygenation in children with acute respiratory distress syndrome.[36] The effectiveness of inhaled NO therapy was also shown in asthma. Previously inhaled NO was successfully used for the treatment of severe asthma attack. The authors suggested that inhaled NO appeared to have a direct relaxing effect on the bronchial smooth muscle.[37]

PDE5 predominates in airway epithelium which plays a key role in airway inflammation.[38] PDE5 is also expressed in pulmonary vascular smooth muscle, bronchial blood vessels and airway smooth muscle.[39,40] It is known that airway smooth muscle is responsible for acute bronchoconstriction, which is potentiated by constrictor hyperresponsiveness, impaired relaxation and length adaptation.[41] Airway hyperresponsiveness (AHR) is a hallmark clinical symptom of asthma.[42] AHR can be evaluated by an exaggerated obstructive response of

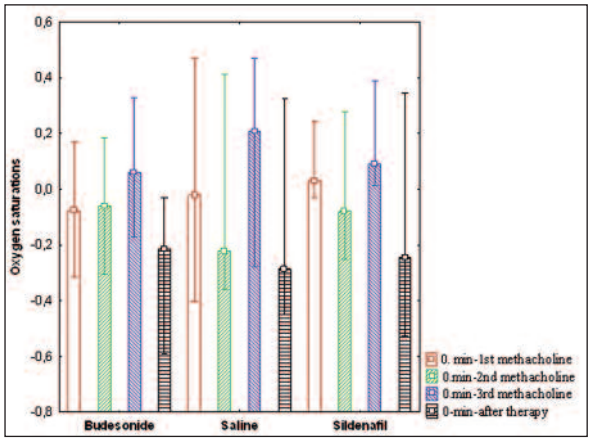

FIGURE 4: Analysis of percent changes of SaO2 according to initial parameters inside and among the groups.
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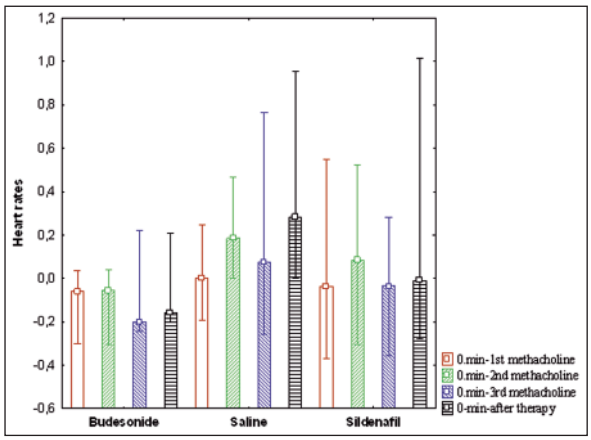

FIGURE 5: Analysis of percent changes of heart rates according to initial parameters inside and among the groups.
(See color figure at http://tipbilimleri.turkiyeklinikleri.com/)

the airways to a variety of pharmacological, chemical and physical stimuli.[9,43] Contractile agonists, like methacholine and histamine can directly activate receptors on airway smooth muscle cells that initiate myocyte contraction and consequent bronchoconstriction.[44] The inhibition of PDE5 leads to an increase in cGMP levels and causes protein kinase G dependent smooth muscle relaxation.[45] Sildenafil inhibited AHR, leucocyte infiltration and exhaled NO levels after allergen exposure in sensitized guinea-pigs, but allergen-induced early and late phase bronchoconstriction were not inhibited in this model.[12] Sildenafil has also been shown to inhibit AHR to inhaled methacholine in individuals with asthma; however, the authors did not measure inflammation in the airways.[46] There was a case report of two patients with COPD taking sildenafil for erectile dysfunction, demonstrating also an improvement in forced expiratory volume in 1 second (FEV_1) of 24% and 12%, respectively.[47] In our study, we measured heart rates and SaO_2 of mice before and after the treatments in order to evaluate the efficacy of nebulized sildenafil in asthma attack. Also blood gas analyses were provided after the treatments. In asthma attack, exacerbation severity is determined based on symptoms and physical examination parameters, as well as lung function and SaO_2.[48] All three therapies did not make difference in heart rates, SaO_2 and blood gas parameters of mice.

Toward et al. reported that sildenafil (1 mg/kg, intraperitoneally) inhibited inflammation in an animal model of airway disease.[12] In another study conducted on mice, sildenafil (3 mg/kg, orally) did not significantly inhibit any markers of inflammation measured.[49] High dose sildenafil (20 mg/kg, intraperitoneally) again showed no positive effect on bronchial hyperreactivity and inflammation.[50] In these studies; different animals, drug doses and routes were used. The efficacy of sildenafil may be

TABLE 3: Blood gas parameters of Group 3, Group 4 and Group 5.

	Nebulized Sildenafil (Group 3)	Nebulized Budesonide (Group 4)	Nebulized Saline (Group 5)	p
pH	7.16 (7.14-7.21)	7.16 (6.93-7.21)	7.08 (7.06-7.22)	0.470
pCO2	54.45 (47.9-61.30)	66.70 (49.10-102.10)	62.60 (56-69.8)	0.131
HCO3	20.85 (17.20-22.20)	22.55 (20.10-25.40)	19.40 (18.6-23.10)	0.295

Data are presented as median (minimum-maximum) of 7 animals in each group.

dose-dependent. In the current study, we used nebulized sildenafil because it is known that inhalation therapy has fewer systemic side effects and increased local efficacy in the target organ.[51] Nebulized sildenafil citrate dose was chosen similar to another study conducted with BALB/c mice.[19] There is a limited number of studies in the literature using sildenafil in the nebulized form, most of which being for the treatment of pulmonary hypertension.[52]

The pathogenesis of allergic airway inflammation is complex, involving multiple cell types such as T helper 2 cells, regulatory T cells, eosinophils, dendritic cells, mast cells, and parenchymal cells of the lung.[53] Kumar and Foster established a model of an allergen-induced acute exacerbation of chronic asthma. [54] Following a low-level challenge for 4 weeks, animals were exposed to a single challenge with 30mg/m^3 aerosolized antigen (10 fold higher than usual) for a 30 minute period. This challenge was associated with more marked airway inflammation compared to the chronic challenge model. The numbers of eosinophils, lymphocytes and neutrophils were slightly higher in bronchoalveolar lavage fluid in acute exacerbation of chronic asthma. Chronic inflammation in tissue and airway remodeling were the same in these two models. They found distal airway inflammation and AHR originating from distal airways in acute exacerbation which were not absent in chronic challenge model.[54] In our study,the number of the mast cells were significantly smaller in the nebulized sildenafil group compared to the nebulized saline group. Mast cells have been thought to play a key role in the immediate reaction in asthma through their release of a variety of mediators.[55] Increased mast cell numbers in airway smooth muscle may be linked to AHR.[56] The release of pre-formed mediators, including histamine, and rapidly synthesized mediators prostaglandin D2 (PGD2) and leukotriene C4 (LTC4), can induce mucus secretion, mucosal edema, and bronchoconstriction. Proinflammatory cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 may be synthesized and secreted by mast cells.[57]

There were some limitations to our study. We used only a small number of animals and we were unable to assess inflammatory cells other than mast cells and cytokine levels which play important roles in asthma pathogenesis, and also lung function tests.

CONCLUSION

In our study, the model of chronic asthma was successfully established. Nebulized sildenafil significantly decreased number of mast cells compared to placebo. We thought that sildenafil, as a PDE5 inhibitor, might provide clinical benefit in asthma attack because of the expression of PDE5 in airway epithelium, pulmonary vascular smooth muscle, bronchial blood vessels and airway smooth muscle. Larger histopathologic studies combined with analysis of inflammatory cells, cytokine levels and lung function tests are required in order to evaluate the efficacy of sildenafil in the treatment of asthma attack.

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