Turkish Journal of Thoracic and Cardiovascular Surgery 2018;26(4):588-597
http://dx.doi.org/doi: 10.5606/tgkdc.dergisi.2019.15149

Original Article / *Özgün Makale*

Protective effect of cysteinyl leukotriene receptor antagonist montelukast in bleomycin-induced pulmonary fibrosis

Bleomisin ile indüklenmiş pulmoner fibroziste sistenil lökotrien reseptör antagonisti montelukastın koruyucu etkisi

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ABSTRACT

Background: This study aims to investigate the early- and late-term effects of pharmacological inhibition of cysteinyl leukotriene activity by using montelukast in bleomycin-induced inflammatory and oxidative lung injury in an animal model.

Methods: The study included 48 male Wistar albino rats (weighing 250 g to 300 g). Rats were administered intratracheal bleomycin or saline and assigned into groups to receive montelukast or saline. Bronchoalveolar lavage fluid and lung tissue samples were collected four and 15 days after bleomycin administration.

Results: Bleomycin resulted in significant increases in tumor necrosis factor-alpha levels (4.0±1.4 pg/mL in controls vs. 44.1±14.5 pg/mL in early-term vs. 30.3±5.7 pg/mL in late-term, p<0.001 and p<0.001, respectively), transforming growth factor beta 1 levels (28.6±6.6 pg/mL vs. 82.3±14.1 pg/mL in early-term vs. 60.1±2.9 pg/mL in late-term, p<0.001 and p<0.001, respectively), and fibrosis score (1.85±0.89 in early-term vs. 5.60±1.14 in late-term, p<0.001 and p<0.01, respectively). In bleomycin exposed rats, collagen content increased only in the late-term (15.3±3.0 µg/mg in controls vs. 29.6±9.1 µg/mg in late-term, p<0.001). Montelukast treatment reversed all these biochemical indices as well as histopathological alterations induced by bleomycin.

Conclusion: Montelukast attenuates bleomycin-induced inflammatory and oxidative lung injury and prevents lung collagen deposition and fibrotic response. Thus, cysteinyl leukotriene receptor antagonists might be regarded as new therapeutic agents for idiopathic pulmonary fibrosis.

Keywords: Bleomycin; collagen; glutathione; interstitial lung disease; malondialdehyde; myeloperoxidase; transforming growth factor beta 1; tumor necrosis factor-alpha.

ÖZ

Amaç: Bu çalışmada bir hayvan modelinde bleomisin ile indüklenmiş enflamatuvar ve oksidatif akciğer hasarında montelukast kullanılarak sistenil lökotrien aktivitesinin farmakolojik inhibisyonunun erken ve geç dönem etkileri araştırıldı.

Çalışma planı: Çalışmaya 48 erkek Wistar albino sıçan (ağırlık 250 g-300 g) dahil edildi. Sıçanlara intratrakeal bleomisin veya serum fizyolojik uygulandı ve sıçanlar montelukast veya serum fizyolojik alacak şekilde gruplara ayrıldı. Bleomisin uygulamasından dört ve 15 gün sonra bronkoalveolar lavaj sıvısı ve akciğer doku örnekleri alındı.

Bulgular: Bleomisin tümör nekroz faktör-alfa düzeylerinde (kontrollerde 4.0±1.4 pg/mL'ye karşı erken dönemde 44.1±14.5 pg/mL, geç dönemde 30.3±5.7 pg/mL, sırasıyla, p<0.001 ve p<0.001), transforme edici büyüme faktörü-beta 1 düzeylerinde (28.6±6.6 pg/mL'ye karşı erken dönemde 82.3±14.1 pg/mL, geç dönemde 60.1±2.9 pg/mL, sırasıyla, p<0.001 ve p<0.001) ve fibrozis skorunda (erken dönemde 1.85±0.89'a karşı geç dönemde 5.60±1.14, sırasıyla, p<0.001 ve p<0.01) anlamlı artışlara yol açtı. Bleomisine maruz kalan sıçanlarda kolajen içeriği sadece geç dönemde arttı (kontrollerde 15.3±3.0 µg/mg'a karşı geç dönemde 29.6±9.1 µg/mg, p<0.001]. Montelukast tedavisi tüm bu biyokimyasal işaretlerle beraber bleomisinin indüklediği histopatolojik alterasyonları tersine çevirdi.

Sonuç: Montelukast, bleomisin ile indüklenmiş enflamatuvar ve oksidatif akciğer hasarını hafifletmekte ve kolajen depolanmasını ve fibrotik yanıtı azaltmaktadır. Dolayısıyla, sistenil lökotrien reseptör antagonistleri idyopatik pulmoner fibrozis için yeni teröpatik ajanlar olarak dikkate alınabilir.

Anahtar sözcükler: Bleomisin; kolajen; glutatyon; interstisyel akciğer hastalığı; malondialdehit; miyeloperoksidaz; transforme edici büyüme faktörü-beta 1; tümör nekroz faktör-alfa.

Received: September 25, 2017 *Accepted:* February 01, 2018

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Cite this article as:
Topaloğlu N, Olgun Yıldızeli Ş, Şener G, Laçin T, Şehirli Ö, Bozkurtlar E, et al. Protective effect of cysteinyl leukotriene receptor antagonist montelukast in bleomycin-induced pulmonary fibrosis. Turk Gogus Kalp Dama 2018;26(4):588-597.

Idiopathic pulmonary fibrosis (IPF) is a chronic, inflammatory and usually fatal interstitial lung disease. In patients with IPF, lung biopsy shows the characteristic histological patterns of usual interstitial pneumonia.[1,2] On the basis that IPF is an inflammatory process that evolves to fibrosis, the natural choice for treatment has been the use of corticosteroids and other immunosuppressive drugs; however, these agents are not curative and disease tends to progress to respiratory failure and death within several years of diagnosis.[3] Lung transplantation is still the only curative treatment for IPF.

The exact mechanisms underlying the development of IPF remain unknown.[4] Dysregulated healing process of lung, or, in other words, loss of healing response is the most popular theory for development of IPF.[5] In animal models of pulmonary fibrosis and in humans with IPF, it has been shown that various cytokines are produced locally in the course of these processes, and believed that these cytokines may participate in various steps of the pathogenesis.[1,6,7] Since no satisfactory treatment is currently available for this progressive disease, researches have centered on these cytokines. Some of these mediators, particularly tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta 1 (TGF-β1,) platelet-derived growth factor (PDGF) and reactive oxygen metabolites might be important in the pathogenesis of IPF.[1,4,6,8] However, a group of these mediators, leukotrienes (LTs), have received little attention. Recently, leukotriene B4 (LTB4) levels in bronchoalveolar lavage fluid (BALF) and LTB4 and cysteinyl LT (cys LT) levels in lung homogenates have been reported to be higher in patients with IPF than those in normal volunteers.[9,10] Lung homogenates LT levels correlated significantly with the extent of fibrosis, suggesting a possible causal relationship between LTs and fibrotic phase of this disease.[10] Lung fibrosis promoted by leukotrienes secondary to increased amount of TGF-β in the lungs was also reported in a mouse model.[11] Thus, targeting LTs has emerged as a novel potential therapeutic strategy of pulmonary fibrosis.

Bleomycin, a glycopeptide antibiotic, is commonly used in cancer treatment and it may cause dose-dependent interstitial pneumonitis.[12] Intratracheal bleomycin administration has been widely used as a model of IPF in animal models and can provide useful insights into the biology of lung injury, fibrosis, and repair. Bleomycin is known to generate reactive oxygen metabolites, which result in deoxyribonucleic acid (DNA) damage, lipid peroxidation, alteration in lung eicosanoid synthesis and glutathione (GSH) content, and an increase in collagen synthesis in lung tissue.[13-15] In an animal model of bleomycin-induced IPF, it has been shown that malondialdehyde (MDA; an end-product of lipid peroxidation) and myeloperoxidase (MPO; an indirect evidence of neutrophil infiltration) levels are increased while GSH (major antioxidant) level is decreased in lung tissue as a result of oxidative injury.[15,16] Montelukast is a cys LT1 receptor antagonist that has been found to reduce subepithelial fibrosis and airway remodeling in an animal model of asthma.[17] However, the therapeutic effect of montelukast on pulmonary fibrosis and oxidant injury, which have important roles in IPF, remains unclear. Therefore, in this study, we aimed to investigate the early- and late-term effects of pharmacological inhibition of cys LT activity by using montelukast in bleomycin-induced inflammatory and oxidative lung injury in an animal model.

MATERIALS AND METHODS

Animals

A total of 48 male Wistar albino rats (weighing 250 g to 300 g) were housed in an air-conditioned room with 12-hour light and dark cycles, where the temperature (22±2°C) and relative humidity (65-70%) were kept constant. The study was conducted at Marmara University School of Medicine between May 2007 and July 2007. All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Guide for the care and use of laboratory animals was successfully used in this study.

Experimental groups

Rats were randomly divided into six groups to investigate early- and late-term effects: *(i)* early C (control) group: rats were subjected to intratracheal and intraperitoneal saline (n=8), *(ii)* early B (bleomycin) + saline group: rats were subjected to intratracheal bleomycin and intraperitoneal saline (n=8), *(iii)* early B+M (montelukast) group: rats were subjected to intratracheal bleomycin and intraperitoneal montelukast (n=8), *(iv)* late C group: rats were subjected to intratracheal and intraperitoneal saline (n=8), *(v)* late B+saline group: rats were subjected to intratracheal bleomycin and intraperitoneal saline (n=8), *(vi)* late B+M group: rats were subjected to intratracheal bleomycin and intraperitoneal montelukast (n=8). Rats were sacrificed four and 15 days after intratracheal administration in order to evaluate the early and late groups (Figure 1).[18,19]

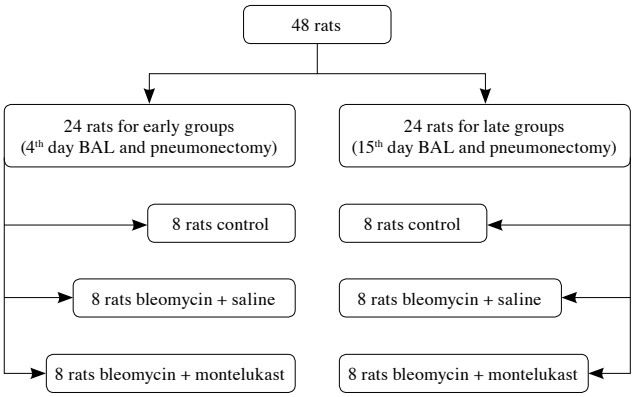

Figure 1. Experimental groups.
BAL: Bronchoalveolar lavage.

Experimental model of pulmonary fibrosis and treatment protocols.

Following an overnight fasting, rats were anesthetized by using 0.5 mg/kg ketamine hydrochloride and 1 mg/kg xylazine. Midline incision was performed in the neck and reached to the trachea. A tracheal cannula was inserted into the trachea by the transoral route. Rats received a single dose of 5 mg/kg bleomycin (dissolved in 0.25 mL saline) via the tracheal cannula to produce pulmonary fibrosis while control rats were administered 0.25 mL saline.

Montelukast treatment (10 mg/kg/day intraperitoneally) was started five days before intratracheal bleomycin or saline administration and continued until sacrification. Saline was administered intraperitoneally instead of montelukast in control groups.

Bronchoalveolar lavage fluid

By the previously described procedure, early and late groups were cannulated four and 15 days after intratracheal administration, respectively. Airways were lavaged four times with 2 mL phosphate-buffered saline through a tracheal cannula. Bronchoalveolar lavage fluid was centrifuged at 350 × g for 10 minutes to separate the cells and the supernatant. The supernatant was harvested for cytokine analysis. Total cell count was performed by hemocytometer and differential cells were counted on cytospin preparations stained with Wright. Four hundred cells were counted for determination of the differential cell count. TGF-β1 and TNF-α in BALF were quantified using enzyme-linked immunosorbent assay (BioSource International, Nivelles, Belgium).

Biochemical analysis in lung tissue

After bronchoalveolar lavage (BAL) procedure, excised lungs were washed in 0.9% sodium chloride and stored at -70°C for measurement of tissue MPO, MDA and GSH levels and fixed in 10% formalin for tissue collagen contents and histopathological evaluation.

Tissue samples were homogenized with ice-cold 150 mM potassium chloride for the determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously.[20] Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×105/M/cm and results were expressed as nmol MDA/g tissue. Glutathione measurements were performed using a modification of the Ellman procedure.[21] Briefly, after centrifugation at 2000 g for 10 minutes, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/L Na2HPO4 sodium phosphate dibasic dihydrate ($2H_2O$) solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels were calculated using an extinction coefficient of 1.36×105/M/cm. Results were expressed in μmol GSH/g tissue.

Table 1. Total cell counts and differential cell percentages in bronchoalveolar lavage fluid

	Control	Early bleomycin+saline	Early bleomycin+montelukast	Late bleomycin+saline	Late bleomycin+montelukast
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Total cells (×10^6 mL^{-1})	0.2±0.1	1.1±0.2*	0.6±0.1§*	0.9±0.1*	0.54±0.1§*
Neutrophils (%)	8.3±3.8	47.1±6.7*†	23.0±4.9§,*,‡	33.8±3.0*	15.1±1.8§**
Lymphocytes (%)	22.4±11.3	20.1±4.1	17.2±5.2	26.6±10.4	27.0±5.9
Macrophages (%)	58.0±8.3	27.3±3.8*	55.0±9.6§	30.2±7.4*	51.0±7.2§

SD: Standard deviation; * p<0.001; ** p<0.05, vs. control group; § p<0.001 vs. bleomycin + saline group in same term; † p<0.001 vs. late bleomycin + saline; ‡ p<0.001 vs. late bleomycin + montelukast groups. Data are means ± standard deviations from eight rats for each group.

Table 2. Tumor necrosis factor-alpha and transforming growth factor beta 1 levels in bronchoalveolar lavage fluid

	Control	Early bleomycin+saline	Early bleomycin+montelukast	Late bleomycin+saline	Late bleomycin+montelukast
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TNF-α (pg/mL)	4.0±1.4	44.1±14.5*†	11.8±4.7§	30.3±5.7*	14.3±4.1¶
TGF-β1 (pg/mL)	28.6±6.6	82.3±14.1*‡	32.0±5.8§	60.1±2.9*	34.8±3.8§

TNF-α: Tumor necrosis factor-alpha; TGF-β1: Transforming growth factor beta 1; * p<0.001 vs. control; ¶ p<0.01; § p<0.001 vs. B+saline groups in same term; † p<0.05 vs. late bleomycin + saline group; ‡ p<0.001 vs. late bleomycin + saline group. Data are means ± standard deviations from eight rats for each group.

Myeloperoxidase is an enzyme that interacts with hydrogen peroxide (H_2O_2) to form the highly toxic hydroxyl radicals and is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMNs). Tissue MPO activity correlates significantly with the number of PMN determined histochemically in inflamed tissues,[22] and therefore, it is frequently utilized to estimate tissue PMN accumulation. Myeloperoxidase activity was measured in tissues in a procedure similar to that documented by Hillegass et al.[23] Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 minutes); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 minutes. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 minutes. Myeloperoxidase activity was expressed as U/g tissue.

Tissue collagen was measured as a bleomycin-induced fibrosis marker. Tissue samples were excised, immediately fixed in 10% formalin, then samples were embedded in paraffin and sections approximately 15 μm thick were obtained. The evaluation of collagen content was performed using the method published by Lopez de Leon and Rojkind,[24] which is based on selective binding of the dyes Sirius Red and Fast Green to collagen and noncollagenous components, respectively. Both dyes were eluted readily and simultaneously using 0.1N sodium hydroxide (NaOH) methanol (1:1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

Histopathological analysis

Excised lung tissues were fixed in 10% formaldehyde and processed routinely in paraffin. Tissue sections were stained with hematoxylin and eosin for general morphology and stained with Masson's trichrome for evaluation of fibrosis. The severity of lung fibrosis was semi-quantitatively assessed according to Ashcroft et al.[25] Briefly, the grade of lung fibrosis was scored on

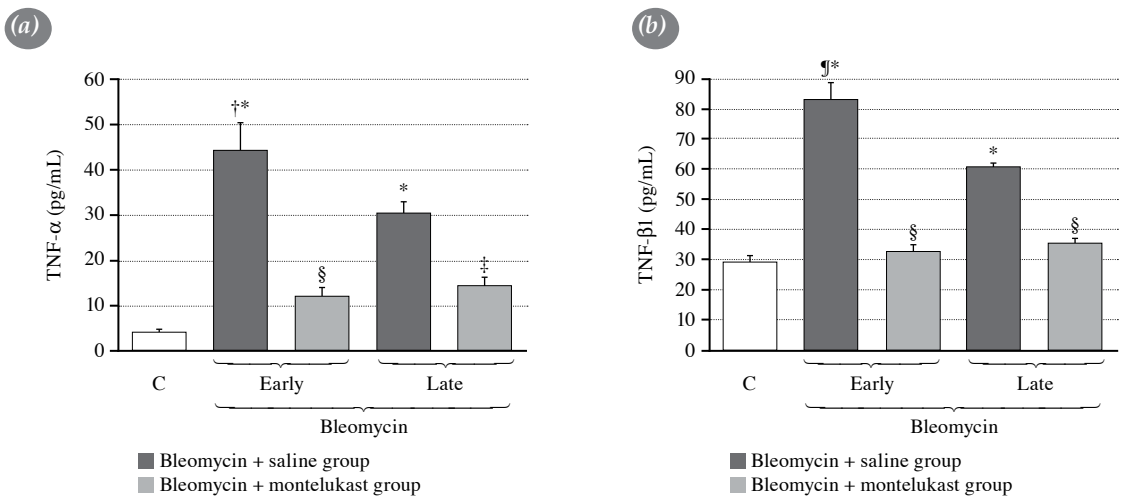

Figure 2. (a) Tumor necrosis factor-alpha and **(b)** transforming growth factor beta 1 levels in bronchoalveolar lavage fluid.

TNF-α: Tumor necrosis factor-alpha; TGF-β1: Transforming growth factor beta 1; B: Bleomycin; * p<0.001 vs C (control group). ‡ p<0.01, § p<0.001 vs B (Bleomycin) + saline groups in same term; † p<0.05 vs late B (Bleomycin) + saline group; ¶ p<0.001 vs late B (Bleomycin) + saline group.

Table 3. Malondialdehyde, myeloperoxidase, glutathione and collagen levels in lung tissue

	Control	Early bleomycin+saline	Early bleomycin+montelukast	Late bleomycin+saline	Late bleomycin+montelukast
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
MDA (nmol/g)	29.2±6.6	46.8±7.7*	32.0±6.6‡	43.0±5.7**	27.2±6.5‡
MPO (U/g)	10.8±2.7	32.8±7.8***	20.1±4.9‡	24.5±8.7*	13.6±2.4‡
GSH (μmol/g)	1.8±0.3	0.8±0.3***	1.4± 0.2**‖	0.9±0.3***	1.8±0.3§
Collagen (μg/mg)	15.3±3.0	20.8±2.3†	14.5±2.1	29.6±9.1***	17.2±2.8§

MDA: Malondialdehyde; MPO: Myeloperoxidase; GSH: Glutathione; * p<0.01, ** p<0.05, *** p<0.001 vs. control group; ‡ p<0.05, ‖ p<0.01, § p<0.001 vs. bleomycin+saline group in same term; † p<0.05 vs. late bleomycin + saline; Data are means ± standard deviations from eight rats for each group.

a scale from 0 to 8 by examining randomly chosen fields of the left middle lobe at a magnification of ×100. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of fields. Grades 2, 4 and 6 were used as intermediate pictures between the aforementioned criteria. All sections were scored by a single investigator in a blinded fashion.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± standard deviation. All results were analyzed by one way analysis of variance followed by Tukey's multiple comparison tests. P values of <0.05 were regarded as statistically significant.

RESULTS

Total and differential cell counts in BALF

As shown in Table 1, intratracheal bleomycin caused significant increases in the BALF total cell count and neutrophil percentage while the macrophage percentage was significantly decreased in early and late B+saline groups. Increases of neutrophil percentage in B+saline groups were most prominent on day four and remained high on day 15. On the other hand, montelukast treatment reversed these changes significantly. There was no significant difference in the BALF lymphocyte percentage between groups (Table 1).

Tumor necrosis factor-alpha and TGF-β 1 levels in BALF

Bleomycin-induced cellular infiltration in BALF was accompanied by an elevation of proinflammatory

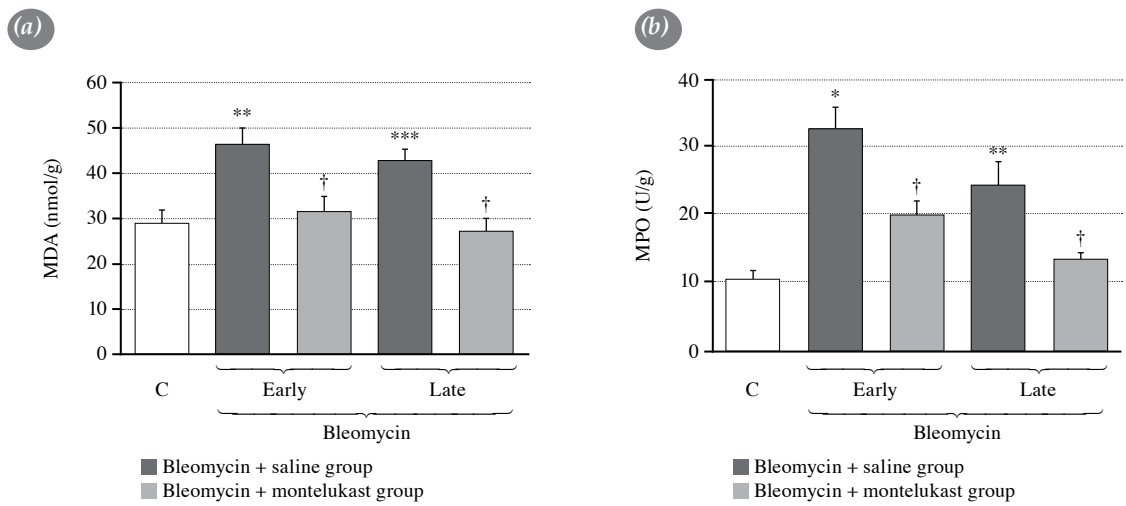

Figure 3. (a) Malondialdehyde and **(b)** myeloperoxidase levels in lung tissue.

MDA: Malondialdehyde, MPO: Myeloperoxidase; * p<0.001, ** p<0.01, *** p<0.05 vs C (control group); † p<0.05 vs B (Bleomycin) + saline group in same term.

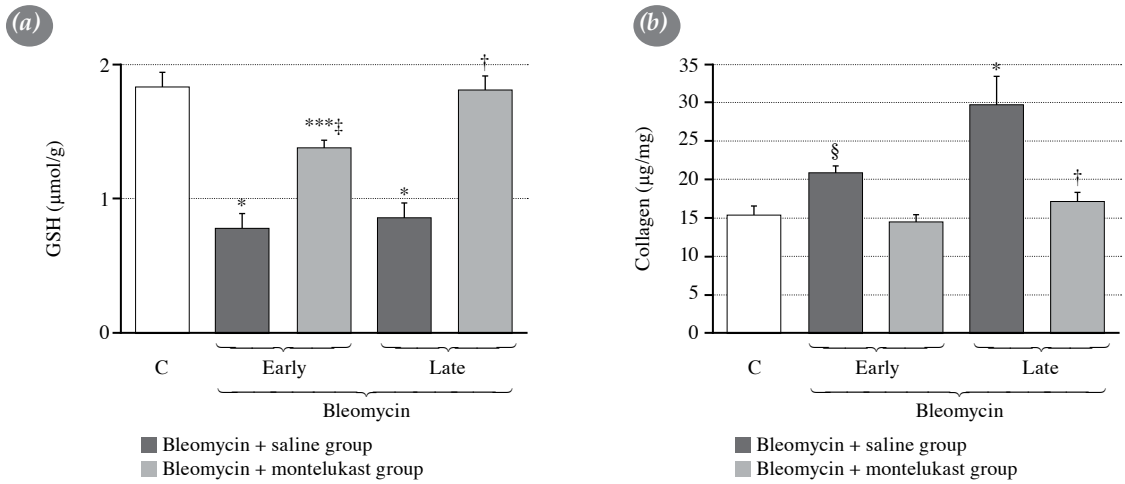

Figure 4. (a) Glutathione and **(b)** collagen levels in lung tissue.

GSH: Glutathione; * p<0.001, ** p<0.01, **** p<0.05 vs C (control group); † p<0.001, ‡ p<0.01 vs B (Bleomycin) + saline group in same term; § p<0.05 vs late B (Bleomycin) + saline.

cytokines, TNF-α and TGF-β1. Compared with the control, levels of these cytokines were significantly increased in early and late B+saline groups (p<0.001). Also, montelukast treatment caused significant decreases in these cytokine levels in BALF (Table 2, Figure 2).

Biochemical indices in lung tissue

Malondialdehyde is a marker of lipid peroxidation secondary to oxidative damage. The lung tissue MDA levels in early and late B+saline groups were increased significantly when compared with control groups (p<0.01, p<0.05, respectively). Montelukast reversed MDA level back to control levels (Table 3, Figure 3).

Bleomycin-induced inflammatory response is characterized by the accumulation of neutrophil infiltration in the lung tissue. Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in early and late B+saline groups when compared with the control group (p<0.001, p<0.01, respectively). Montelukast treatment significantly decreased lung tissue MPO levels, which was not found to be statistically different from that of the control (Table 3, Figure 3).

Bleomycin-induced oxidative injury caused an increase in MDA accompanied by a decrease in GSH levels; as a major antioxidant in lung tissue. Glutathione levels were significantly decreased in early and late B+saline groups. This effect was abrogated with montelukast treatment in early and late B+M groups (Table 3, Figure 4).

Collagen content in the lung tissue was not different from control groups in early B+saline and early B+M groups. However, collagen content in the late B+saline group was markedly increased indicating enhanced tissue fibrotic activity as compared to control (p<0.001). Montelukast significantly reduced lung collagen content to the control values 15 days after bleomycin administration (Table 3, Figure 4).

Histopathological analysis

In histological examination of lung tissues, no fibrotic changes were observed in control groups.

Table 4. Grading of lung fibrosis

	Control	Early bleomycin+saline	Early bleomycin+montelukast	Late bleomycin+saline	Late bleomycin+montelukast
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Fibrosis score	0.0	1.9±0.9*†	1.4±0.5**‡	5.6±1.1*	4.3±0.5*§

* p<0.001, ** p<0.01 vs control group; § p<0.01 vs B (Bleomycin) + saline group in same term; † p<0.001 vs late B (Bleomycin) + saline group; ‡ p<0.001 vs late B (bleomycin) + M (Montelukast) group; Data are means ± standard deviations from 8 rats for each group.

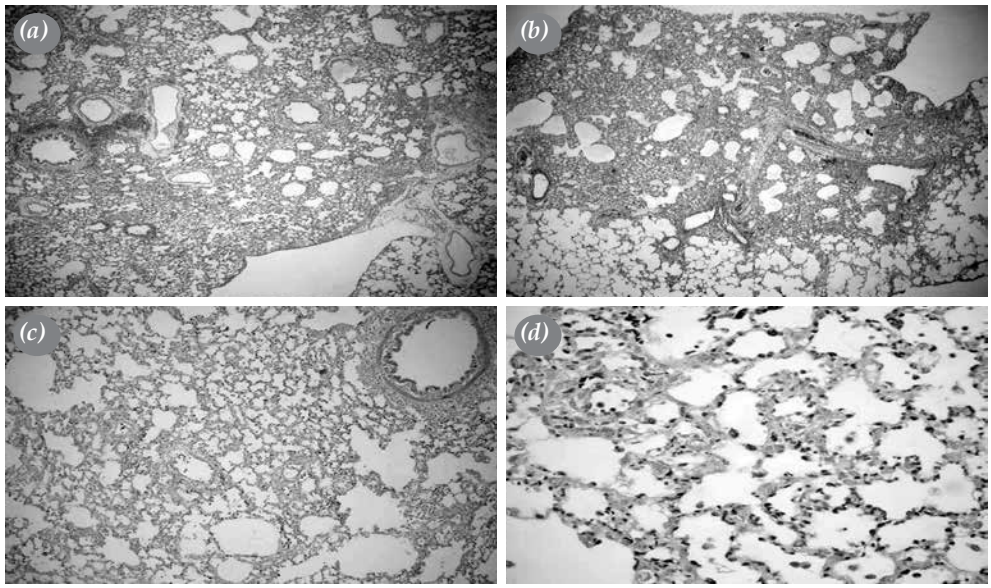

Figure 5. In early bleomycin + saline group. **(a)** Peribronchiolar and interstitial lymphocytic infiltration (H-E×200). **(b)** Focal interstitial inflammation and fibrosis. Fibrosis score: 3 (×200 Masson's trichrome). In early bleomycin + montelukast group: **(c)** Minimal interstitial collagen accumulation. Fibrosis score: 1 (×200 Masson's trichrome). **(d)** ×400 Masson's trichrome.

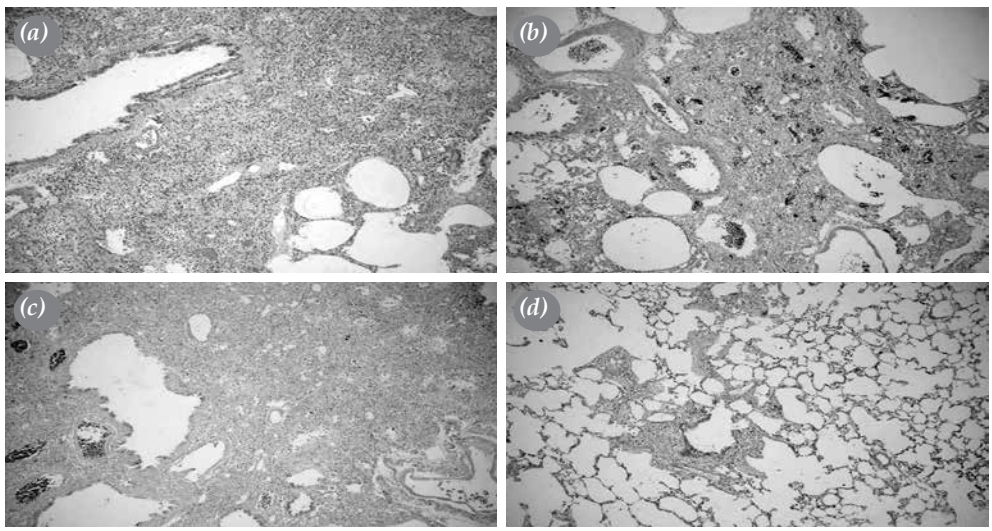

Figure 6. In late bleomycin+saline group: **(a)** Peribronchiolar dense inflammatory cellular infiltration (H-E×400). **(b)** Distortion of lung architecture and severe fibrosis. Fibrosis score: 6 (×400 Masson's trichrome). **(c)** Peribronchial and interstitial dense fibrosis. Fibrosis score: 7 (×200 Masson's trichrome) In late bleomycin+montelukast group: **(d)** Focal interstitial fibrosis with no architectural distortion. Fibrosis score: 4 (×100 Masson's trichrome).

The bleomycin administration produced an almost two- and five-fold increases in fibrosis score in early and late B+saline groups as compared to control group, respectively ($p<0.001$, $p<0.001$, respectively). Montelukast treatment significantly attenuated the fibrotic response in late B+M groups ($p<0.01$), while failed to significantly decrease in early B+M group (Table 4, Figures 5 and 6).

DISCUSSION

In the present study, we showed that a cys LT1 receptor antagonist, montelukast, attenuated inflammatory

cellular inflammation, profibrotic cytokine production, oxidative injury, and fibrotic response in a bleomycin-induced lung fibrosis model.

Development of pulmonary fibrosis is closely related to inflammatory cellular infiltration in lung parenchyma in human and animal models. Intratracheally instilled antitumor agent bleomycin is the most commonly used agent in animal models for pulmonary fibrosis. Giri et al.[18] showed that the total cell counts in the BALF of bleomycin treated hamsters, as compared with controls, were increased 4.4 and 1.6-fold at fourth and 14th days after treatment, respectively. The predominant cell types in the BALF of control animals were macrophages, while PMNs are predominant cell types in bleomycin treated animals.[26] Previous studies have supported that cys LTs upregulate endothelial cell expression of adhesion molecules which are necessary for leukocyte migration into tissues.[27] In accordance with this evidence, in our study, intratracheal bleomycin caused significant increases in BALF total cell count and neutrophil percentage in acute and chronic phase of pulmonary fibrosis. Furthermore, montelukast treatment attenuated inflammatory cells and neutrophil accumulation in bronchoalveolar space in early- and late-term in the present study. Similarly, a novel dual antagonist of the cyst LT and thromboxane A2 receptors significantly decreased cell numbers in BALF on a bleomycin-induced pulmonary fibrosis model in mice on day seven and 21.[28] Consistent with lung tissue MPO activity, in which an enzyme is predominantly found in the azurophilic granules of polymorphonuclear leukocytes, increased following bleomycin administration, this increase was effectively reversed by montelukast, concomitantly with BALF neutrophil accumulation. Similarly, Izumo et al.[29] reported decreased number of inflammatory cells in BALF of montelukast treated mice on seventh day. More recently, Failla et al.[30] demonstrated the first evidence that inhibition of LT activity by using zileuton or sodium salt (MK-571) attenuates bleomycin-induced neutrophil infiltration as evaluated by MPO activity assay and lung fibrosis.

It has been shown that TNF-α and TGF-β1 are the most important factors in mediating pulmonary fibrosis. TNF-α orchestrates the cytokine networking which implicates IPF pathogenesis and amplifies the inflammatory response and drives the progression to fibrosis. In animal models, it has been revealed that over expression of TNF-α in the lung induces inflammation, fibrosis, and secretion of TGF-β1, and TNF-α knockout mice fail to develop fibrosis in spite of treatment with a fibrotic agent.[31,32] TNF-α and TGF-β1 gene expressions were demonstrated to have increased in bleomycin-induced lung fibrosis rat models and also in human IPF studies.[33,34] Furthermore, it has been reported that over expression of TGF-β1 results in prolonged and severe lung fibrosis in animal models, which, in turn, is inhibited by the blockade of this cytokine with soluble receptors.[35,36] Shimbori et al.[37] have shown that montelukast exhibited its beneficial effect against bleomycin-induced pulmonary fibrosis in mice by inhibiting overexpression of TGF-β1. In our study, we confirmed that bleomycin administration increased TNF-α and TGF-β1 in BALF both in early- and late-terms of the process. Montelukast treatment reduced these proinflammatory cytokines levels. This data have been supported by Failla et al.[30] since lung tissue TNF-α level was reduced by cys LT1 receptor antagonist.

Reactive oxygen radicals have been claimed to be a major cause of tissue damage in IPF that result in lipid, protein and DNA injuries.[38] Several studies have shown elevated levels of MDA as an index of lipid peroxidation and reduced GSH levels as a major antioxidant in the BALF and lung tissue of bleomycin exposed rats.[15,16] It has been reported that cys LTs facilitate inflammatory cellular infiltration, which is an important source of oxidant radicals and stimulate the profibrotic cytokine production from these cells.[26,39] The efficacy of cys LT1 receptor blockage in bleomycin-induced oxidative damage in lung tissue, particularly the mechanism of antioxidant activity of montelukast, has not been well-defined. Sener et al.[19] demonstrated that montelukast attenuated burn-induced oxidative injury of the skin and remote organs and reduced MDA and MPO with increased GSH levels in lung, liver, kidney and skin tissues. Similarly, Otunctemur et al.[40] showed decreased tubular necrosis and fibrosis in montelukast received and ureteral obstructed rats. In our study, elevation of MDA and depletion of GSH contents following bleomycin administration were restored by montelukast in early- and late-term.

It has been previously shown that bleomycin-induced fibroblast proliferation and extracellular matrix synthesis are initiated four-14 days after challenge and collagen content elevated approximately two-fold three weeks following challenge.[41,42] These fibroblasts are activated directly and indirectly by bleomycin-induced cytokines such as fibroblast growth factor, PDGF, TNF-α.[42-44] Tumor necrosis factor-alpha is one of the central mediators in the process of collagen production by fibroblasts, as has been shown in animal models.[45,46] Recently, a study confirmed that administration of montelukast results in decreased amount of lung

rejection in lung transplanted rats, related with the anti-inflammatory effects of the drug.[47] In our study, collagen content started to increase in the acute phase, and was significantly elevated 15 days after bleomycin administration. Similarly, in animals treated with montelukast, collagen contents were effectively reduced back to control levels in late-term, accordingly with the studies of Izumo et al.[19] and Failla et al.[30] In accordance with these results, Shaker and Sourour[48] showed that montelukast was therapeutically effective for inhibiting further progression of lung fibrosis through inhibition of alpha-smooth muscle actin positive myofibroblasts while prednisone failed to ameliorate lung fibrosis.

The main limitation of our study is that the optimal time for detecting early fibrosis is not clear. In this study, we accepted 15 days for early fibrosis period. Furthermore, new effective antifibrotic agents were not included in the study because of timing and effects of montelukast versus new agents were not evaluated.

In conclusion, our data demonstrated that montelukast, which is commonly used to treat asthma and allergic rhinitis with anti-inflammatory properties, was able to prevent acute lung inflammation and subsequent development of fibrotic changes related to bleomycin administration in an animal model. Possible explanations for such protective effects of montelukast are inhibition of cellular infiltration, reduction of proinflammatory cytokines production from inflammatory cells, prevention of bleomycin-generated oxygen radicals and protection of lung tissue antioxidant capacity and inhibition of fibroblast proliferation and collagen synthesis. New generation leukotriene receptor antagonists and inhibitors of leukotriene biosynthesis that target 5 lipooxygenase, (5-LO), 5-LO activating protein, leukotriene A4 (LTA4) hydrolase, and leukotriene C4 (LTC4) synthase shall be developed in the future. Thus, the inhibition of cysteinyl leukotriene activity might provide a novel therapeutic approach for idiopathic pulmonary fibrosis.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The authors received no financial support for the research and/or authorship of this article.

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