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Original Article

The anti-inflammatory and anti-remodeling effect of tiotropium bromide in the subacute cigarette exposure mouse model

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Abstract: Chronic obstructive pulmonary disease is an inflammatory lung disease mainly caused by cigarette smoke inhalation. We aimed to evaluate the effect of tiotropium bromide, which is an anticholinergic bronchodilator, on inflammation and remodeling in the subacute cigarette exposure mice model. Thirty-five healthy 25-40 g male balb-c mice were categorized into 3 groups: control group (n=7), those exposed to cigarette smoke (n=18), and those exposed to cigarette smoke and treated with tiotropium bromide (n=10). After 5 weeks, tiotropium or saline was administered to mice for a period of two weeks by inhalation. The mice were anesthetized, bronchoalveolar lavage was performed and lung tissues removed. Interleukin-1β (IL-1β), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor α (TNF-α) measurements were made in BAL fluid. The lung tissues were fixated and sections were stained for morphological evaluation of the lung tissues. The presence of cells undergoing apoptosis and the macrophages were determined by immunohistochemical detection of caspase-3 and MAC387. We found that cigarette exposure significantly increased the IL-1β, TNF-α and MIP-1α levels. Furthermore, tiotropium significantly improved the IL-1β, TNF-α and MIP-1α levels ($P<0.05$). The smoke exposure group exhibited an increased thickness of the alveolar wall, pulmonary edema and hemorrhage, as well as infiltration of the inflammatory cells into the alveolar spaces. In the Thio group the interstitial fibrosis and inflammation were decreased compared to the smoke group. The numbers of MAC387-labeled cells and the caspase-3-labeled cells were higher in the smoke group than in the other groups ($P<0.0001$). Besides its bronchodilator effect, tiotropium may be a promising therapeutic choice to control inflammation and remodeling due to cigarette exposure.

Keywords: Chronic obstructive pulmonary disease, tiotropium bromide, smoking, bronchodilator agents, airway remodelling, inflammation

Introduction

Chronic obstructive pulmonary disease (COPD) is a disease characterized by progressive airway obstruction accompanied by increased chronic inflammation in the airways and pulmonary parenchyme [1]. An inflammatory response to harmful particles or gases is observed. Cigarette smoke-related airway inflammation and remodeling, in particular, widening of the alveolar spaces and remodeling in the small airways are the causes of airway obstruction [2, 3]. Inflammation in the airways is known to develop before appearance of the typical pathological findings [4]. Oxidative stress observed as a result of pulmonary infiltration by neutrophils and macrophages contributes to the irreversible damage in the parenchyme and the airways, and molecular mechanisms start the pulmonary and systemic inflammations [5].

The effects of acetylcholine, a parasympathetic neurotransmitter, on the bronchomotor tonus and mucus secretion in submucosal glands within the respiratory tract are well known [6]. There are data present suggesting that acetylcholine regulates various functions about the inflammation and remodeling within the respiratory tract in pulmonary diseases [7, 8]. Furthermore, acetylcholine is known to be syn-

Table 1. Smoke exposure test paramaters

Number of cigarettes	2
Puff time per minute for each cigarette	1
Puff drawing duration	2 seconds
Mainstream flow rate	1, 1 lt/min
Mainstream (puff) volume	37 cm^3
Sidestream flow rate	4 lt/min
Clean air flow rate	8 lt/min

thesized particularly from the inflammatory cells and the airway epithelium in the neurons [9, 10].

Tiotropium bromide is a long-acting muscarinic antagonist (LAMA), which has been used in the treatment of COPD. Tiotropium prevents the binding of acetylcholine to muscarinic receptors. Its muscarinic M3 receptor binding half-life is longer, which induces bronchoconstruction [11]. The effects of tiotropiumun on pulmonary functions, COPD exacerbations and the quality of life in patients with COPD have been demonstrated in many clinical studies [12, 13]. Although various experimental studies have recently been conducted on the effects of tiotropiumun on pulmonary inflammation and remodeling, these effects are yet unclear.

We aimed to evaluate the anti-inflammatory and anti-remodeling effects of tiotropium bromide, which is a long-acting muscarinic antagonist in the subacute cigarette exposure model.

Material and methods

Animal groups and treatments

Thirty-five healthy 25-40 gram male balb-c mice were purchased from the Experimental Animal Centre of Uludag University Medical Faculty. The present study was approved by the Ethics Committee of The Kocaeli University Committee on the Use and Care of Animals approved the experiments (Ethical Committee no: KOÜ HADYEK 4/4-2015). All of the investigations conformed to the 1996 National Academy of Science's Guide for the Care and Use of Laboratory Animals.

The mice were randomized into three groups. Control group (C) (n=7), the group exposed to cigarette smoke (CS) (n=18), and the group exposed to CS treated with tiotropium (Tio) (n=10). The mice were bred in SPF conditions and were maintained at a constant temperature and humidity, with *ad libitum* access to food and water.

Cigarette smoke exposure

In the CS and tiotropium groups, the mice were exposed to cigarette smoke for 2 h twice daily, 5 days per week for 5 weeks by using whole-body Smoke Exposure System. Our whole-body Smoke Exposure System is similar to the system designed and developed by the GMT R&D company. The mice were exposed to CS using available filtered cigarettes (12 mg tar/1.0 mg nicotine, Philip Morris, Richmond, VA). The system basically included a cigarette smoke machine, mixing and dilution areas and two exposure areas. All areas were constructed using Plexiglas. The smoke exposure test conditions conducted in this study have been presented in **Table 1**.

Drug administration

After the 5th week, tiotropium was applied to the tiotropium group, and saline was administered to the control group as well as CS group once daily for 2 weeks. For inhalation, the mice were placed into an acrylic box (15×21×15 cm) with 5-8 animals in one box, and tiotropium bromide diluted in sterile saline (5 micrograms/kg/day; Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) or sterile saline only were administered via ultrasonic nebulizer. In order to provide the air flow and exhaust of the aerosols, two exhaust holes of 1 cm were opened on one side of the box [14].

Collection of bronchoalveolar lavage fluid (BALF) and preparation of the lung tissue

For BALF analysis, the mice were anesthetized with pentobarbital (50 mg/kg^{-1}, intraperitoneally), and, after having performed a tracheotomy, a custom-built cannula was inserted into the trachea. The lungs were lavaged three times with 0.5 ml of PBS. Following the lavage procedure, the lungs were removed for histopathological evaluation.

ELISA for Inflammatory cytokines

The BALF was centrifuged at 3000 rpm for 20 min, and the supernatant was collected and stored at -40°C for subsequent measurement of IL-1β, MIP-1α, TNF-α.

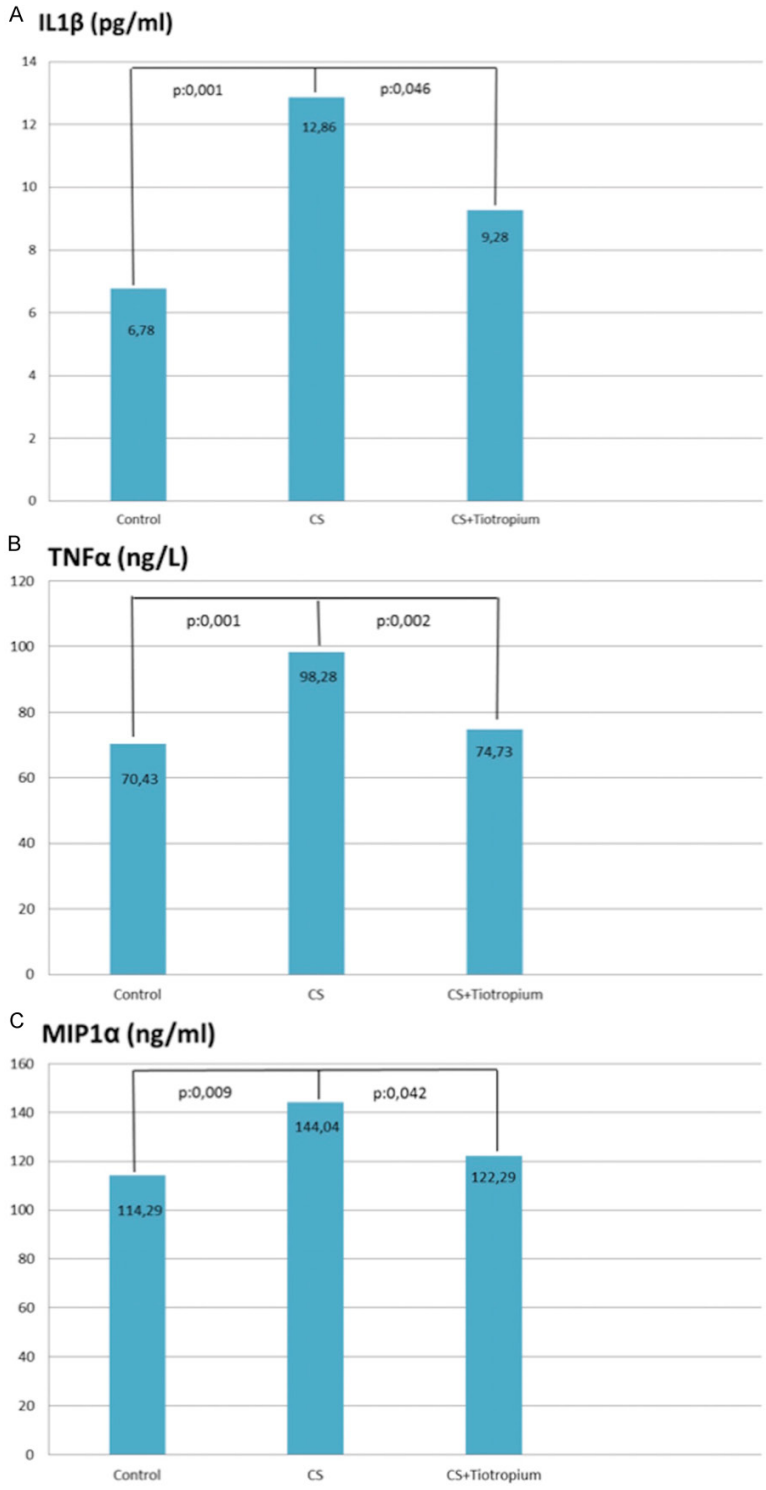

Figure 1. IL1β in the BALF (P=0.002) (A), TNFα levels in the BALF (P<0.001) (B), and MIP1α in the BALF (P=0.007) (C).

The IL-1β, MIP-1α, and the TNF-α levels were analyzed with the ELISA kits using the Alisei Quality System Seac Radin Company analyser (Sunred, Shanghai).

Immunohistochemical and Masson trichrome stainings

The lungs of all experimental groups were fixed with buffered in 4% paraformaldehyde. Following fixation, the lung tissues were embedded in paraffin and sectioned at 5 μm. The paraffin sections were processed with caspase-3 (Santa Cruz Biotechnology, Inc., sc-98785), Macrophage Marker Antibody (MAC387, Santa Cruz Biotechnology, Inc., sc-66204), immunostainings and Masson trichrome staining as described below.

Paraffin sections were immunostained with ImmunoCruz™ rabbit LSAB Staining System (sc-2051) or ImmunoCruz™ mouse ABC Staining System (sc-2017) for the caspase-3 (1:200 dilution) or MAC387 (1:200 dilution) antibodies. The paraffin-embedded tissue slices were deparaffinized with xylene. The endogenous peroxidase activity was stopped by incubation in 0.3% hydrogen peroxide in methanol. The tissue slices were hydrated with graded alcohol, treated with 10% normal serum, and then incubated with the primer antibody at 4°C overnight. They were then incubated with the biotinylated anti-rabbit IgG or anti-mouse IgG for 30 min at room temperature, and then with avidin D-horseradish peroxidase or avidin and biotinylated horseradish peroxidase in 10% normal goat serum for 30 min at room temperature. The slices were then visualized using 3,3'-Diaminobenzidine (DAB) or 3-amino-4-ethylcarbazole (AEC) as chromogen. The negative controls comprised tissue sections incubated without primary antibody. Finally, the sections were mounted for counting [15, 16].

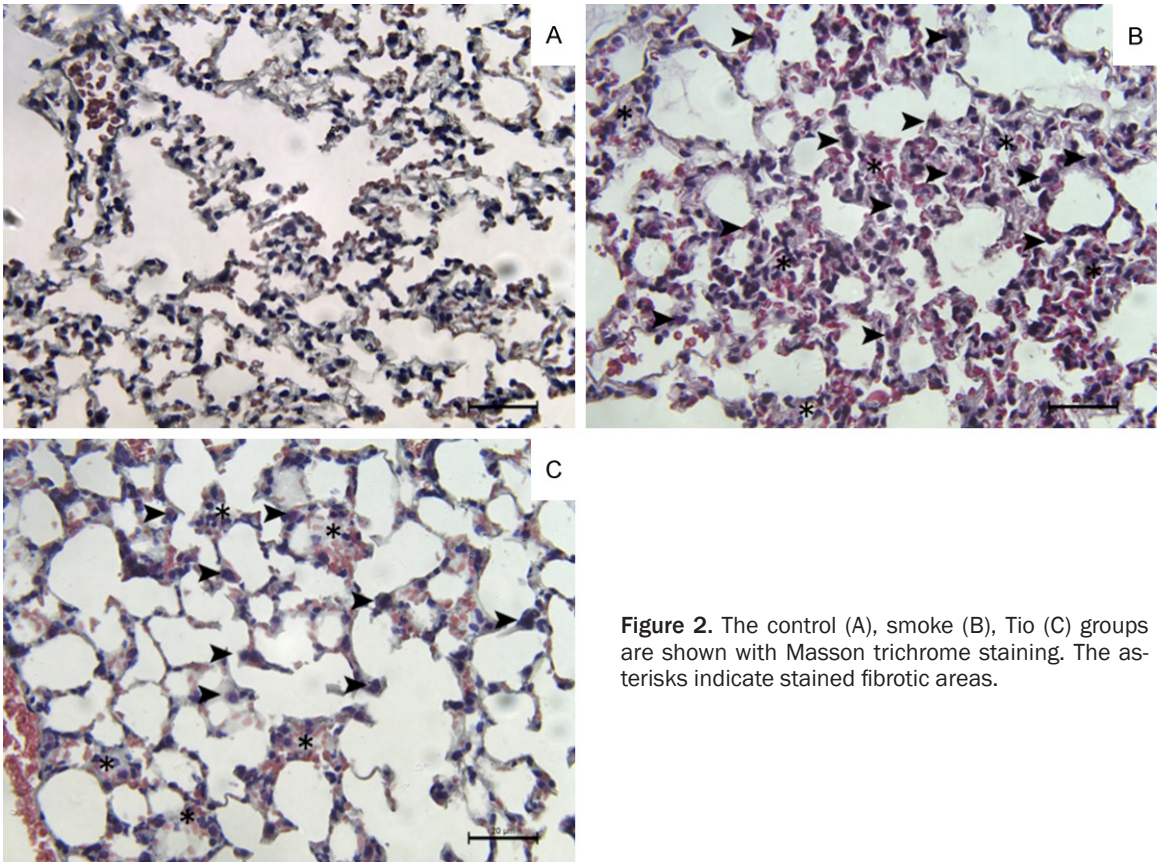

Figure 2. The control (A), smoke (B), Tio (C) groups are shown with Masson trichrome staining. The asterisks indicate stained fibrotic areas.

The sections were stained with Masson's trichrome and Hematoxylin and Eosin (HE) for morphological evaluation of the lung tissues. The sections were processed for conventional histology using the Masson's Trichrome stain. Finally, images of the stained sections were captured with a Leica DFC295 HD color digital camera mounted on a Leica DM2500 microscope and stored as Tagged Image File Format images. The images were processed with the Image analysis program to the area measurements.

Counting of caspase-3 and MAC387 labelled cells

The presence of cells undergoing apoptosis and the macrophages were determined by immunohistochemical detection of caspase-3 and MAC387. We randomly selected four fields from the three sections through the lung tissue for each mouse. Caspase-3 and MAC387-labeled cells were counted.

Statistical analysis

Statistical analysis was performed using the computer software program SPSS for Windows (Version 17.0, SPSS Inc., USA). The data of this study were expressed as a mean ± standard deviation (SD). The differences among multiple groups were analyzed using the one-way ANOVA followed by post hoc Tukey test calculations for the inter-group comparisons. Significance was defined as a *p*-value of 0.05 (two-tailed).

Results

The IL1β within the BAL fluid was determined to be 6.78±3.88 in the control group, 12.86±0.83 in the smoke exposure group, and 9.28±3.49 in the tiotropium group (P=0.02). The MIP within the BAL fluid was observed to be 114.29±14.18 in the control group, 144.04±20.03 in the smoke exposure group, and 125.46±19.024 in the tiotropium group (P=0.012). TNFα within the BAL fluid was determined to be 70.43±6.36 in the control group, 98.28±5.15 in the smoke exposure group and 74.73±21.20 in the tiotropium group (P<0.0001). When the IL1β, MIP1α and the TNFα levels within the BAL fluid were evaluated, increased cytokine levels were observed in smoke groups, whereas improvement was observed in tiotropium groups (**Figure 1**).

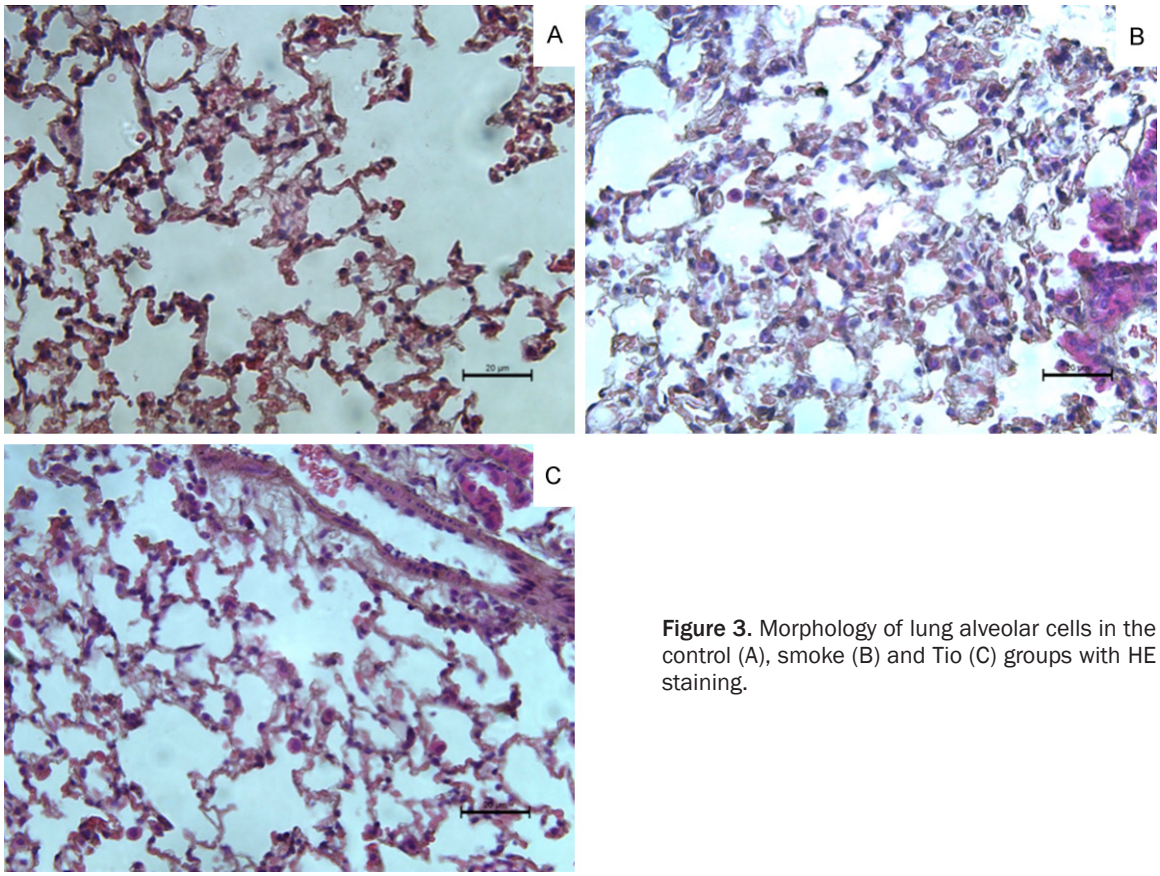

Figure 3. Morphology of lung alveolar cells in the control (A), smoke (B) and Tio (C) groups with HE staining.

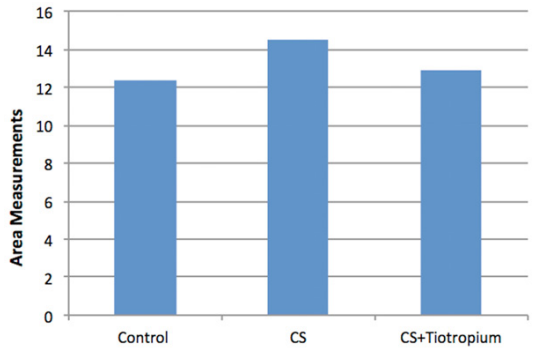

Figure 4. Area measurements using ImageJ with Masson's trichrome staining.

We performed Masson trichrome staining for alveolar architecture and collagen to understand the development of fibrosis. The control group demonstrated normal alveolar architecture with the Masson trichrome staining (**Figures 2A**, **3A**).

The smoke exposure group exhibited an increased thickness of the alveolar wall, pulmonary edema and hemorrhage, as well as infiltration of inflammatory cells into the alveolar spaces (**Figures 2B**, **3B**). The alveolar spaces were filled with large, vacuolated and foamy macrophages. The macrophages in the alveolar spaces were larger than normal macrophages and described as hypertrophic and proliferative macrophages. The smoke exposure group exhibited a significant increase in the interstitial fibrosis and inflammation. In addition, many multi-nucleated giant cells were identified within the alveolar spaces. In the Thio group, the interstitial fibrosis and inflammation were decreased compared to the smoke group (**Figures 2C**, **3C**, **4**).

Caspase-3 labeling was undetectable in the control group (**Figure 5A**). The number of caspase-3-labeled cells was higher in the smoke exposure group than in the other groups (**Figure 5B**, $P<0.0001$). There was a significant decrease in the number of caspase-3 labeled cells in the tiotropium group compared to smoke group (**Figures 5C**, **6**).

The MAC387-labeled cells were greater in the number of the smoke group than in the control group (**Figure 7B**, $P<0.0001$). There was a sig-

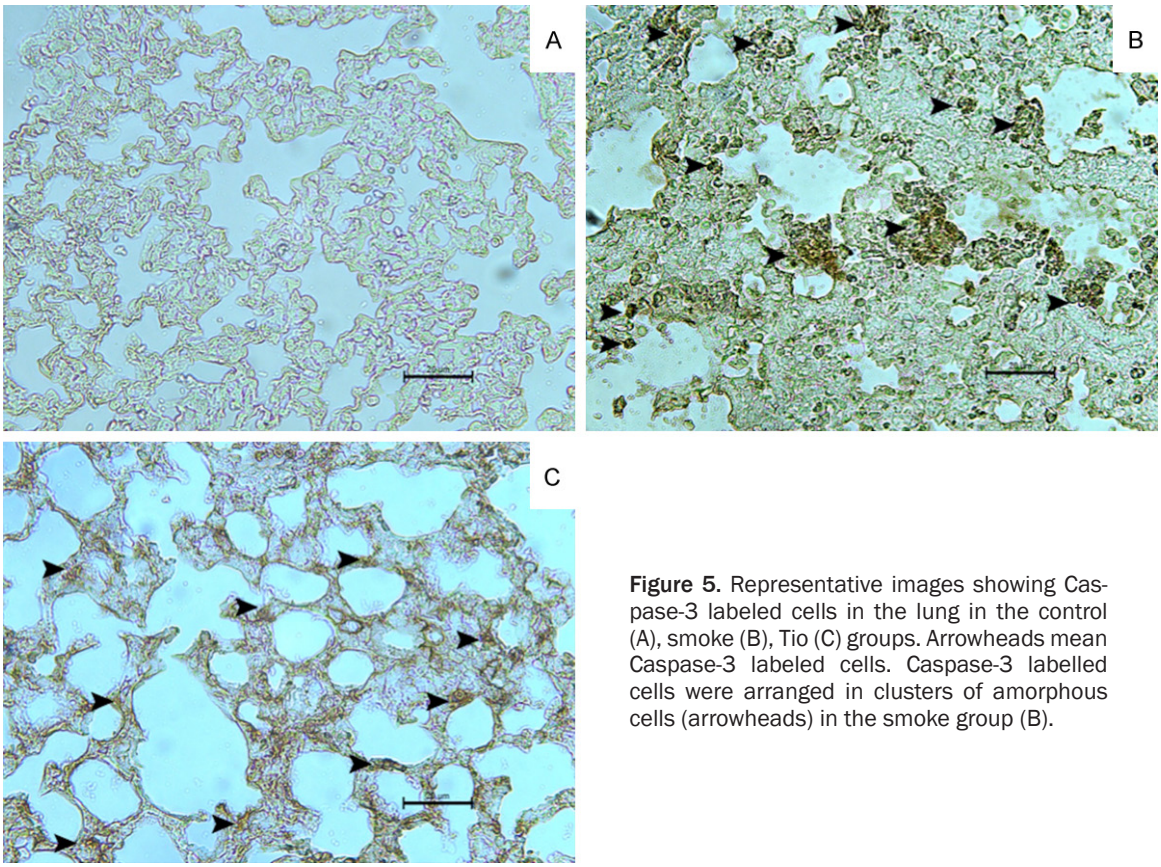

Figure 5. Representative images showing Caspase-3 labeled cells in the lung in the control (A), smoke (B), Tio (C) groups. Arrowheads mean Caspase-3 labeled cells. Caspase-3 labelled cells were arranged in clusters of amorphous cells (arrowheads) in the smoke group (B).

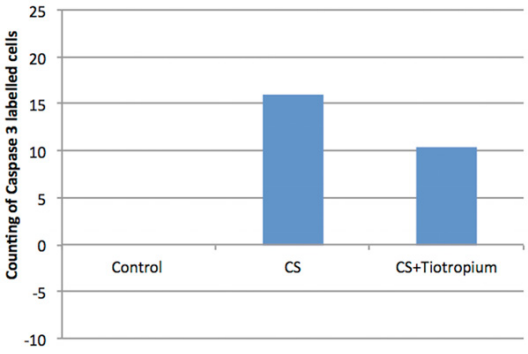

Figure 6. The Caspase-3 labeled cell counts of groups in the lung for apoptotic cells.

nificant decrease in the number of MAC387-labeled cells in the tiotropium group compared to smoke group (**Figures 7C**, **8**).

Discussion

In our study, tiotropiumun-related improvement was observed in the levels of inflammatory cytokines IL-1β, MIP-1α, TNF-α in the BALF, which were increased due to smoking, and a decrease in the alveolar MAC387-labeled macrophage count, apoptosis, alveolar Wall thickness and interstitial fibrosis were observed.

COPD has a chronic inflammatoy course. Chronic airway obstruction is believed to develop as a result of inflammatory responses leading to the remodeling and stenosis of the airways in response to various particles and gases and primarily, cigarette smoke. Various studies have been conducted on the role of inflammatory cell types and cytokines within the airways, and these have been demonstrated to be related to the development of COPD [17-19]. There are data suggesting the contribution of monocyte chemo-attractant protein (MCP-1), matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, mediators secreted from these epithelial cells and the macrophage inflammatory protein (MIP)-1α COPD development [20-22].

TNFα has an important role in the pathophysiology of COPD [23]. TNFα is produced primarily in the alveolar macrophages and many other cells including mast cells, epithelial cells and T

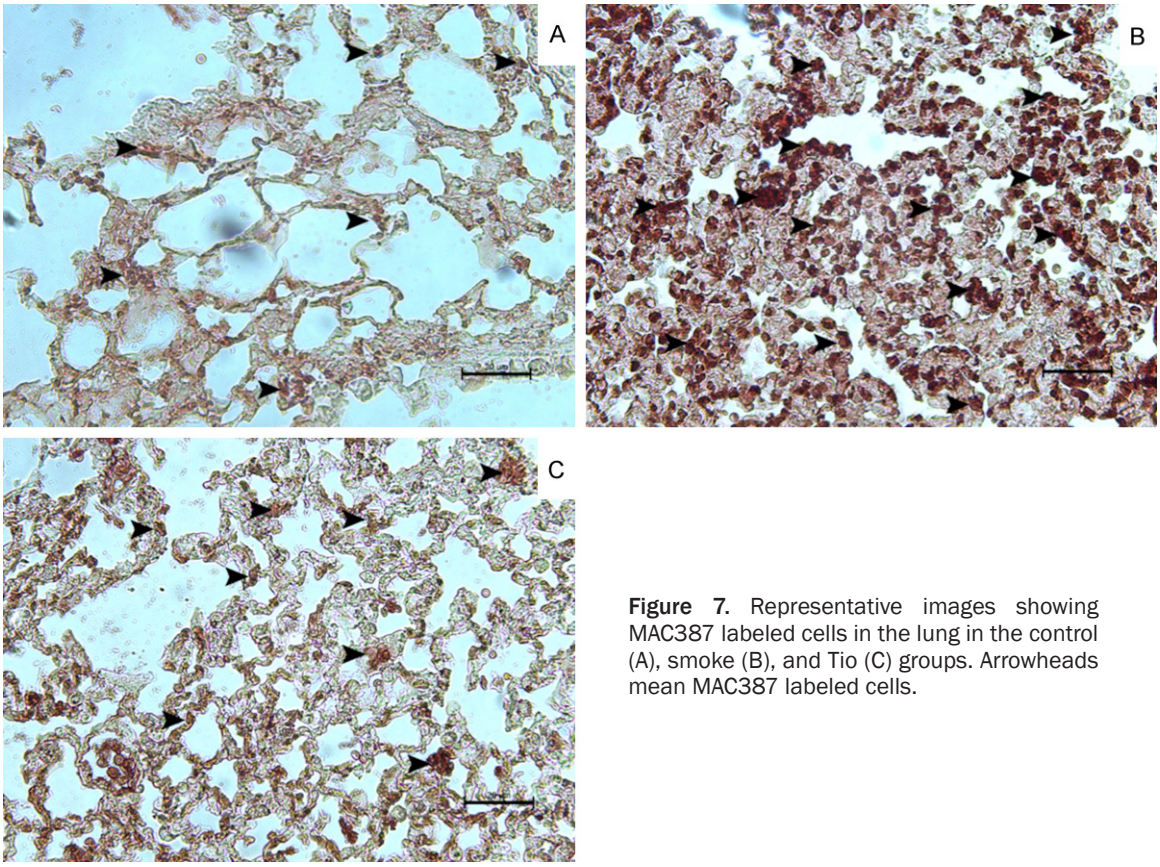

Figure 7. Representative images showing MAC387 labeled cells in the lung in the control (A), smoke (B), and Tio (C) groups. Arrowheads mean MAC387 labeled cells.

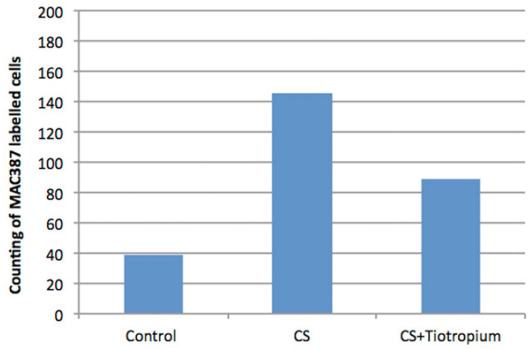

Figure 8. The MAC387 labeled cell counts of groups in the lung for macrophage cells.

cells. TNFα induces IL8 expression and up-regulation of endothelial adhesion molecules, and provides neutrophil chemotaxis and migration. Additionally, CCL13 expression contributes to the chemotactic activities of eosinophils, monocytes, T lymphocytes, and basophils [24, 25]. TNFα also activates the epithelial and smooth-muscle cells [25, 26]. Increased TNFα levels have been determined in the sputum and blood samples of patients with COPD [27, 28].

IL-1α and β are proinflammatory cytokines that are produced within monocytes, macrophages and fibroblasts, which bind to the IL-1 receptor. IL1β is believed to have a role in smoke-induced emphysema and airway remodelling. It is known to be increased in the serum, sputum and BALF samples of patients with stable COPD [29, 30].

Macrophage inflammatory protein 1α (MIP-1α) induces monocyte and macrophage chemotaxis and t lymphocyte subset [31]. MIP-1α mRNA expression has been shown to be increased in the pulmonary tissues and bronchial epithelium of smoker patients with emphysema [32].

In our study, TNF, IL1β and MIP-1α levels were observed to be increased by the smoke exposion compared to the control group. The levels of these cytokines were regressed with tiotropium application.

Vagal tone is increased in COPD. Various studies have shown that acetylcholine and the enzyme synthesizing it, choline-acetyl transferase (ChAT), have been dispersed in all of the

airway epithelium and throughout the airways including inflammatory cells as well [33]. Muscarinic receptors have been known to be located in various cell types including epithelial and inflammatory cells. Macrophages secrete M3 receptor more frequently than M1 and M2 in COPD, and the M1 and M3 receptors within the neutrophils tend to increase as well [34]. The acetylcholine-mediated autocrine and paracrine post-junctional target cell stimulation of muscarinic receptors leads to various inflammatory processes and results in remodeling. Through activation of the muscarinic receptor, acetylcholine stimulates the proliferation of the smooth muscle cells fibroblasts within the lungs and collagen synthesis in the fibroblasts [35, 36].

Tiotropium is a synthetic muscarinic receptor inhibitor and resmbles ipratropium structurally; however, it is cleaved at a slower rate and has higher receptor affinity [37, 38]. Thus, bindings to M3 and M1 receptors are provided with a single dose and prolonged improvement in clinical respiratory parameters is provided [39, 40]. In addition to the bronchodilatator effects of tiotropium, the inflammation reducing effect oftiotropium has begun to be investigated in experimental studies.

In the allergic asthma model formed in Guinea pigs, tiotropiumun has been shown to inhibit airway smooth cell hypertrophy, mucus gland hypertrophy, goblet cell hyperplasia and inflammation as well [41]. In the study of Cui et al., a chronic gastro-oesophageal reflux disease (GORD) model was formed by causing pulmonary infection via HCL administration, and the data obtained indicated that muscarinic receptor blockage via atropine or tiotropium prevented the airway inflammation and remodeling similar to dexamethasone. IL8 and ICAM1 levels were shown to have increased, and epitelial and smooth muscle thicknesses were demonstrated to be decreased with tiotropium application. No reduction was observed in the total cell count or macrophages, lymphocyte and neutrophil counts [42]. In another study on Guinea pigs, parenchymal neutophilia and goblet cell count were observed to have decreased, and a positive effect was observed in the muscularized vessels within the adventitia layer or the cartilaginous airways, and no effect was observed on emphysema in the LPS-induced COPD model. Hydroxyproline and airway wall collagen deposition formed by repetitive lipopolysaccharide (LPS) application was observed to regress with thitropium treatment [43]. In another experimental study, goblet cell metaplasia was observed to regress with tiotropium, and the total cell count and neutrophil count within BALF and LTB4 level were observed to decrease in a NE-induced COPD model. Although the macrophage count was decreased, the decrease was not statistically significant [14]. In the study of Ohta et al., improvement was observed in goblet cell metaplasia, thickness of airway smooth muscle and airway fibrosis, and a decrease was observed in the increased TGF b1 level in BALF with tiotropium in the asthma mice model [44]. In the study of Wollin et al., tiotropium treatment with 4-day-exposure to cigarette smoke caused a decrease in BAL leukotriene B4, interleukin-6, keratinocyte derived chemokine, monocyte chemotactic protein 1 (MCP1), macrophage inflammatory protein-1alpha and 2, and TNF alpha levels compared to the control group [45]. Kolahian et al. administered tiotropium treatment and 4-day heavy cigarette smoke exposure in cats, and observed improvement in BAL IL6, 8, MCP1 and TNFα. Furthermore, improvement was observed in the decreased total antioxidant level in the BALF, serum and pulmonary tissue due to cigarette smoke exposure with tiotropium treatment [46]. In a recent study, the mice were exposed to cigarette smoke and infected with viruses, and observed a significant reduction in IL6, IFNγ, TNFα and KC levels and the neutrophil count in BALF with tiotropium treatment [47].

Tiotropium, which has been demonstrated to have various positive effects on pulmonary inflammation and remodeling evaluated by different GORD related views in smoke exposure, COPD or asthma models, was observed to decrease inflammation and to have positive effects on remodeling in the subacute cigarette smoke exposure model in our study.

In our study, the increased inflammatory cytokines IL1β, MIP1α and TNFα in BALF due to smoke exposure were observed to recover with tiotropium treatment. Moreover, the increased alveolar wall thickness, collagen deposits, inflammatory cell infiltration and increased macrophage infiltration in the smoke exposure group were observed to decrease with tiotropium treatment. Increased cell apoptosis with

smoke exposure was also regressed with tiotropium.

One of the limited number of human studies evaluating the anti-inflammatory effect of thiortopium was the randomized, placebo-controlled study of Powrie et al. which investigated the effect of thotropium on the presence of inflammatory markers in sputum and blood, and on the frequency of the attacks. Although a reduced attack frequency was observed with tiotropium treatment, no improvement was observed in BAL IL6, MP0, IL8 or serum IL6 and CRP levels [48]. In another study, the effects of tiotropium and salmeterol fluticasone propionate (SFP) on IL8 and MMP9 were compared in induced sputum, and a significant cytokine level reducting effect of SFP was observed; however, no effect was observed on the total cell count or blood CRP [49]. In these studies, inflammation was evaluated using limited numbers of markers.

Nevertheless, in various studies evaluating the anti-inflammatory effects of tiotropium in human, it was observed that these studies were based on the cytokines within the sputum. Although there is no studiy evaluating the effect of tiotropium on mucus secretion, oxitropium-induced cholinergic stimulus inhibition was observed to reduce the mucus production [50]. Thus, the concentrations of the cytokines within the mucus may be observed to be increased. We believe that BALF examination and using many cytokines and markers would be useful in evaluating the pulmonary inflammation in human studies.

In conclusion, tiotropiumun was observed to regulate the smoke exposure related increase in the levels of IL-1β, MIP-1α and TNF-α in the BALF, and to reduce the alveolar macrophage count, apoptosis, alveolar thickness and interstitial fibrotic process in our study. Tiotropium, having a known bronchodilatator effect, is believed to effective with its anti-inflammatory and anti-remodeling effects. Further studies investigating the effects of tiotropium on inflammation and remodeling on human should also be carried out.

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Disclosure of conflict of interest

None.

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