

Azithromycin Attenuates Pulmonary Inflammation and Emphysema in Smoking-Induced COPD Model in Rats

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INTRODUCTION: The role of inflammation and immunity in COPD treatment is increasingly being recognized. The relationship between anti-inflammation/immunoregulation and emphysema in COPD lungs remains to be elucidated. The aim of this study was to investigate the effects of azithromycin (Azm) on the development of emphysema in smoking-induced COPD in rats. **METHODS:** Sprague-Dawley rats ($n = 50$) were randomly assigned to normal, COPD, saline-treated, Azm-treated, and levofloxacin-treated (Lev) groups. The effects of treatment were assessed by measuring the levels of vascular endothelial growth factor (VEGF) by enzyme-linked immunosorbent assay and measuring the numbers of neutrophil and macrophage in bronchoalveolar lavage fluid, vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR2) protein expression by western blotting. Lung function measurements and histopathological evaluations (mean linear intercept and destructive index) were performed. **RESULTS:** FEV_{0.3}/FVC and peak expiratory flow were lower in the COPD group than in the normal group. Mean linear intercept and destructive index were lower in the Azm-treated group than in the COPD, saline-treated, and Lev-treated groups. The numbers of neutrophil and macrophage in bronchoalveolar lavage fluid were lower in the Azm-treated group than in the COPD, saline-treated, and Lev-treated groups. As confirmed by western blotting, the levels of VEGF in lung homogenates were higher in the Azm-treated group than in the COPD, saline-treated, and Lev-treated groups. VEGFR2 protein expression was higher in the Azm-treated group than in the COPD, saline-treated, and Lev-treated groups. **CONCLUSIONS:** Azm attenuates pulmonary emphysema by partly reversing the decrease in the numbers of inflammatory cells (neutrophil and macrophage) and VEGF secretion and VEGFR2 protein expression in smoking-induced COPD in rats. *Key words:* chronic obstructive pulmonary disease; emphysema; VEGF; azithromycin; cigarette smoking. [Respir Care 2015;60(1):128–134. © 2015 Daedalus Enterprises]

Introduction

COPD is currently listed as the fourth leading cause of death in the world and affects more than 200 million people worldwide. It is also an important cause of chronic

disability and permanent impairment, representing a major economic and social burden worldwide^{1,2}. Although cigarette smoking is a common etiologic factor, other injurious stimuli also contribute to the deterioration of airway function. Chronic exposure to cigarette smoke (CS) leads to lung inflammation with an increase of inflammatory cells

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such as macrophages,³ neutrophils,^{4,5} dendritic cells,^{6,7} and CD8⁺ T lymphocytes.⁸ Release of elastase from these cells, as well as their production of reactive oxygen species, have long been considered to play important roles in disease development. Degradation of extracellular matrix components within alveolar walls by these proteases leads to the development of emphysema, the main characteristic of COPD.⁹

Macrolide antibiotics have immunomodulatory, anti-inflammatory, and antibacterial effects.¹⁰ Actions observed with macrolides include inhibition of neutrophil accumulation, chemokine and cytokine production, as well as inhibition of adhesion molecule expression. Such effects account, at least partially, for the essential role of macrolide antibacterials in the treatment of patients with diffuse panbronchiolitis, bronchiolitis obliterans syndrome, and bronchiectasis.¹¹⁻¹³ Beneficial clinical effects have also been observed in patients with chronic sinusitis, asthma, and COPD.¹⁴⁻¹⁶ Long-term azithromycin (Azm) therapy was associated with significant reductions in the rate of exacerbations compared with placebo.^{17,18} However, the mechanism of the anti-inflammatory/immunomodulatory actions of macrolides remains unclear. There are few reports on the role of Azm in the treatment of CS-induced COPD in rats. The aim of this study was to investigate whether oral administration of azithromycin has any effect on the development of smoking-induced COPD in rats, in terms of lung function, bronchoalveolar lavage fluid, and morphometry.

Methods

Animals

Fifty male Sprague-Dawley rats, each weighing 192–243 g, were purchased from the Animal Centre of Yangzhou University, Yangzhou, China. The rats were randomly assigned to 5 groups of 10: normal, COPD, saline-treated, Azm-treated, and levofloxacin (Lev)-treated groups. The last 4 groups were exposed to smoke from 20 commercial unfiltered cigarettes (Dafengshou, Jiangsu Cigarette Factory, Huaian, Jiangsu, China) for 1 h each day, 7 d a week, for a total of 90 d. The smoke exposure box was 70 × 50 × 40 cm. Rats in the Azm-treated group were administered intragastrically with Azm (Pfizer, New York, New York; 50 mg/kg, once/day), and rats in the Lev-treated group were lavaged with Lev (Yangzijiang Pharmaceutical, Taizhou, China; 50 mg/kg, once/d), while rats in the saline-treated group were instilled with saline solution after 30 d of smoking exposure. One rat in the COPD group died on d 55. Two rats in the Azm-treated group died on d 36 and 44, and 1 rat in the Lev-treated group died on d 75. The lungs of the 4 dead rats were filled with fluid. Instillation of reagent solutions into the lungs

QUICK LOOK

Current knowledge

COPD is the fourth leading cause of death in the world and an important cause of chronic disability, representing a major economic and social burden worldwide. Chronic inflammation in COPD is increasingly recognized as a major factor in disease burden.

What this paper contributes to our knowledge

In an animal model of smoking-induced COPD, azithromycin attenuated pulmonary emphysema by reversing the decrease in the numbers of inflammatory cells and vascular endothelial growth factor secretion. Vascular endothelial growth factor receptor-2 protein expression was elevated in animals treated with azithromycin.

may have resulted in the deaths of 4 rats. Experiments were approved by the animal ethics committee and were performed under strict government and international guidelines.

Pulmonary Function

Rats from all groups were randomly selected for lung function measurements (PFT, Buxco Research Systems, Wilmington, North Carolina). After induction of anesthesia by intraperitoneal administration of chloral hydrate (3 mL/kg), a Y-type endotracheal cannula was connected to a flow transducer (HX200, Beijing Baianji Corporation, Beijing, China) for measurement of FEV_{0.3}/FVC and peak expiratory flow (PEF).

Preparation of Bronchoalveolar Lavage Fluid and Lung Tissue

Twenty-four h after the last smoke exposure, rats were weighed and sacrificed with an overdose of pentobarbital, and a tracheal cannula was inserted. Three mL of sterile phosphate-buffered saline, free of ionized calcium and magnesium but supplemented with 0.05 mM sodium ethylenediaminetetraacetic acid, was instilled 3 times via the tracheal cannula and recovered by gentle manual aspiration. The 3 lavage fractions were centrifuged (2,000 g, 10 min), and the cell-free supernatants were stored at –70°C for subsequent cytokine analysis. The cell pellet was washed twice and finally resuspended in 1 mL of phosphate-buffered saline. A total cell count was performed in a Bürcker chamber, and the differential cell counts (at least

400 cells) were performed on cytocentrifuged preparations using standard morphologic criteria after Giemsa staining.

The chest was opened, and the cardiopulmonary block was quickly isolated and excised. The right main bronchus was cross-clamped, and the left lung was filled with 0.5% low melting agarose in 10% formalin at a constant pressure of 25 cm H₂O, allowing for homogenous and full expansion of the lung parenchyma.¹⁹ The lungs were then fixed in 10% formalin for 48 h and paraffin-embedded. Tissue sections from upper and lower lobes of the left lung were used for histological analysis.

Vascular Endothelial Growth Factor Determination

Vascular endothelial growth factor (VEGF) levels in bronchoalveolar lavage (BAL) fluid supernatants were measured by sandwich enzyme-linked immunosorbent assay using specific anti-mouse monoclonal antibody for capture and detection (R&D Systems, Minneapolis, Minnesota). Assays were performed according to the manufacturer's protocol.

Western Blotting

Twenty μ g of protein from lung homogenate lysates were electrophoresed and separated on 4–12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, California). The membranes were blocked with 5% skim milk at room temperature for 1 h, and then incubated overnight at 4°C with primary antibodies including mouse VEGF receptor (VEGFR) monoclonal IgG, rabbit polyclonal VEGF IgG, goat anti-rabbit antibody, and goat anti-mouse antibody (all from Santa Cruz Biotechnology, Santa Cruz, California). Membranes were re-probed for β -actin (Santa Cruz Biotechnology) to confirm equal protein loading and transfer. The experiments were performed with at least 5 animals per study group.

Quantification of Emphysema

Emphysema is a structural disorder characterized by destruction of the alveolar walls and enlargement of the alveolar spaces. We determined destruction of alveolar walls by measuring the destructive index (DI)²⁰ and enlargement of alveolar spaces by quantifying the mean linear intercept (Lm)²¹ in air- and CS-exposed rats, as described previously.²²

Quantification of air space enlargement was determined after 90-day air or CS exposure by measuring the Lm using image analysis software (ImageJ 1.47, National Institutes of Health, Bethesda, Maryland). Only sections without cutting artifacts, compression, or hilar structures (airway or blood vessel with a diameter larger than 50 μ m) were used in the analysis. The Lm was measured by plac-

ing a 100 \times 100 μ m grid over each field. The total length of each line of the grid divided by the number of alveolar intercepts gives the average distance between alveolated surfaces, or the Lm.²¹

The destruction of alveolar walls was quantified by the DI.²⁰ A grid with 42 points that were at the center of hairline crosses was superimposed on the lung field. Structures lying under these points were classified as normal (N) or destroyed (D) alveolar and/or duct spaces. Points falling over other structures, such as duct walls and alveolar walls, were not included in the calculations. The DI was calculated from the formula: $DI = D/(D + N) \times 100$.

Statistical Analysis

Continuous data were expressed as mean \pm SD. Differences were evaluated for statistical significance by analysis of variance using the Kruskal-Wallis test. A *P* value $< .05$ was considered statistically significant. All analyses were performed using the statistical software SPSS 16.0 (SPSS, Chicago, Illinois).

Results

Lung Function

Half of the rats from all groups were randomly selected for lung function measurements. The levels of FEV_{0.3}/FVC and PEF were lower in the COPD group compared with the normal and Azm-treated groups (*P* $< .05$). FEV_{0.3}/FVC and PEF were higher in the Azm-treated group compared with the saline- and Lev-treated groups (*P* $< .05$) (Table 1).

Determination of Relative Cell Number in Bronchoalveolar Lavage Fluid

An increase in the numbers of neutrophil and macrophage in airways (BAL fluid) was observed in the COPD group compared with those in the normal group. As expected, the numbers of neutrophil and macrophage in Azm-treated group were significantly decreased compared with those in the COPD, saline-treated, and Lev-treated groups (Table 2).

Vascular Endothelial Growth Factor Determination

The levels of VEGF in BAL fluid were lower in the COPD group (855 \pm 150 pg/mL) than in the normal group (1,568 \pm 276 pg/mL) or the Azm-treated group (1,093 \pm 192 pg/mL) (*P* $< .05$). There were also significant differences of VEGF levels in BAL fluid in the Azm-treated group compared with those of the Lev-treated group

Table 1. Comparison of Lung Function Parameters in the 5 Groups of Rats

	<i>n</i>	FEV _{0.3} /FVC %	PEF mL/s
Normal	5	85.3 ± 5.2	46.8 ± 5.9
COPD	4	63.5 ± 6.1*†	18.1 ± 2.7*†
Saline-treated	5	64.3 ± 6.4*	19.0 ± 3.2*†
Azm-treated	4	75.3 ± 5.7*	34.1 ± 5.5*
Lev-treated	5	65.0 ± 4.7*	21.4 ± 5.6*†

Values are mean ± SD.
 * *P* < .05 compared with normal group.
 † *P* < .05 compared with azithromycin (Azm)-treated group.
 Lev = levofloxacin

Table 2. Comparison of Neutrophil and Macrophage Cells in BAL Fluid of the 5 Groups of Rats

	<i>n</i>	Neutrophil	Macrophages
Normal	10	17.6 ± 3.34	150.5 ± 17.7
COPD	9	174.4 ± 21.5*†	81.1 ± 15.8*
Saline-treated	10	169.5 ± 25.0*†	81.0 ± 16.1*
Azm-treated	8	135.6 ± 24.4*	95.0 ± 16.6*
Lev-treated	9	172.0 ± 18.6*†	81.7 ± 14.5*

Values are mean ± SD.
 * *P* < .05 compared with normal group.
 † *P* < .05 compared with azithromycin (Azm)-treated group.
 Lev = levofloxacin

(875 ± 169 pg/mL) (*P* < .05) and saline-treated group (864 ± 166 pg/mL) (*P* < .05).

Western Blotting

VEGF and VEGFR2 protein expression was analyzed by western blotting in lung homogenates from rats in the 5 experimental groups (Fig. 1). Significant decreases were observed in VEGF and VEGFR2 expression in the COPD group and the saline- and Lev-treated groups compared with the normal group. Treatment with Azm led to an increase in lung VEGF and VEGFR2 protein expression compared with the COPD, saline-treated, and Lev-treated groups.

Quantification of Emphysema

Pulmonary emphysema is characterized by destruction of alveolar walls due to damage to the lung parenchyma, leading to enlargement of alveolar spaces. Therefore, to quantify emphysematous lesions, it is recommended to evaluate both the air space enlargement (quantified by the

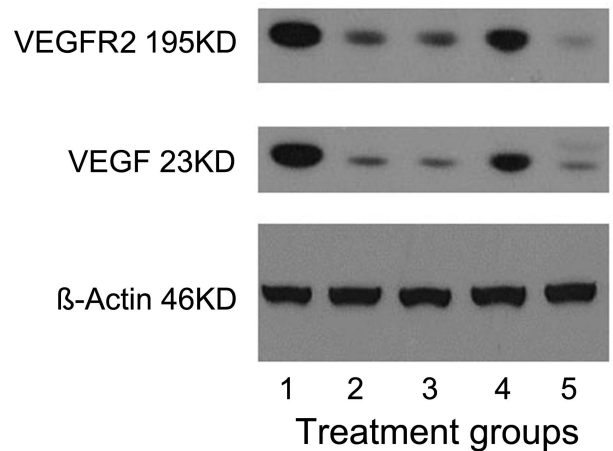


Fig. 1. Western blot analysis of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR2) protein expression in lung homogenates from the 5 groups of rats.

measurement of the Lm) and the destruction of the alveolar walls (quantified by the measurement of the DI).^{20,21}

Chronic CS exposure clearly induced pulmonary emphysema in SD rats, evidenced by a significant increase in Lm (air, 43.32 ± 4.78 μm vs cigarette smoke, 77.8 ± 14.76 μm, *P* = .00) and DI (air, 17.23 ± 5.60 vs smoke, 56.40 ± 8.41, *P* = .00). Furthermore, the Lm and DI of the chronic CS exposure with azithromycin-treated rats were significantly higher than the air-exposed rats (*P* = .00). However, in azithromycin-treated animals with chronic CS exposure, the induction of emphysema, evidenced by a significant decrease in Lm (Azm-treated, 57.98 ± 10.12 μm vs saline-treated, 75.28 ± 10.30 μm, *P* = .01; and vs Lev-treated, 75.39 ± 10.99 μm, *P* = .01) and DI (Azm-treated, 46.58 ± 7.44 vs saline-treated, 55.05 ± 7.02, *P* = .03; and vs Lev-treated, 55.1 ± 6.94, *P* = .04), indicating a partial protection against pulmonary emphysema in the Azm-treated rats (Fig. 2, A and B). The significant air space enlargement due to chronic CS exposure and the attenuated emphysema in chronic CS exposure with Azm-treated rats is illustrated with H&E-stained lung tissue sections (Fig. 3).

Discussion

COPD is mainly caused by cigarette smoking, and is characterized by pulmonary emphysema and inflammation with an increase of inflammatory cells of both the innate and the adaptive immune system. In this study, chronic exposure to CS resulted in a substantial accumulation of inflammatory cells in the airways of the rats. However, the CS-induced neutrophils and macrophages in the BAL fluid were significantly decreased in the Azm-treated rats, compared with COPD littermates. A significant increase of VEGF level was observed in the serum and lung

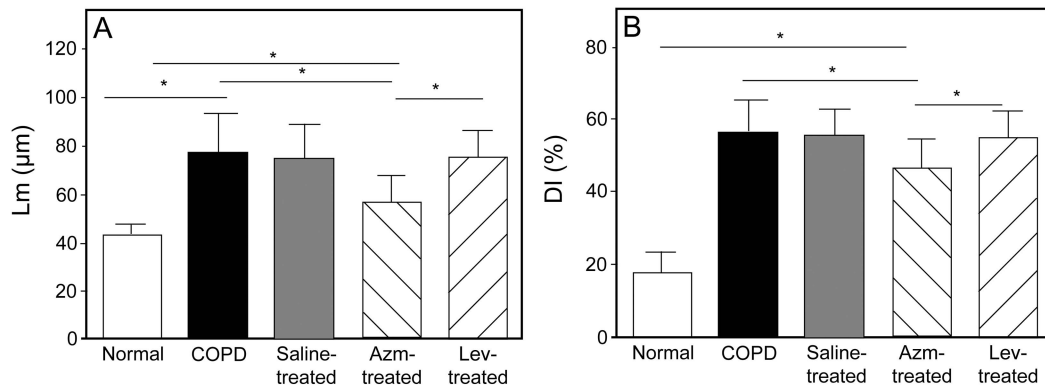


Fig. 2. Quantification of pulmonary emphysema in the 5 groups of rats. A, mean linear intercept (μm); B, destructive index ($*P < .05$). Lm = mean linear intercept; DI = destructive index.

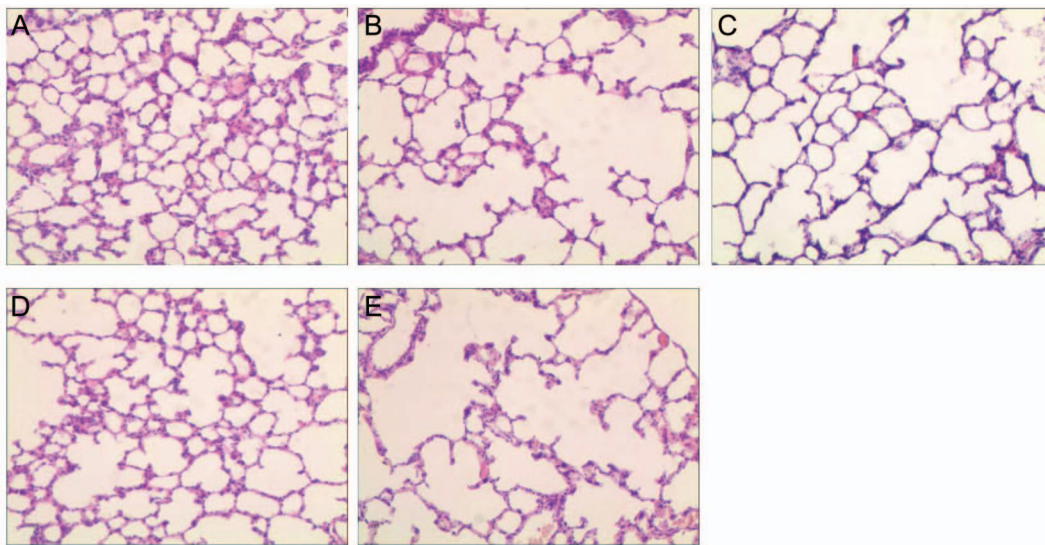


Fig. 3. Hematoxylin and eosin staining of lung tissue sections from rats. A, normal group; B, COPD group; C, saline-treated group; D, azithromycin-treated group; E, levofloxacin-treated group (magnification $\times 100$).

homogenates of azithromycin-treated rats, compared with the COPD, saline-treated, and Lev-treated groups. The exposure to CS resulted in development of pulmonary emphysema in model littermates. A partial protection against pulmonary emphysema was discovered in the Azm-treated rats. In contrast, levofloxacin had no effect on the development of airway wall remodeling and airway inflammation.

COPD is characterized by an accelerated decline in lung function, expressed as the FEV_1 and its ratio to the FVC, namely FEV_1/FVC . The present study showed that exposure of rats to cigarette smoking decreased $FEV_{0.3}/FVC$ and PEF, which is similar to clinical observations in patients with COPD. COPD is characterized by air flow limitation that is not fully reversible due to airway inflammation and changes of airway anatomy. Airway remodeling in COPD consists of several structural changes like

changes in bronchial epithelium, bronchial glands hypertrophy, and smooth muscle hyperplasia and hypertrophy. Those changes are related to an increase in various cytokines, mucus-containing cell number, and proteolytic burden in animal models of cigarette smoke exposure²³⁻²⁵ and in COPD.²⁶ In this study, the Azm-treated group showed greater attenuation of emphysema than the COPD group, and $FEV_{0.3}/FVC$ and PEF were higher in the Azm-treated group than in the COPD group but lower than those of the normal group. Therefore, azithromycin can partially improve lung function and attenuate emphysematous changes in COPD rats.

Investigations in our experimental findings presented here with the use of cell counts show that azithromycin treatment efficiently reduced neutrophil and macrophage numbers in bronchoalveolar lavage fluid. Neutrophils and macrophages have an important role in the pathogenesis of

airway inflammation in COPD. Activated neutrophils and macrophages cause lung destruction through the release of oxygen radicals and proteolytic enzymes such as neutrophil elastase and matrix metalloproteinases, including matrix metalloproteinases 8, 9, and 12 (formerly called macrophage elastase).²⁷ Neutrophil elastase-induced production of mucin occurs via proteolytic cleavage of transforming growth factor α , a ligand of epidermal growth factor receptor. Excessive mucus production and impaired mucociliary clearance contribute to airway obstruction in patients with COPD.²⁸ In addition, neutrophil and macrophages can release cytokines and chemokines, which can potentiate inflammation and trigger an immune response.²⁹

We have shown in the present study that cigarette smoke reduces VEGF expression in a lung model of smoking in rats. VEGF, which is highly expressed in the normal lung, plays a critical role in lung development and the maintenance of alveolar structure in the adult lung.³⁰ VEGF expression is decreased in emphysematous lungs concomitant with increased endothelial cell apoptosis, and the inhibition of VEGFRs leads to the development of emphysema-like changes in the lung.³¹ VEGF levels are also reduced in the bronchoalveolar lavage fluid of smokers.³² Interestingly, VEGFR inhibition leads to the enlargement of the air spaces, increased endothelial cell death, and decreased capillary density, characteristic of emphysema.^{30,33} The comparison of the expressions of VEGF and VEGFR2 protein in the samples from rats with and without emphysema demonstrated that samples from emphysematous lungs contained a lower amount of the growth factor and its receptor. In addition, the levels of VEGF in BAL fluid of the Azm-treated group were lower than those of the normal group but higher than those of the COPD group. The results above suggest that azithromycin can partially reverse the cigarette smoke-induced decrease of VEGF and VEGFR2 expression.

Conclusions

We have demonstrated that azithromycin leads to beneficial VEGF expression and a decreased number of neutrophils and macrophages upon CS exposure, which is reflected in a partial protection against the development of pulmonary emphysema. In addition, the difference in the inhibitory profile of azithromycin from levofloxacin highlights the potential of the azithromycin scaffold for the development of new or combination therapies for COPD.

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