International Journal of Chronic Obstructive Pulmonary Disease Dovepress
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ORIGINAL RESEARCH

RETRACTED ARTICLE: Effect of Doxofylline on Reducing the Inflammatory Response in Mechanically Ventilated Rats with Chronic Obstructive Pulmonary Disease

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Objective: To evaluate the effect of doxofylline on reducing the inflammatory response in mechanically ventilated rats with chronic obstructive pulmonary disease (COPD).

Methods: A total of 40 eight-week-old male Sprague-Dawley rats were randomly divided into four groups of 10 rats each: a control group (group C), a model group (group M), a model + natural saline group (group N), and a doxofylline group (group D). Then mechanical ventilation, drug intervention, and the extraction of the experimental material were performed in each group. Pulmonary tissue samples were taken after 120 minutes of mechanical ventilation and the pulmonary histopathological changes and the wet/dry (W/D) weight ratio of the pulmonary tissue were identified. The levels of tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10) were detected using an enzyme-linked immunosorbent assay, and the expression levels of c-Jun-N-terminal kinase (JNK) and phosphorylated c-Jun N-terminal kinase (p-JNK) were detected using immunohistochemistry.

Results: Compared with group C, the pulmonary histopathology in groups M, N, and D showed typical changes associated with COPD. Furthermore, the W/D weight ratio and levels of TNF-α, JNK, and p-JNK in the pulmonary tissue increased in groups M, N, and D ($P < 0.05$), while the levels of IL-10 decreased ($P < 0.05$). Compared with group M, no statistically significant changes in the above indicators were detected in the pulmonary tissue of group N ($P > 0.05$). Compared with group N, the W/D weight ratio and levels of TNF-α, JNK, and p-JNK in the pulmonary tissue decreased in group D ($P < 0.05$), while the levels of IL-10 increased ($P < 0.05$).

Conclusion: Doxofylline might attenuate pulmonary inflammatory responses in mechanically ventilated rats with COPD, and the JNK/stress-activated protein kinase signaling pathway is involved in doxofylline's inhibition of inflammatory responses in the pulmonary tissue of rats with COPD.

Keywords: chronic obstructive pulmonary disease, TNF-α, IL-10, JNK, p-JNK

Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death worldwide, killing at least 3 million people globally each year,[1] and it is predicted to become the third leading cause of death by 2030.[2] The airflow limitation caused by COPD is usually progressive and is associated with an abnormal inflammatory response of the pulmonary tissue to harmful gases or particles. Patients with COPD experience local infections, injuries, and other inflammatory lesions as well as systemic reactions. With advances in clinical anesthesia and surgical techniques, there has been an increase in the number of

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Received: 16 April 2021
Accepted: 2 August 2021
Published: 16 August 2021

patients with COPD undergoing surgery, during which they are anesthetized and mechanically ventilated.[3] This ventilation can lead to increased airway pressure and cause an increased inflammatory response in the lungs.

Doxofylline, a xanthine derivative, has a good and long-lasting bronchodilator effect. It inhibits the activity of phosphodiesterases in the cells, thereby increasing the intracellular concentration of cyclic adenosine monophosphate and reducing the airway pressure and incidence of air-pressure injury in mechanically ventilated patients.[4] Its effects have been well demonstrated in patients with asthma and COPD.[5,6] A study by Zhang et al[4] showed that doxofylline can also affect the progression of inflammation and achieve anti-inflammatory effects. Compared to aminophylline, doxofylline has a reduced affinity for the adenosine A1 and A2 receptors[7] and has fewer adverse cardiovascular effects, offering a better safety profile and a better user experience. The results of a clinical meta-analysis conducted by Cazzola et al[8] support these findings.

Smoking is a major causative factor of COPD. Lipopolysaccharide (LPS) can induce the release of tumor necrosis factor α (TNF-α) and other inflammatory mediators in the pulmonary tissue in patients with COPD and promote the activation process of neutrophils and eosinophils, which then promotes the infiltration of local inflammatory cells and a further inflammatory injury response. The repeated airway inflammatory responses induced by LPS can mimic the pathophysiological state of COPD. The cigarette smoke and LPS-induced rat model is a widely used animal model of COPD that can reliably simulate the pathophysiological process of COPD.[9–11] Preclinical studies indicate that doxofylline can inhibit bacterial LPS-induced neutrophil infiltration in mouse lungs, suggesting that it might affect leukocyte exudation and therefore be involved in modulating the inflammatory response in COPD,[12] which corresponds with the international approach in the COPD rat model.

Based on this research background, the present study aimed to provide a theoretical basis for the mechanism of the action of doxofylline. To achieve this, it evaluated the protective effect of doxofylline on the lungs of mechanically ventilated rats with COPD by investigating the inflammatory indicators in the pulmonary tissue in the cigarette smoke- and LPS-induced COPD rat model. In addition, it investigated the role of the c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in reducing the inflammatory response through the use of doxofylline in mechanically ventilated rats with COPD.

Materials and Methods

Experimental Material

Experimental Material and Apparatus

The following materials and apparatus were used in this study: doxofylline (batch number 20160115, Jiangsu Enhua Pharmaceutical Co., China), Fu Jian cigarettes (Fujian Province China Tobacco Industry Co., China), LPS (batch number L2880-10MG, Sigma, USA), rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit and rat interleukin 10 (IL-10) ELISA kit (Shanghai Preferred Biotechnology Co., China), rabbit anti-mouse JNK monoclonal antibodies (Abcam, UK), rabbit anti-mouse p-JNK monoclonal antibodies (Cell Signaling Technology, USA), rabbit anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibodies (Shanghai Po Wan Biotechnology Co., China), horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) antibodies (Shanghai Biyuntian Institute of Biotechnology, China), an Image-Pro Plus 6.0 image analysis software system (Media Cybernetics, USA), and an ImageQuant LAS-4000 mini imager (GE, USA).

Selection of Animals

A total of 64 eight-week-old clean-grade male Sprague Dawley rats, weighing 200–250 g, were purchased from Fuzhou Minhou County Wu's Laboratory Animal Trade Co. (laboratory animal certificate number: SCXK, Shanghai, 2012–0002). The rats were kept in Quanzhou Medical College's animal center laboratory at a constant temperature, with free access to food and water. All experiments were evaluated and approved by the Ethics Committee of the Fujian Medical University (No. 37, 2018) and complied with the National Institutes of Health Guides for the Care and Use of Laboratory Animal Use.

Methods

Construction of the COPD Rat Model and Grouping

The rats were randomly divided into two groups: a control group (n = 16) and a model group (n = 48). Using the modeling method of Tang[13] and Luo et al,[14] the rats in the model group were placed in a 4 L glass resin container and exposed to smoke from three lit unfiltered cigarettes for 30 minutes a day for 60 days. They received 200 μg of LPS (200 μg/200 μL) intratracheally on day 1 and day 30. The rats in the control group were kept in normoxia for 60 days and received

0.2 mL of natural saline (NS) intratracheally on day 1 and day 30. The feeding conditions were the same in each group.

Mechanical Ventilation, Drug Intervention, and the Extraction of the Experimental Material

After the successful construction of the model, 30 rats were randomly selected from the surviving rats in the model group and divided into a model group (group M), a model + natural saline group (group N), and a doxofylline group (group D), with 10 rats in each group. A further 10 rats were randomly selected from the surviving rats in the control group to create a new control group (group C). All the rats were anesthetized with 1% pentobarbital sodium (40 mg/kg) by intraperitoneal injection and underwent intubation of the right femoral vein. Intratracheal intubation was conducted and connected to a small-animal artificial ventilator.

Parameters for the mechanical ventilation: tidal volume = 8 mL/kg, inhalation-to-expiration ratio = 1:1, frequency of respiration = 80 times/min, inhaled oxygen concentration = 0.5, and positive end-expiratory pressure = 0 (Figure 1).

After intubation, the rats in group D were immediately given an intravenous injection of 50 mg/kg of doxofylline (dissolved in 0.2 mL of NS), following Rao et al,[7] while the rats in groups C and N were immediately given an intravenous injection of an equal volume of NS. The rats in group M were not given an intravenous injection. All the rats were then mechanically ventilated for 120 minutes. At the end of the experiment, the rats were euthanized through the bleeding of the abdominal aorta. The experiment was approved by the Experimental Animal Ethics Committee of Fujita Medical University (No. 37, 2018).

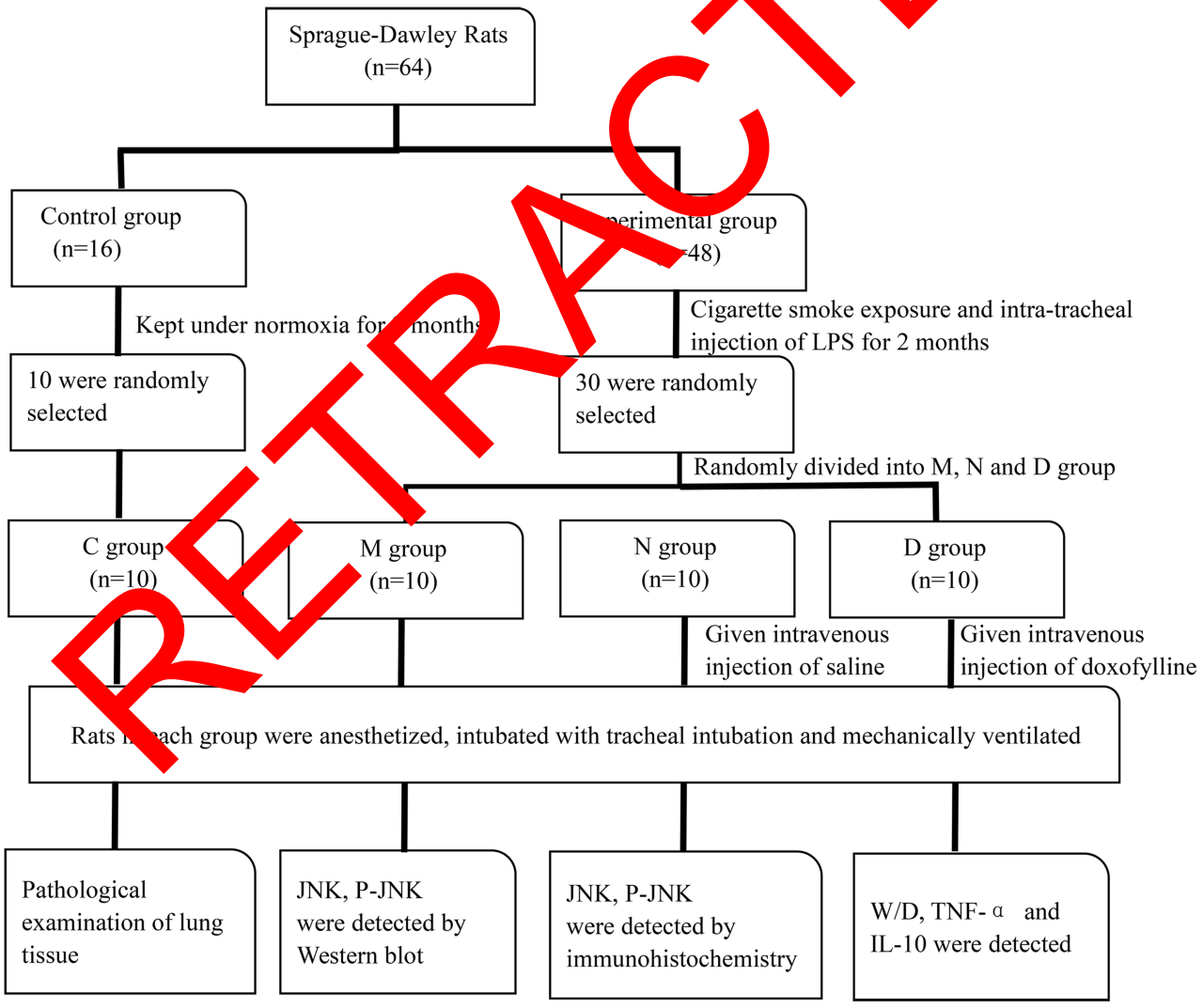

Figure 1 Flowchart of the study design of the animal model.

Measurement of the Wet/Dry (W/D) Weight Ratio and the Pathology of the Pulmonary Tissue

Following the full exposure of the thoracic and abdominal cavities of the rats, the wet weight of the lung was determined by weighing the upper lobe of the right lung. This lobe was then placed in a drying oven at 70°C for 24 hours to reach a constant weight, and the dry weight of the lung was weighed and the W/D ratio calculated. The separated middle lobe of the right lung was fixed in 10% formalin solution, embedded in paraffin, and serially sectioned into three sets. One set was taken for routine hematoxylin and eosin (H&E) staining to analyze and observe the histopathological changes in the lung.

Determination of the TNF-α and IL-10 Concentrations via ELISA

The lower lobe of the right lung was removed from the freezing tube, and the tissue homogenate was prepared. Following the preparation of the standard solution, the sample was added and the plate was washed. The working solutions containing the primary antibody, the enzyme-labeled antibody, the substrate, and the termination solution were added successively, and the absorbance value was measured at 450 nm with a microplate reader.

Detection of the Changes in JNK and p-JNK via Immunohistochemistry

A total of 30 mg of the lower lobe tissue of the right lung was added to a sterile Eppendorf tube, and the protein concentration of the lung tissue was measured using a bicinchoninic acid assay. Next, 30 μg of the protein sample was loaded into SDS-polyacrylamide gel, and constant pressure electrophoresis was conducted. The protein was transferred to a polyvinylidene fluoride membrane using the semi-dry method. After blocking and washing, the samples were cut according to the corresponding molecular weight and refrigerated overnight with rabbit anti-mouse GAPDH primary antibodies (1:5000), JNK primary antibodies (1:500), and p-JNK primary antibodies (1:500) at 4°C. The next morning, after washing the membrane, goat anti-rabbit secondary antibodies (1:5000) were added, and the samples were incubated at room temperature. Incubation with electrogenerated chemiluminescence solution, X-ray exposure and development, and washing was then conducted. The gray value of the target protein was represented by the ratio of the gray value of the target protein to the gray value of the internal reference GAPDH.

Two sets of paraffin-embedded pulmonary tissue underwent routine deparaffinization until hydration, and the expression of JNK and p-JNK was detected according to the instructions of the immunohistochemistry kit. A total of 0.01 mmol/L of phosphate-buffered saline was used instead of the primary antibody in the negative control group. A multifunctional true-color cell image analysis and management system (Image-Pro Plus v. 5.1) selected five visual fields, and the average optical density of each field was calculated. These averages were used as the final average optical density value of each slice.

Statistical Analysis

SPSS 20.0 software was used for the data analysis. After a normality test and variance homogeneity test, the measurement results were expressed as mean ± standard deviation (± SD). A one-way analysis of variance (ANOVA) was used to conduct the comparison between the groups. Before performing the ANOVA, a homogeneity of variance test was carried out to ensure that the prerequisite requirements for using the ANOVA were met. When the ANOVA appeared to be different, a post-test was used to further analyze the differences between the two groups in detail. $P < 0.05$ was considered to be statistically significant.

Results

W/D Ratio and Levels of TNF-α and IL-10

Compared with group C, the W/D ratio of the pulmonary tissue and levels of TNF-α increased in groups M, N, and D ($P < 0.05$), while the levels of IL-10 decreased ($P < 0.05$). There was no statistical significance in the above indicators between group M and group N ($P > 0.05$). Compared with group N, the W/D ratio of the pulmonary tissue and levels of TNF-α in group D decreased ($P < 0.05$), while the levels of IL-10 increased ($P < 0.05$). See Table 1.

Table 1 The Comparison of the Wet/Dry Weight Ratio, and the Levels of TNF-α and IL-10 in the Pulmonary Tissue in Various Groups of Rats (n=10, ±s)

Groups	W/D Levels	TNF-α (pg/mL)	IL-10 (pg/mL)
Group C	3.66±0.043	199.19±3.25	132.45±3.37
Group M	4.80±0.10^{c}	247.58±6.01^{c}	83.07±9.50^{c}
Group N	4.83±0.08^{cd}	243.22±2.06^{cd}	89.53±14.80^{cd}
Group D	4.24±0.02^{cn}	219.26±8.41^{cn}	113.23±5.84^{cn}

Notes: Compared with group C, $^{c}P < 0.05$; compared with group N, $^{n}P < 0.05$, compared with group D, $^{d}P<0.05$.

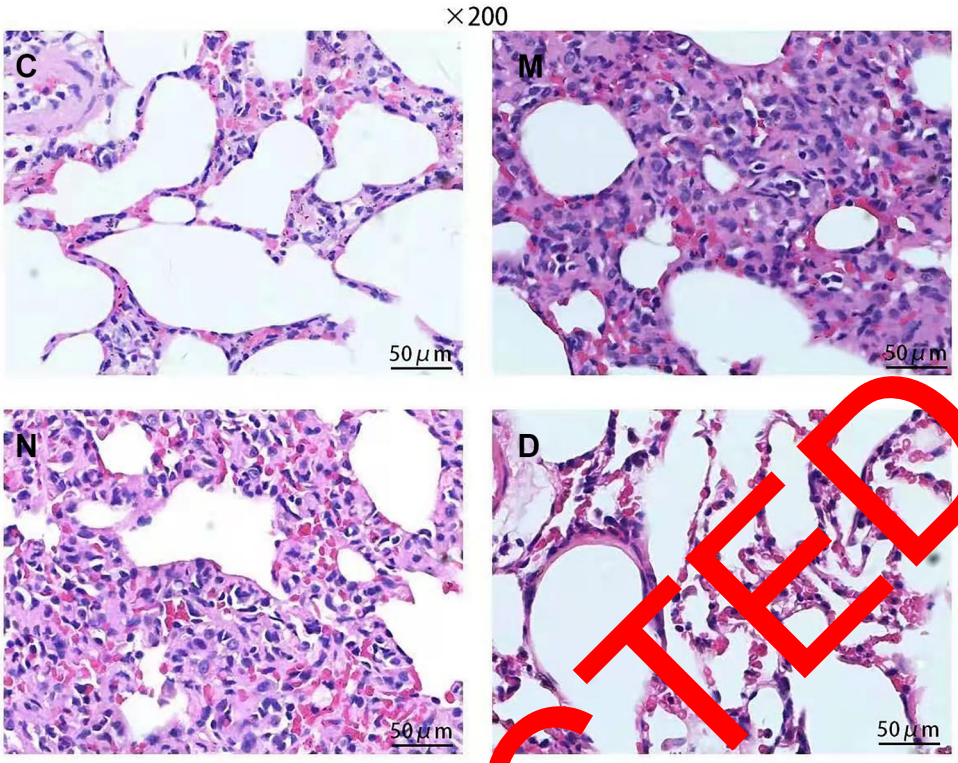

Figure 2 H&E staining of the lung histopathology (magnification ×200). The blue-stained cells are the inflammatory cells (mainly neutrophils, lymphocytes, and monocytes). The other cells appear to have larger, pink cytoplasm and small, blue or dark-blue nuclei. In the pathological sections of the C, M, N, and D groups, the majority of the inflammatory cells were blue. C: a control group (group C), M: a model group (group M), N: model + natural saline group (group N), D: a doxofylline group (group D).

Changes to the Small-Airway Mucosa

The structure of the small-airway mucosa of the pulmonary tissue in group C was normal, without obvious inflammatory cell infiltration, and the alveolar cavity size was also normal. There were significant differences between the pathological results of the control group and those of groups M and N, which exhibited small-airway mucosal edema, a large number of inflammatory cell infiltrations on the airway mucosa, and hyperplasia and hypertrophy of the submucosal gland. Alveolar rupture was also visible in some individuals, with the forming of bullae by fusion and a reduced number of alveoli. There were no significant differences in the histological changes in groups M and N.

Group D exhibited reduced small-airway mucosal edema and inflammatory cell infiltration compared with group N, with mild hyperplasia and hypertrophy of the submucosal gland, a decreased number of alveoli, and the formation of bullae by a few ruptured alveoli. See Figure 2.

JNK and p-JNK Expression

Figures 3 and 4 show the immunohistochemistry results, with the brown–yellow particles representing the positive staining cell components of immunohistochemistry. The average optical density values of JNK and p-JNK in the lung tissue of the rats in each group are shown in Table 2, while the protein expression levels are shown in Figures 5 and 6. Compared with group C, the expression of JNK and p-JNK in the pulmonary tissue in groups M, N, and D were up-regulated ($P < 0.05$), and the corresponding average optical density increased ($P < 0.05$). There were no significant differences in the expression of JNK and p-JNK or the corresponding average optical density in groups M and N ($P > 0.05$). Compared with group N, the protein expression of JNK and p-JNK in the pulmonary tissue in group D decreased ($P < 0.05$).

Discussion

Pathological changes are a key feature in the confirmation of the development of COPD. A pathological examination can determine whether the pathological changes in the airway and lung parenchyma are in line with the typical changes associated with COPD. The typical airway pathological manifestations of COPD are as follows: airway submucosal gland hyperplasia and hypertrophy, hypersecretion, an increase in goblet cells, and focal hyperplasia and squamous metaplasia of the mucosal epithelium. In

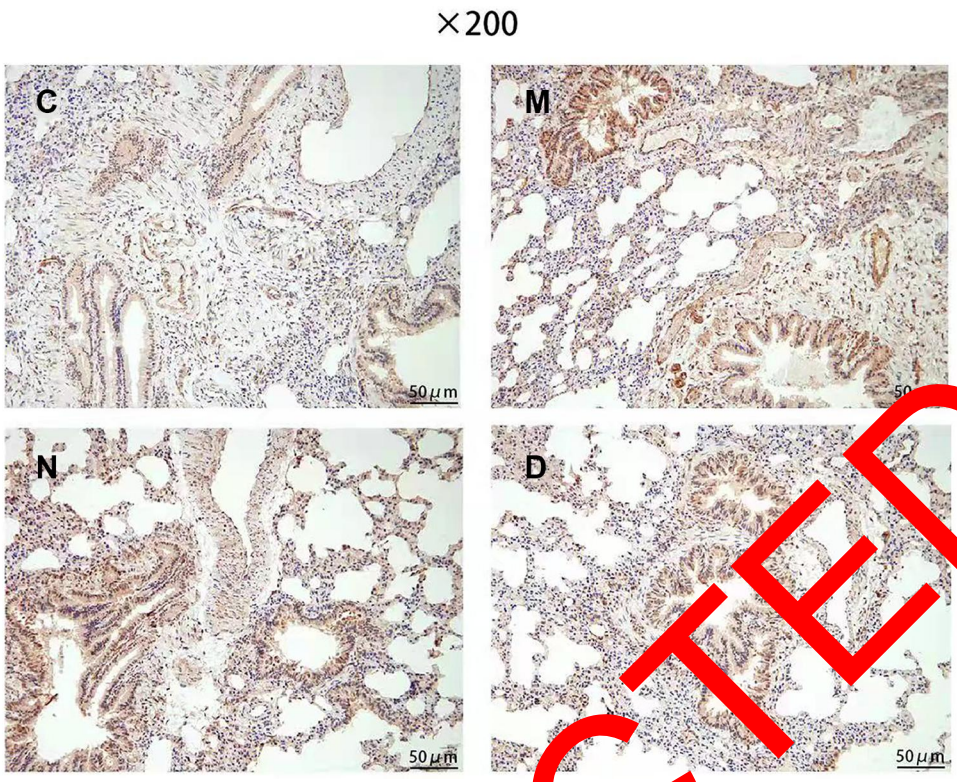

Figure 3 Measurement of JNK expression using the immunohistochemistry of lung tissue (magnification ×200). The brownish-yellow particles indicate the positive staining protein components of immunohistochemistry. C: a control group (group C), M: a model group (group M), N: model + natural saline group (group N), D: a doxofylline group (group D).

addition, respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli are significantly enlarged in the lungs and the alveolar walls are mostly broken, while some alveoli merge with each other to form pulmonary bullae.[15,16]

Compared with the control group, groups M, N, and D showed significant pathological changes associated with COPD, indicating that the COPD model had been successfully constructed. COPD causes repeated airway remodeling, expansion of the pulmonary tissue gaps, destruction of the alveolar walls, airway obstruction, airway inflammation and injury, and increased alveolar–capillary permeability. The edema fluid that passes through the endothelial barrier and accumulates in the pulmonary interstitium may result in diffused edema of the pulmonary tissue, which can manifest as an increase in the W/D ratio of the pulmonary tissue. In the present study, the W/D ratios of groups M, N, and D increased compared with those of group C, indicating that the constructed rat model conformed to the edema of the pulmonary tissue in COPD. The W/D ratio of the rats in group D after treatment decreased compared with those in group N, indicating that doxofylline was able to reduce the tissue edema caused by the pulmonary inflammation and have a therapeutic effect on COPD.

TNF-α is mainly produced by the activation of monocytes and macrophages. Excessive TNF-α can increase the permeability of microvascular walls and directly activate neutrophils and macrophages, leading to the synthesis and excessive release of inflammatory mediators such as IL-8, which mediates the continuous accumulation of a large number of neutrophils in the lungs and promotes the degranulation of neutrophils and the release of elastase and metalloproteinases. This causes local tissue damage, triggers the respiratory storm of neutrophils, enhances local lymphocyte infiltration and proliferation in the inflammation, and kills invading pathogens. Fu et al[17] found that TNF-α directly mediates the inflammatory response in COPD and that the inflammatory mediators produced locally in the lungs are transferred into blood circulation, which causes pulmonary injury, aggravates the development of emphysema, and ultimately leads to the remodeling of the airway structure.

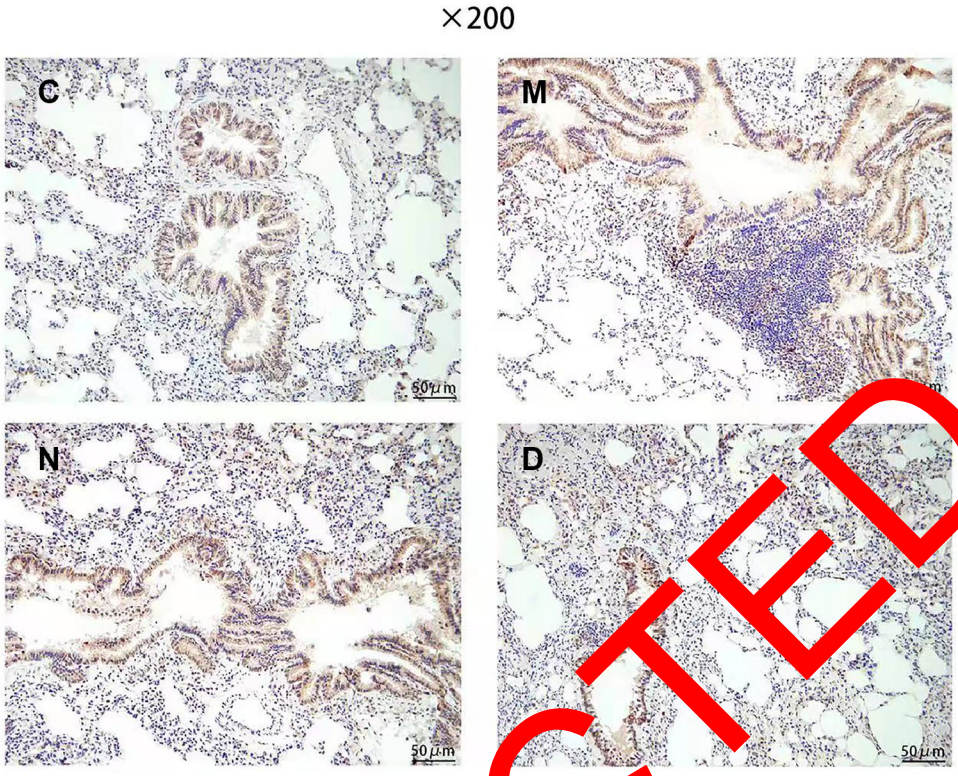

Figure 4 Measurement of p-JNK expression using the immunohistochemistry of lung tissue (magnification ×200). Brownish-yellow particles indicate the positive staining protein components of immunohistochemistry. C: a control group (group C), M: a model group (group M), N: a model + natural saline group (group N), D: a doxofylline group (group D).

IL-10 is the most important anti-inflammatory cytokine in the body, and it plays a particularly dominant role in reducing immune-mediated inflammation. In COPD, especially at the acute exacerbation stage caused by infection, the number of apoptotic T cells ingested by macrophages increases with the severity of injury. IL-10 deactivates macrophages by inhibiting the production of interferon γ, IL-2 and other cytokines.[18] Major histocompatibility complex II is down-regulated, and the production of anti-inflammatory mediators IL-10 and TGF-β (transforming growth factor β) increases, which further leads to immunosuppression and reduces the inflammatory reaction. In the present study, the levels of TNF-α and IL-10 in the pulmonary tissue in groups M, N, and D increased compared with the control group, while in group D they decreased compared with group N. This indicates that doxofylline is able to reduce the levels of the inflammatory factor TNF-α and increase the levels of the anti-inflammatory cytokine IL-10 in rats with COPD, thereby alleviating the pulmonary injury caused by inflammation and improving the condition of the rats.

The JNK/SAPK signaling pathway is a member of the mitogen-activated protein kinase (MAPK) family of signaling systems. Inflammation, stress stimulation, and LPS are among the conditions that can activate the JNK

Table 2 The Comparison of the Levels of JNK and p-JNK in the Pulmonary Tissue in Various Groups of Rats (n=10, ±s)

Groups	The Relative Grayscale of JNK	The Relative Grayscale of p-JNK	The Average Optical Density of JNK	The Average Optical Density of p-JNK
Group C	1.515±0.042	0.829±0.013	0.200±0.004	0.184±0.007
Group M	1.930±0.088^{c}	1.961±0.030^{c}	0.384±0.005^{c}	0.364±0.007^{c}
Group N	1.897±0.071^{cd}	1.958±0.011^{cd}	0.390±0.006^{cd}	0.363±0.011^{cd}
Group D	1.636±0.025^{cn}	1.372±0.011^{cn}	0.278±0.011^{cn}	0.232±0.009^{cn}

Notes: Compared with group C, $^{c}P < 0.05$; compared with group N, $^{n}P < 0.05$, compared with group D, $^{d}P<0.05$.

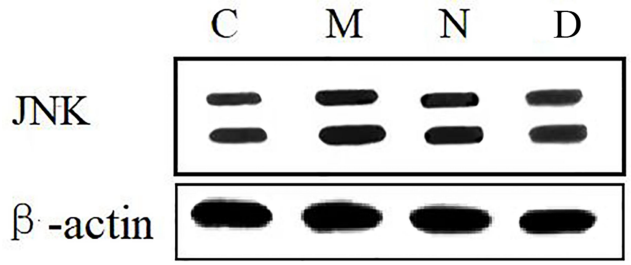

Figure 5 The protein expression levels of JNK were determined by the Western blot. C: a control group (group C), M: a model group (group M), N: a model + natural saline group (group N), D: a doxofylline group (group D).

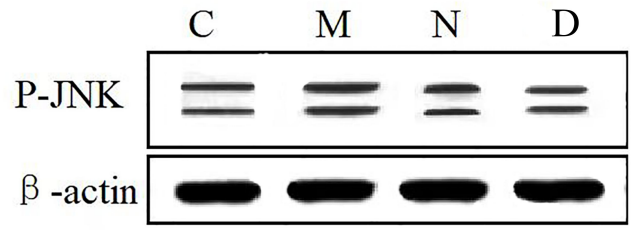

Figure 6 The protein expression levels of p-JNK were determined by the Western blot. C: a control group (group C), M: a model group (group M), N: a model + natural saline group (group N), D: a doxofylline group (group D).

signaling pathway. TNF-α and IL-6, which are secreted by the mononuclear macrophages under the action of extern factors, can activate the MAPK signaling pathway through a three-stage enzymatic cascade reaction to form the upstream regulators of the JNK signaling pathway, resulting in JNK activation. This activates the downstream target gene c-Jun, which helps cells respond to various stimuli, such as cytokines and bacterial and viral infections, by regulating gene expression. In addition, it enhances the activity of intranuclear transcription factors, such as c-fos and c-Jun, in combination with p38 and participates in enhancing the expression of pro-apoptotic proteins, such as TNF-α, to regulate the inflammatory response. In the present study, the levels of JNK and p-JNK in the pulmonary tissue of the rat model were measured to investigate the extent of inflammatory injury in rats with COPD and to identify whether doxofylline has a protective effect on pulmonary inflammatory response. The results showed that the expressions of JNK and p-JNK in groups M, N, and D increased compared with those of group C and that they were lower in group D than in group N. This suggests that doxofylline has an inhibitory effect on the JNK/MAPK pathway in COPD, improving the JNK/MAPK pathway-mediated inflammatory response and alleviating the repeated airway remodeling process caused by the activation and aggregation of neutrophils in the airway mucosa. This then alleviates airflow restriction and contributes to the control of COPD.

This experiment has a number of limitations. We did not observe the effect of doxofylline on the mechanical ventilation pressure during the mechanical ventilation of the COPD rats nor the macrophages, lymphocytes, neutrophils, and eosinophils in the lung tissue. It is hoped that the influence of the ratio of acidic granulocytes will be further explored in future studies.

Conclusion

By constructing a COPD rat model, the effects of doxofylline on the inflammatory response process in the lungs of rats with COPD were investigated. First, smoke exposure and the intratracheal injection of LPS were used to construct a COPD rat model, and mechanical ventilation and the injection of doxofylline into the femoral vein were used as pharmacological interventions to observe the pathological changes of the pulmonary tissue in each group of rats. The W/D ratio and the expressions of TNF-α, IL-10, JNK, and p-JNK in the pulmonary tissue were detected. The results of HE staining showed that the COPD rat model was successfully constructed. Comparing the differences in the W/D ratio and the levels of TNF-α, IL-10, JNK, and p-JNK in the pulmonary tissue of the rats in groups N and D, it was found that doxofylline might attenuate the pulmonary tissue edema caused by increased capillary permeability and edema fluid accumulation in the pulmonary interstitium, which were induced by the inflammatory response. In addition, doxofylline might cause a decrease in the inflammatory cytokine TNF-α and an increase in the anti-inflammatory cytokine IL-10, which would inhibit the inflammatory process triggered by the JNK/SAPK pathway, reduce small-airway inflammatory cell infiltration and airway mucus secretion, improve the small-airway obstruction caused by mucus, alleviate airflow limitation, and improve ventilation in patients with COPD.

The JNK/SAPK signaling pathway might be involved in the process of the control of doxofylline in the pulmonary tissue inflammatory response and the reduction of the small-airway inflammatory cell infiltration in rats with COPD, which might be correlated with the mechanism of the therapeutic effect of doxofylline on COPD.

Ethics Approval and Consent to Participate

All experiments were evaluated and approved by the Ethics Committee of the Fujian Medical University (No. 37, 2018) and complied with the National Institutes of Health Guides for the Care and Use of Laboratory Animal Use.

Acknowledgments

We are particularly grateful to all the people who have given us help on our article.

Funding

This study was funded by the Fujian Natural Science Foundation (2017J01275) and Guiding (key) project of social development in Fujian Province (2017Y0029).

Disclosure

The authors declare that they have no competing interests.

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