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ORIGINAL RESEARCH

RETRACTED ARTICLE: The Mechanism of Penehyclidine Hydrochloride and Its Effect on the Inflammatory Response of Lung Tissue in Rats with Chronic Obstructive Pulmonary Disease During Mechanical Ventilation

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Background: Penehyclidine hydrochloride is a selective antagonist of M1 and M3 receptors. Clinical studies suggest that it is a potential drug for the treatment of chronic obstructive pulmonary disease (COPD). The purpose of this study was to evaluate the effect of penehyclidine hydrochloride on the inflammatory response of lung tissue during mechanical ventilation in rats with COPD and explore the role of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway.

Methods: Eight-week-old male Sprague Dawley rats were exposed to cigarette smoke for 30 minutes every day for three months, and on the first and thirtieth days, 200 ug of lipopolysaccharide was injected into the trachea. Two months later, the rats were randomly divided into the control group (C), model group (M), model + normal saline group (N), and penehyclidine hydrochloride group (H) to undergo anesthesia and mechanical ventilation. In group H, 1 mg/kg of penehyclidine hydrochloride was injected intravenously.

Results: The results showed that: ① Compared with group C, the other groups all showed typical chronic obstructive pathological changes in the lung tissue; their wet/dry weight ratio (W/D), TNF-α, JNK, and p-JNK levels increased ($P < 0.05$), and their interleukin (IL)-10 levels decreased ($P < 0.05$). ② Compared with group M, there was no significant change in the lung tissue indexes in group N ($P > 0.05$). ③ Compared with group N, the W/D, TNF-α, JNK, and p-JNK levels in group H decreased ($P < 0.05$), while the levels of IL-10 increased ($P < 0.05$).

Conclusion: Penehyclidine hydrochloride can alleviate the pulmonary inflammatory response in rats with COPD undergoing mechanical ventilation. The JNK/SAPK signaling pathway may be involved in this process.

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

Background

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow restriction.[1] It is a highly prevalent and long-term disease that often leads to breathing problems and many other complications. Smoking and age are factors in the pathogenesis of this progressive disease, and immune cells participate in the response.[1] It is also related to the abnormal inflammatory response of lung tissue to harmful gases or particles.[2] With the progress of clinical anesthesia and operation

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technology, the number of patients with COPD undergoing surgery is increasing. Mechanical ventilation during general anesthesia can lead to an increase in airway pressure, an imbalance in the pulmonary ventilation/blood flow ratio, and the release of inflammatory mediators, which can result in lung injury.[3] Therefore, it is necessary to further study the mechanism of lung protection drugs that may reduce inflammation-related lung complications.

Penehyclidine hydrochloride is a selective anticholinergic drug that can act on M1 and M3 receptors in the body, thus inhibiting cholinergic nerve activity, reducing the mucus secretion of airway mucosa, relaxing airway smooth muscle, reducing airway resistance, and improving lung compliance and respiratory function. It also activates NF-kβ in lung tissue, inhibits the release of inflammatory factors (eg, interleukin [IL]-6 and IL-8), and reduces the permeability of pulmonary capillaries and the edema of extrapulmonary tissues.[4] Clinical studies have found that penehyclidine hydrochloride can also significantly reduce airway pressure and the release of inflammatory mediators during mechanical ventilation in patients with COPD, thereby inhibiting the inflammatory response, reducing lung damage, and helping improve the prognosis.[5] The purpose of this study is to investigate the effects of penehyclidine hydrochloride on lung injury in rats with COPD undergoing mechanical ventilation as well as the role of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in reducing the inflammatory response of rats with COPD initiated with penehyclidine hydrochloride to provide theoretical basis for research on the mechanism of this drug.

Methods

Materials and Reagents

The following materials and reagents were used: penehyclidine hydrochloride injection (Batch No.: 170922, Chengdu Lishite Pharmaceutical Co., Ltd.); lipopolysaccharide (Batch No.: l2880-10MG, Sigma Company, U.S.A.); Fujian cigarettes (Fujian Zhongyan Industry Co., Ltd.); rat TNF-α and rat IL-10 ELISA test kits (Shanghai Preferred Biotechnology Co., Ltd.); rabbit anti-rat GAPDH polyclonal antibody (Shanghai Bowan Biotechnology Co., Ltd.); rabbit anti-mouse JNK monoclonal antibody (Abcam Company, U.K.); rabbit anti-mouse p-JNK monoclonal antibody (Cell Signaling Technology Company, U.S.A.); horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) antibody (Shanghai Biyuntian Biotechnology Research Institute).

Apparatus

The following apparatus was used: Las4000 Mini Imager (GE Company, U.S.A.); Image Pro Plus 6.0 Image Analysis System (Media Cybernetics Company, U.S.A.); Reward R407 small animal ventilator (Shenzhen Reward Life Technology Co., Ltd., China).

COPD Animal Model Evaluation Criteria

The ideal COPD animal model, with evaluation criteria in line with clinical reality, should have common causes consistent with the human disease, pulmonary function changes characterized by continuous airflow obstruction, and pathological airway remodeling and emphysema changes.[6] Pathological examination can determine whether the pathological changes of the airway and lung parenchyma are in line with the typical COPD changes, which are as follows: airway submucosal gland hyperplasia, hypertrophy, hypersecretory function increased goblet cells, mucosal epithelial focal hyperplasia, and squamous epithelial metaplasia; bronchiole and terminal bronchiole in the lung appearance of chronic inflammatory cells of varying degrees, infiltration of neutrophils and lymphocytes, blockage of lumens by secretions, degeneration and necrosis of ciliated cells; significant enlargement of respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli, with many alveolar walls. Fracture occurs, and some of the alveoli merge to form bullae. Morphological measurements to evaluate the air cavity expansion and lung parenchymal destruction of emphysema are commonly used to evaluate the average lining interval proportional to the amount of emphysema to evaluate air cavity expansion, and the damage index is calculated by macroscopic microscopy to evaluate the degree of lung parenchymal destruction.[7,8]

Animal Selection

A total of 64 clean eight-week-old male Sprague Dawley rats weighing 200–280 g were selected and purchased from the laboratory animal trade company Wu Ltd. of Minhou County, Fuzhou. The certificate number of the laboratory animals was SCXK (Shanghai) 2012–0002. The animals were raised in the animal experimental center of Quanzhou Medical College and kept in a constant-temperature environment. This study was conducted with approval from the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (No.37–2018). All animals were treated in compliance

with the National Research Council' s Guide for the Care and Use of Laboratory Animals (1996).

Construction and Grouping of the COPD Rat Model

The 64 rats were randomly divided into two groups: a control group (n = 16) and a model-making group (n = 48). In line with the model-making methods of Tang et al[9] and Luo et al,[10] the rats in the latter group were placed in a custom-made 4-L glass resin container every day, wherein they were exposed to smoke generated by three lit unfiltered cigarettes for 30 min every day for a period of two months. The trachea was injected with 200 μg lipopolysaccharide (LPS) on the first and thirtieth days. The rats in the control group were exposed to normal oxygen for two months, and an equal volume of normal saline solution was injected into the trachea on the first and thirtieth days. The feeding conditions of each group were the same. This animal experiment was approved by the experimental animal ethics committee of Fujian Medical University (2018 No. 37), and the study was conducted in June 2018.

Mechanical Ventilation, Drug Intervention, and Experimental Material Extraction in Rats

In the pre-experimental animal modeling stage, we found that the animals died during the breeding process with a mortality rate of about 85%. To randomly select the designated number of rats were needed from the actual surviving rats, during the modeling phase, the number of rats was increased in the same proportion in the control group and the model-making model. The remaining animals not randomly selected to participate in the experiment were kept normally until they died. After the model was successfully prepared, 24 rats in the model-making group were randomly divided into the model group (M), model + normal saline group (N), and penehyclidine hydrochloride group (H), while 12 rats in the control group were randomly selected to construct a new control group (C). The rats in all groups were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg) and then via right-side lateral femoral vein catheterization. They were intubated, connected to an artificial ventilator for small animals, and given mechanical ventilation in accordance with Li et al[11] as follows: tidal volume = 8 mL/kg, respiratory ratio = 1:1, respiratory frequency = 80 times/min, oxygen concentration = 0.5, and positive end expiratory pressure = 0. After intubation, the rats in group H were given 1.0 mg/kg of penehyclidine hydrochloride (dissolved in 0.3 mL normal saline) via a real-time intravenous injection, following Shen et al.[11] Meanwhile, the rats in groups C and N were given an equal volume of normal saline via a real-time intravenous injection, and the rats in group M were not given any intravenous injection at all. The rats in each group were given mechanical ventilation for two hours and then killed by being bled from the abdominal aorta.

Measurement of the Wet/Dry Weight Ratio (W/D) and Pathological Examination of Lung Tissue

The chest and abdominal cavities of the rats were fully exposed. The wet weight of the right upper lobe of the lung was measured and then placed in a 70°C drying oven for 24 hours. Afterward, the dry weight of the lung was recorded and the W/D ratio calculated. Then, the right middle lobe was fixed in a 10% formalin solution, embedded in paraffin, and sectioned. One set of lung tissue was taken for routine hematoxylin and eosin (HE) staining and the pathological changes of the tissue were observed and analyzed.

Determination of TNF-α and IL-10 by ELISA

Once the TNF- α/IL-10 ELISA kit and required reagents were ready to use, the right lower lobe of the lung tissue was removed from the cryopreservation tube, and the tissue homogenate was prepared. The standard solution was also prepared; the sample was added, and the plate was washed. The first antibody working solution, the enzyme-labeled antibody working solution, the substrate working solution, and the termination solution were added successively, after which the absorbance value at 450 nm was measured with the enzyme-labeled instrument.

Changes in JNK and p-JNK Measured by Western Blotting and Immunohistochemistry

A total of 30 mg of the right inferior lobe of the lung was taken and placed in a sterile electropolished tube. The protein concentration of the lung tissue was measured using the BCA method. Then, 30 SDS-G protein was injected into the gel, after which the gel was used for

constant pressure electrophoresis. The protein was transferred to a PVDF membrane using the semi-dry method. After sealing and washing, the protein was cut into sections according to the corresponding molecular weight and placed in a refrigerator at 4°C with the rabbit anti-rat GAPDH antibody (1:5000), JNK antibody (1:600), and p-JNK antibody (1:500). After the morning wash, the goat anti-rabbit antibody (1:5000) was added. The protein concentration was incubated at room temperature in an ECL chemiluminescent solution, and the X film was then exposed and developed. The expression level of the target protein was reflected by the gray value of the target protein and gray value of the internal gap DH band. The expression of JNK and p-JNK was detected following the immunohistochemistry kit's instructions. In the negative control group, 0.01 mmol/l PBS buffer was used instead of the first antibody. After the analysis of the multi-functional true color cell image analysis management system (Image Pro Plus V 5.1 software, Media Cybernetics, U.S.A.), the system automatically selected five meaningful fields, calculated the average optical density value of each field, and took the average optical density value of the five fields as the final value of each slice.

Statistical Analysis

The SPSS 20.0 software was used for anal After a normal test and a homogeneity test of varianc the results were expressed as the mean ± sta ard d (x ± s). The comparisons among the oups w analyzed by a single-factor analysis of vari P < 0.05 dicated that the difference was statistically sig cant.

Results

The Pathological tures of Lung Tissue in Each G oup

In group C he stru re of the small airway mucosa was normal, there o obvious inflammatory cell infiltration, and the size of th alveolar cavity was normal. Compared with group C, there were significant differences in the pathological results of groups M and N. There was edema of the small airway mucosa, a large number of inflammatory cells, infiltration of airway mucosa, and hyperplasia and hypertrophy of the submucous glands. Some tissue also saw alveolar rupture and fusion forming pulmonary bullae, and the number of alveoli was decreased. There was no significant difference in the histological changes between groups M and N. Compared with group N, the edema and inflammatory cell infiltration of the small airway mucosa in group H were reduced, and slight submucosal gland hyperplasia and hypertrophy were observed. In addition, the number of alveoli was reduced, and a few alveoli ruptured and fused to form bullae (Figure 1).

Lung Tissue W/D, TNF-α, and IL-10 in Each Group

Compared with group C, the TNF-α and W/D levels in groups M, N, and H were higher ($P < 0.05$) and the IL-10 levels lower ($P < 0.05$). There was no significant di ference tween groups M and N ($P > 0.05$). Compared wi group N, the NF-α and W/D levels in group H were l wer (< 0.05) an the IL-10 levels higher ($P < 0.05$) (F ure 2).

JNK and p-JNK in t Lung Tissue of Each Group w th Western Blotting

Compared with gro C, the expression of JNK and p-JNK in the lung tiss of groups M, N, and H was upr gulated ($P < 0.05$), and the corresponding mean optical ensity was creased ($P < 0.05$). There was no significa differe e in the expression of JNK or p-JNK or corresponding mean optical density between groups M and ($P > 0.05$). However, compared with group N, the expression of JNK and p-JNK in the lung tissue of roup H was downregulated, and the density was decreased ($P < 0.05$) (Figures 3 and 4).

Discussion

It is currently believed that the potential pathogenesis of COPD includes the following: genetic susceptibility, harmful suckers, protease imbalance, and mediator and oxidative imbalance. The chronic inflammatory response of the airways and lungs to toxic particles or gases is the main factor.[1] The disease is characterized by persistent airflow limitation and progressive development. According to clinical statistics, about 90% of patients with COPD are smokers, and thus exposure to cigarette smoke is the closest way to simulate the human disease environment. This method has been used in the preparation of various COPD models[12] with animals such as rats, mice, guinea pigs, and dogs. Lipopolysaccharide is the main active component of endotoxin in Gram-negative bacteria. It can stimulate monocytes, endothelial cells, and neutrophils to synthesize and release a series of inflammatory mediators, mediate the inflammatory

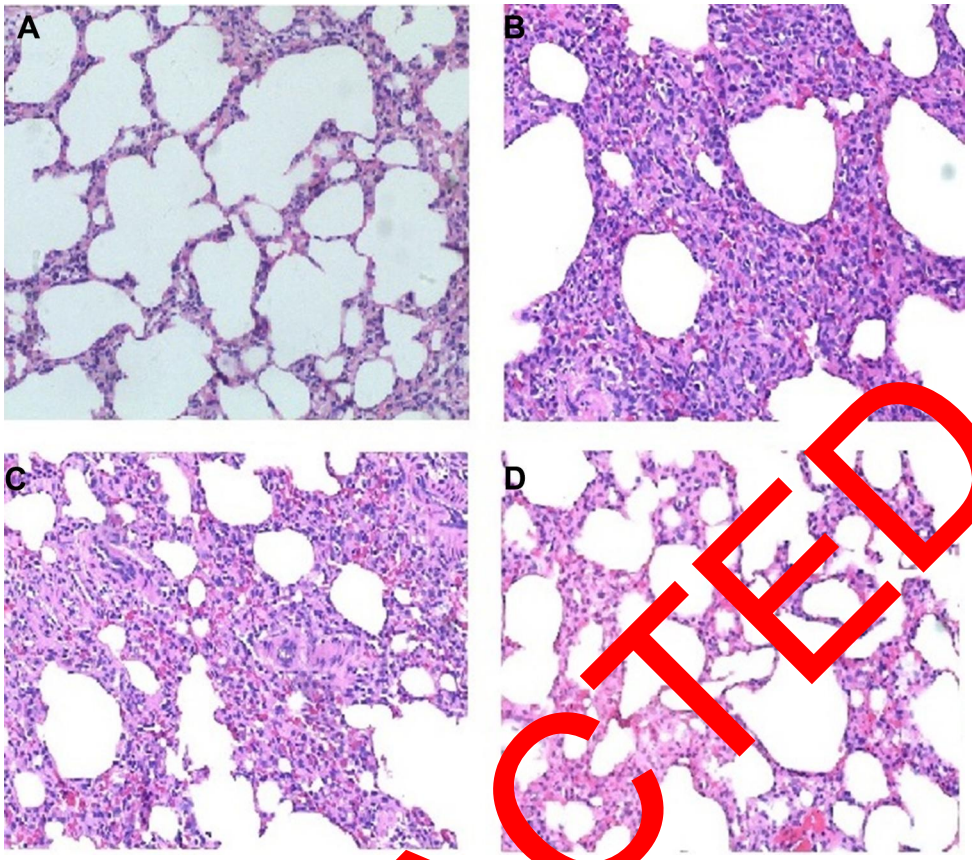

Figure 1 (**A**) Pathological section image of the lung tissue of rats in control group (C, HE staining × 200). (**B**) Pathological section image of the lung tissue of rats in model group (M) (HE staining × 200). (**C**) Pathological section image of the lung tissue of rats in model + normal saline group (N) (HE staining × 200). (**D**) Pathological section image of the lung tissue of rats in penehyclidine hydrochloride group (H) (HE staining × 200).

reaction of airway and lung tissue, destroy the balance between protease and anti-protease and form emphysema.[13]

Simultaneous exposure to cigarette smoke and LPS can aggravate inflammatory factor response and cause lung parenchymal damage to accelerate COPD progress.[14] Therefore, this study used a COPD rat model consisting of cigarette exposure combined with LPS airway injection. In the evaluation of the model, pathological examination determined whether pathological changes of the airway and lung parenchyma conformed to typical COPD changes.[15] Compared with the control group, the model group had edema of the small airway mucosa, a large amount of inflammatory cell infiltration in the airway mucosa, and hyperplasia and hypertrophy of submucous glands. Some individuals also had alveolar rupture and fusion, forming pulmonary bullae, and a decreased number of alveoli. Thus, significant pathological COPD changes were demonstrated, indicating the successful construction of the COPD model.

In this experiment, compared with group C, the W/D level in group M was increased. This was mainly caused by repeated airway remodeling, expansion of lung tissue space, even destruction of the alveolar wall, airway obstruction, airway inflammation damage, increased alveolar capillary permeability, and accumulation of edema fluid through the endothelial barrier in the pulmonary interstitium, resulting in diffuse edema of the lung tissue. After the intervention with penehyclidine hydrochloride, compared with group N, the W/D level in group H decreased, indicating that penehyclidine hydrochloride can reduce the inflammation caused by mechanical ventilation injury and has a protective effect on rats with COPD.

According to Chen et al,[16] TNF-α can be used as an important index to observe the severity of COPD. Excessive TNF-α can increase the permeability of the microvascular wall, directly activate neutrophils and macrophages, lead to the synthesis and excessive release of inflammatory mediators, and mediate the continuous

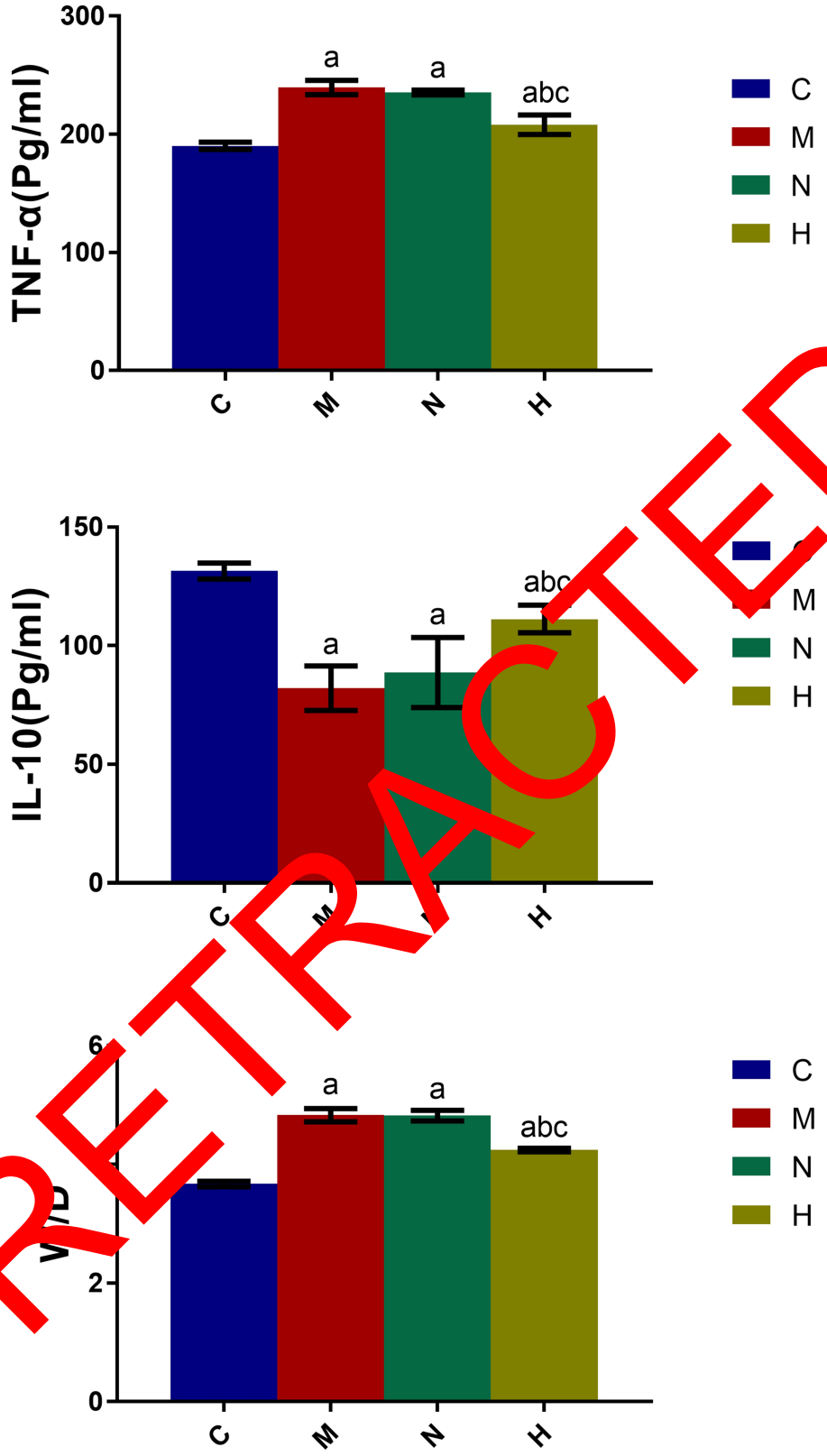

Figure 2 Comparison of the W/D, TNF-α, and IL-10 levels in the lung tissue of rats in each group (n = 12, X ± s).
Notes: Compared with group C, $^{a}P < 0.05$ compared with group M, $^{b}P < 0.05$ compared with group N, $^{c}P < 0.05$.

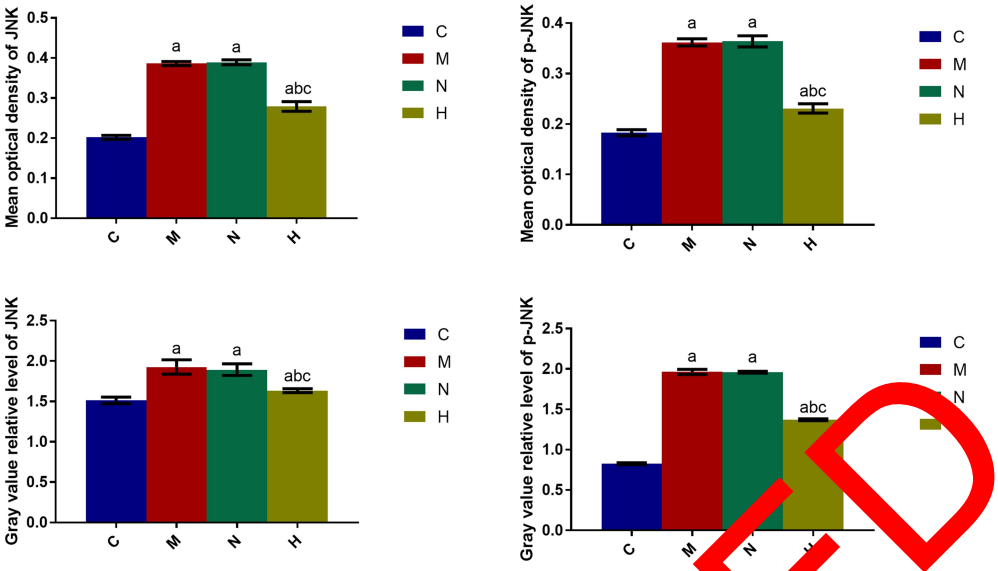

Figure 3 Comparison of JNK and p-JNK levels in the lung tissue of rats in each group (n = 12, X ± s).
Notes: Compared with group C, $^{a}P < 0.05$ compared with group M, $^{b}P < 0.05$ compared with group N, $^{c}P < 0.05$.

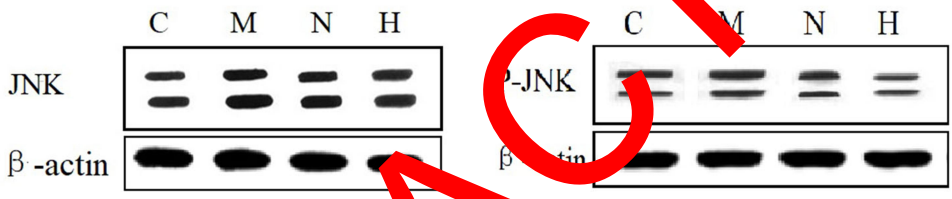

Figure 4 Expression of JNK and p-JNK in the lung tissue of rats in each group detected by Western blotting.

aggregation of a large number of neutrophils in the lung, promoting the degranulation of neutrophils and release of elastase and metal. Protease can cause local tissue damage and lead to a respiratory burst of neutrophils. Fu et al[17] found that TNF-α can directly mediate the inflammatory response of COPD and the inflammatory mediators produced in the lung can also be transferred to the blood circulation, causing lung injury, aggravating the development of emphysema, and, ultimately, leading to remodeling of the airway structure. Interleukin-10 is the most important anti-inflammatory cytokine in the body. In patients with COPD, IL-10 inactivates macrophages by inhibiting the production of interferon γ and IL-2;[18] MHC II decreases, and the production of anti-inflammatory mediators TGF-β and IL-10 increases, leading to immunosuppression and reducing the inflammatory response. Therefore, IL-10 plays an important role in reducing immune-mediated inflammation. The results showed that compared with group C, the TNF-α level of lung tissue in the other groups increased while the IL-10 level decreased. Compared with group N, the TNF-α level of lung tissue in group H decreased whereas the IL-10 level increased, indicating that penehyclidine hydrochloride could reduce TNF-α levels and increase IL-10 levels of anti-inflammatory cytokines in rats with COPD. This could in turn alleviate the inflammatory response caused by mechanical ventilation and improve the level of lung injury in the rats.

The JNK/SAPK signaling pathway is a member of the mitogen-activated protein kinase (MAPK) family signaling system. Inflammation, stress stimulation, and LPS can act on monocyte macrophages and secrete TNF-α and IL-6. They can activate a MAPK signal pathway to form a JNK signal pathway through a three-stage enzymatic cascade reaction. The upstream regulator of the JNK signal pathway can activate JNK. On the one hand, it can activate downstream target gene c-Jun to help cells cope with a variety of stimuli, such as cytokines, bacteria, and diseases.[19] On the other hand, p38 can enhance the activity of transcription factors c-fos and c-jun, participate

in the expression of apoptosis-promoting proteins such as TNF-a, and regulate the inflammatory process. The results showed that the expression of JNK and p-JNK in group H was lower than that in group N, which indicated that penehyclidine hydrochloride could inhibit the JNK/MAPK pathway in mechanically ventilated rats with COPD, improve the inflammatory response mediated by the JNK/MAPK pathway, improve the inflammatory edema of lung tissue caused by neutrophil activation and aggregation in airway mucosa, and improve airflow restriction, all of which would protect the lung function of the rats.

In this study, we constructed a COPD rat model to investigate whether penehyclidine hydrochloride had an inhibitory effect on the pulmonary inflammatory response in the rats. To start, we used smoked and intratracheal injections of LPS to construct the model. Mechanical ventilation and femoral injection of penehyclidine hydrochloride were used as drug interventions to observe the pathological changes of lung tissue in each group. Detection indicators included lung tissue W/D, TNF-α, IL-10, JNK, and p-JNK levels. The HE staining results showed that we successfully constructed a COPD rat model, comparing the W/D, TNF-α, IL-10, JNK, and p-JNK levels in the lung tissue of the model + saline group and the model + penehyclidine hydrochloride group. The differences found indicated that penehyclidine hydrochloride can reduce the increase of capillary permeability caused by inflammatory reaction and the accumulation of edema fluid in pulmonary interstitial lung tissue. It can also reduce inflammatory cytokine TNF-α and improve resistance inflammatory cytokine IL-10 levels, thereby inhibiting the inflammatory response process induced by the JNK/SAPK pathway. This reduces small airway inflammatory cell infiltration and airway mucus secretion, improves small airway obstruction caused by mucus obstruction, and alleviates the airflow limitation of COPD.

Conclusion

In summary, penehyclidine hydrochloride can attenuate lung tissue edema caused by lung tissue inflammation. The JNK/SAPK signaling pathway may be involved in the process of lung tissue inflammation and the reduction of small airway inflammatory cell infiltration in rats with COPD and is related to the therapeutic mechanism of penehyclidine hydrochloride on COPD.

Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (No.37-2018). All animals were treated in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals (1996).

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Disclosure

The authors report no conflicts of interest in this work.

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