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Expression of Endoplasmic Reticulum Stress-Associated Protein IRE1 and JNK in Lung Tissues of Smoking Induced COPD Model Rats

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Abstract

Aim: To investigate the expression of IRE1 (inositol-requiringenzyme 1) and JNK (c-Jun N-terminal kinase) in lung tissue of cigarette smoke (CS) induced chronic obstructive pulmonary disease (COPD) rats.

Methods: Adult male Wistar rats (n=40) were randomly divided into 4 groups with 10 rats in each group: control group, CS-2 group (exposed to CS for 2 months), CS-4 group (exposed to CS for 4 months) and ex-smoking group (quit smoking for 1 month after exposed to CS for 4 months). The percentage of forced expiratory volume in 0.3 seconds to forced vital capacity (FEV0.3/FVC) and peak expiratory flow (PEF) were measured. TUNEL assay was used to detect apoptotic cells. Immunohistochemistry and Western blotting were used to detect the expression of IRE1, P-IRE1, JNK and P-JNK proteins.

Results: The pulmonary of function greatly decreased in the rats exposed to CS for 2 months in comparison with control group ($P<0.05$), markedly decreased in the rats exposed to CS for 4 months as compared with the rats after exposure to CS for 2 months ($P<0.05$) and was improved little in ex-smoking rats ($P>0.05$). The apoptotic cells were markedly increased in the rats exposed to CS for 2 months and were ever more in the rats exposed to CS for 4 months. The apoptotic cells were alveolar epithelial cell I(ACEI), ACE II, vascular endothelial cells and bronchial epithelial cells. The protein level of p-IRE1 (phospho-inositol-requiringenzyme 1) and p-JNK (phospho-c-Jun N-terminal kinase) were remarkably increased in the rats after exposure to CS for 2 months compared with the control rats ($P<0.05$), significantly elevated in the rats exposed to CS for 4 months ($P>0.05$).

Conclusion: CS promotes the development of COPD by inducing the expression of endoplasmic reticulum stress-associated protein IRE1 and JNK.

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Keywords: Cigarette smoke (CS); Endoplasmic reticulum stress; Chronic obstructive pulmonary disease (COPD); Apoptosis

Introduction

Chronic obstructive pulmonary disease (COPD) is a common disease that seriously endangers human health. Its condition often develops progressively and eventually leads to the loss of working ability of patients [1]. Oxidative substances in cigarette smoke (CS) can induce apoptosis of lung structural cells and promote the development of COPD by endoplasmic reticulum stress (ERS) [2-5]. IRE1 (inositol-requiringenzyme 1) and JNK (c-Jun N-terminal kinase) are almost undetectable under normal physiological conditions, but expression are significantly elevated in the ERS state and are hallmark of ERS [6]. In this study, the expression of IRE1 and JNK signaling pathways in COPD model rats was detected to explore the role of smoking-induced endoplasmic reticulum stress in the pathogenesis of COPD and provide a new direction for the prevention and treatment of COPD.

Materials and Methods

Animals

Forty clean-grade male Wistar rats (provided by Experimental Animal Center of Hunan University of Traditional Chinese Medicine) were certified as SCXK (Hunan) 2013-0003, weighing (200 ± 20) g and aged 10 weeks. Rats were randomly divided into 4 groups with 10 rats in each group: control group, CS-2 group (exposed to cigarette smoke for 2 months), CS-4 group (exposed to cigarette smoke for 4 months) and Ex-S group (quit smoking for 1 month after exposed to cigarette smoke for 4 months). The COPD model rat was established by the known method [3]. Rats were exposed to 10 cigarettes twice a day in a self-made contamination box (control group rats were also exposed to the contamination box for false exposure), one hour at a time, two times more than four hours apart. Four cigarettes were burned at first, followed by two cigarettes each time, each cigarette burned for about 12 minutes, and the cigarette change interval was 3 minutes.

The smoking group smoked for 2 months and 4 months respectively, and the smoking cessation group rats were exposed to normoxia for 1 month after smoking for 4 months.

Experimental materials and instruments

Furong brand cigarettes (Hunan Zhongtobacco Industry Company, nicotine 1.0 mg/cigarette, tar 12 mg/cigarette). TUNEL kit purchased from Wuhan Boster Biology Company. The antibodies including IRE1 (inositol-requiringenzyme 1), p-IRE1 (phospho-inositol-requiringenzyme 1), JNK (c-Jun N-terminal kinase) and p-JNK (phospho-c-Jun N-terminal kinase) all come from Santa Cruz, America. Total protein extraction kit, protein concentration detection kit, immunohistochemical kit and in situ hybridization kit were purchased from Wuhan Boster Biology Company. Western blot chemiluminescence agent purchased from Shanghai Bi Yun Tian Biotech Corp. Small animal pulmonary function test system and HX200 respiratory flow transducer were purchased from Beijing Xingye Science and Trade Company. PAS900 pathological image analysis system is purchased from Jiangsu Langa bioengineering company. GIS-2010 gel imaging analysis system is purchased from Shanghai Tian Neng Technology Company.

Determination of pulmonary function

Forced expiratory volume in 0.3 second (FEV 0.3) occupied forced vital capacity (FVC) and peak expiratory flow (PEF)were measured with a small animal pulmonary function test system. Rats were anesthetized by intraperitoneal injection of 1% pentobarbital. The trachea of the rats was exposed and the artificial trachea was inserted into the trachea of the rats. After a period of quiet breathing, at the end of exhalation, the rats were injected with a syringe through a three-way tube into 6 mL air (equivalent to forced inhalation), then immediately released, connected with negative pressure (-25 cmH_2O, 1 cmH_2O=0.098 kPa) to exhale (equivalent to forced exhalation). The indicators of lung function in rats were processed by computer.

Retention of lung tissue specimens

The rats of CS-2 group and CS-4 group were killed by bleeding together with the rats of control group and Ex-S group. The rats' trachea and lungs were exposed, the right main bronchus was ligated, and 4% paraformaldehyde was injected into the left lung through a tracheotomy opening with a syringe. After the left lung was re-expanded, the left main bronchus was ligated. The right lung was removed and placed in liquid nitrogen for rapid freezing. The left lung was removed and immersed in 4% paraformaldehyde, and then paraffin embedded.

Observation of lung tissue morphology

Tissue paraffin blocks were sliced and thickness was 4 μm. Each rat chose 3 paraffin sections of lung tissue. Tissue specimens were dewaxed with xylene and ethanol, stained with hematoxylin, differentiated with ethanol hydrochloride, re-stained with eosin solution, and then dehydrated with transparent seals. Specimens were observed under optical microscope.

The apoptotic cells in lung tissue were detected by TUNEL assay.

Formalin-fixed, paraffin-embedded lung tissue sections were digested with proteinase K for 20 minutes at room temperature and then incubated with 3% H_2O_2 in methanol for 30 minutes at room temperature. This part is incubated with TDT enzyme solution for 1 h at 37°C. In the negative control study, phosphate buffered saline was used in place of the TDT enzyme solution according to the manufacturer's instructions. Peroxidase activity is visualized by the color reaction of diaminobenzidine. Finally, the sections were stained with hematoxylin and then installed. Apoptosis rate was determined by PIPS-2020 pathological image analysis system.

The expression of target protein in lung tissue was detected by immunohistochemistry.

Formalin-fixed, paraffin-embedded lung tissue sections were digested with 3% H_2O_2 for 7 minutes at room temperature and then pre-incubated with 10% non-immune serum. Sections were incubated overnight at 4°C with specific polyclonal antibodies (1:100). In the negative control study, phosphate buffered saline was used instead of the antibody. After washing the unbound antibody, the sections were incubated with the corresponding secondary antibodies and then incubated with streptavidin peroxidase. Subsequently, peroxidase activity was observed by a color reaction using diaminobenzidine. Finally, staining with hematoxylin and sections was performed. Protein expression levels were measured using a PIPS-2020 pathology image analysis system based on the absorption of positive signals in the alveolar wall.

The expression of target protein in lung tissue was detected by Western blot analysis.

Total protein was extracted from lung tissue using a total protein extraction kit according to the manufacturer's instructions. Protein concentration was detected using a protein detection kit. 30 μg of total protein was separated using a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a Hybond-enhanced chemiluminescent nitrocellulose membrane. The membrane was blocked with 5% (w/v) instant skim milk and 0.1% Tween-20 for 1 h at room temperature and then incubated overnight at 4°C with specific antibodies diluted 1:500. The membrane was then incubated with peroxidase-conjugated immunoglobulin G diluted 1:2000. Signal detection is performed using an enhanced chemiluminescence detection kit. TANON Gel Image System version 3.74 was used for quantification.

Statistical analysis

Data were expressed as mean ± SD. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA), and statistical differences between the two groups were analyzed by Newman-Kells test. $P<0.05$ was considered statistically significant.

Results

Changes of lung function in rats

The lung function FEV0.3 (forced expiratory volume in 0.3 second)/FVC (forced vital capacity) and PEF (peak expiratory flow) of the CS-2 group were significantly lower than those of the control group ($P<0.05$), and the lung function of the CS-4 group was further significantly decreased ($P<0.05$). The lung function of Ex-S group was higher than that of CS-4 group, but there was no significant difference (TABLE 1).

TABLE 1. **Results of lung function test in each group.**

Group	n	FEV0.3/FVC (%)	PEF(ml/s)
Control group	10	83.47 ± 4.98	40.11 ± 3.66
CS-2 group	10	75.48 ± 2.57*	33.42 ± 3.21*
CS-4 group	10	66.18 ± 4.12*▲	25.89 ± 2.22*▲
Ex-S group	10	69.35 ± 2.45*▲	28.41 ± 2.30*▲
F	42.624	46.400	
P	<0.01	<0.01	

Note: Results of lung function test in each group. CS-2 group: exposed to cigarette smoke for 2 months. CS-4 group: exposed to cigarette smoke for 4 months. Ex-S group: quit smoking for 1 month after exposed to cigarette smoke for 4 months. Data were expressed as mean ± SD. *$P<0.05$ VS control group; ▲$P<0.05$ VS CS-2 group.

Pathological changes of lung tissue

Compared with the control group, the lung tissue structure of rats in the CS-2 group was disordered, and some alveolar cells were ruptured. The lung tissue structure of rats in the CS-4 group was more disordered, the alveolar wall was ruptured, the alveolar space was enlarged, and it was fused into a large bullae. The alveolar cavity of the rats in the Ex-S group was enlarged, but there was no significant change compared with the CS-4 group (FIG. 1).

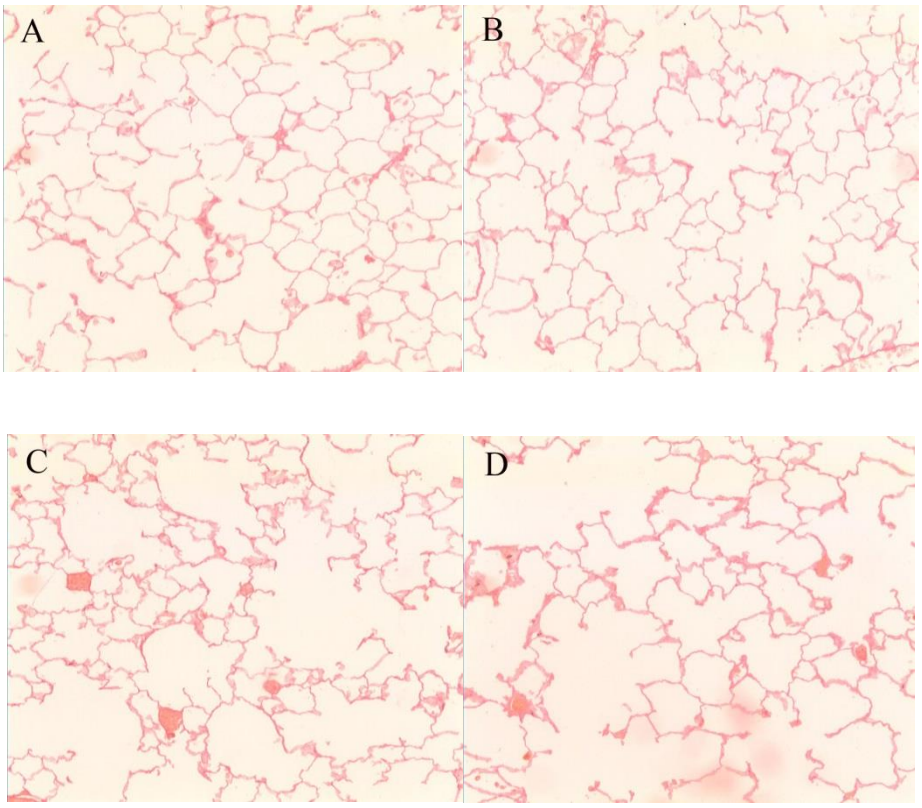

FIG. 1. **Morphological characteristics of lung tissue of each group of rats. HE stains for control group and the goups (×200). A: control group; B: CS-2 group; C: CS-4 group; D: Ex-S group.**

Structure cell apoptosis of lung tissue

Apoptosis of alveolar epithelial cells and endothelial cells increased in rats exposed to cigarette smoke (CS). TUNEL assay showed that the apoptosis rates of control group, CS-2 group; CS-4 group and Ex-S group were 11.445 ± 1.725%, 22.401 ± 1.616%, 34.831 ± 3.202% and 31.37 ± 1.821%.

Apoptotic cells are mainly lung tissue cells, including lung epithelial cells and vascular endothelial cells (FIG. 2).

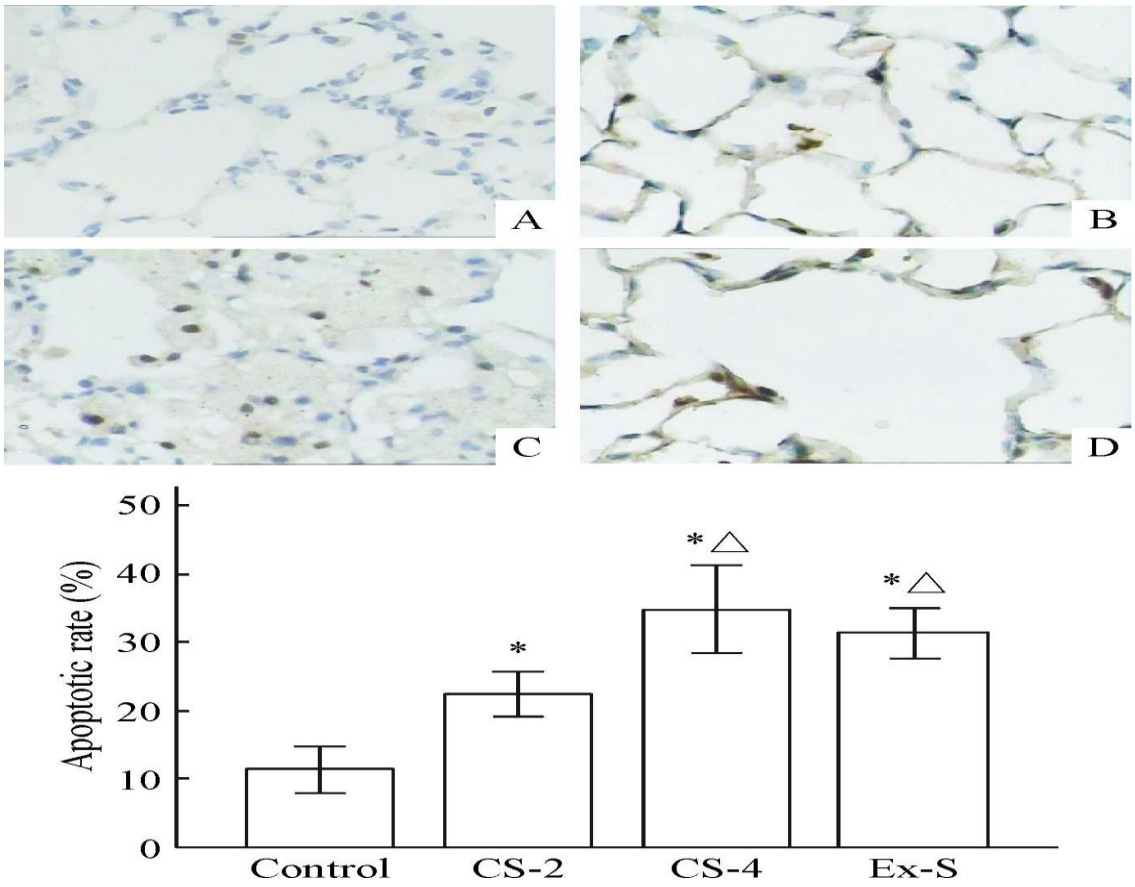

FIG. 2. **Cell apoptotic in rats after exposure to CS (×200). The rat lungs were sectioned and the apoptotic cells (yellow) were observed by the TUNEL method. A: control group; B: CS-2 group; C: CS-4 group; D: Ex-S group. *P<0.05 VS control group; △P<0.05 VS CS-2 group.**

Expression of IRE1, P-IRE1, JNK and P-JNK proteins in lung tissue

Immunohistochemical light microscopy showed IRE1, P-IRE1 protein was mainly expressed in the cytoplasm of vascular endothelial cells and alveolar epithelial cells. There was no difference in the expression of IRE1 protein between the control group and the smoking group. P-IRE1 protein was weakly expressed in the control group, CS-2 group was higher than the control group, there was a significant difference in the expression of CS-4 group was more obvious, Ex-S group and CS-4 group rats expression was not significantly different. JNK protein was mainly found in the cytoplasm of bronchial, vascular endothelial cells and alveolar epithelial cells. There was no significant difference in the expression of each group. P-JNK was mainly expressed in the nucleus, P-JNK protein was weakly expressed in the control group, CS-2 group rats were higher than the control group, there was a significant difference, CS-4 group increased more significantly, EX-S group rats and CS-4 group rats expression was not significantly different (FIG. 3).

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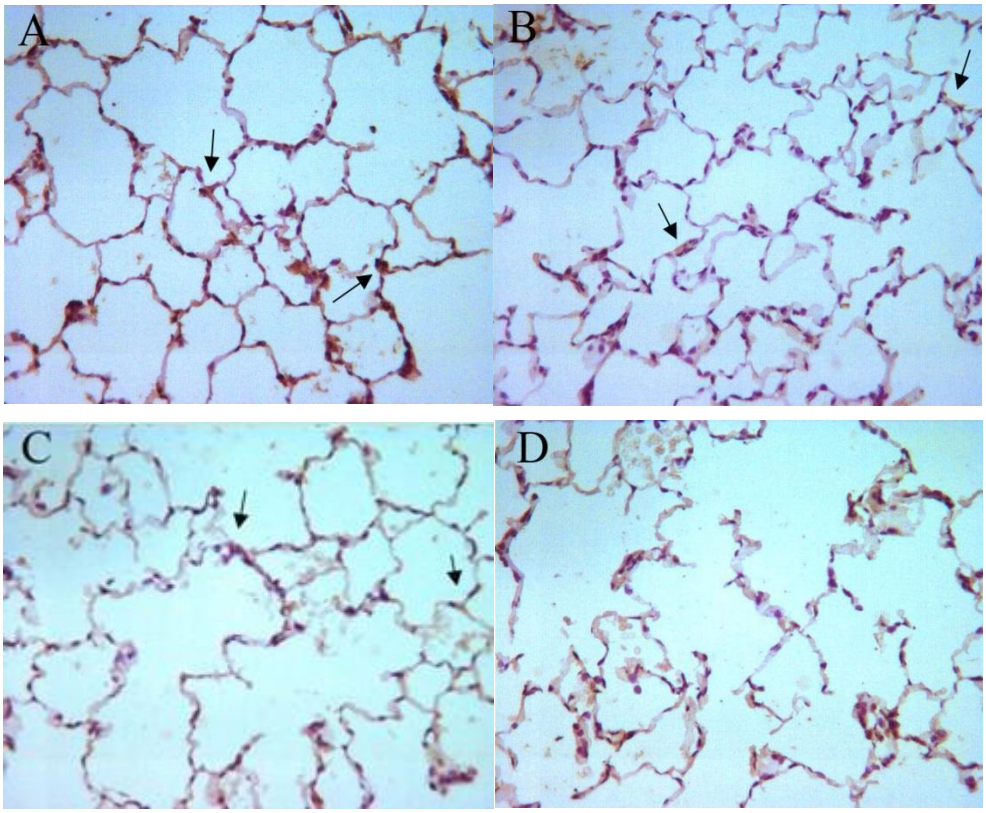

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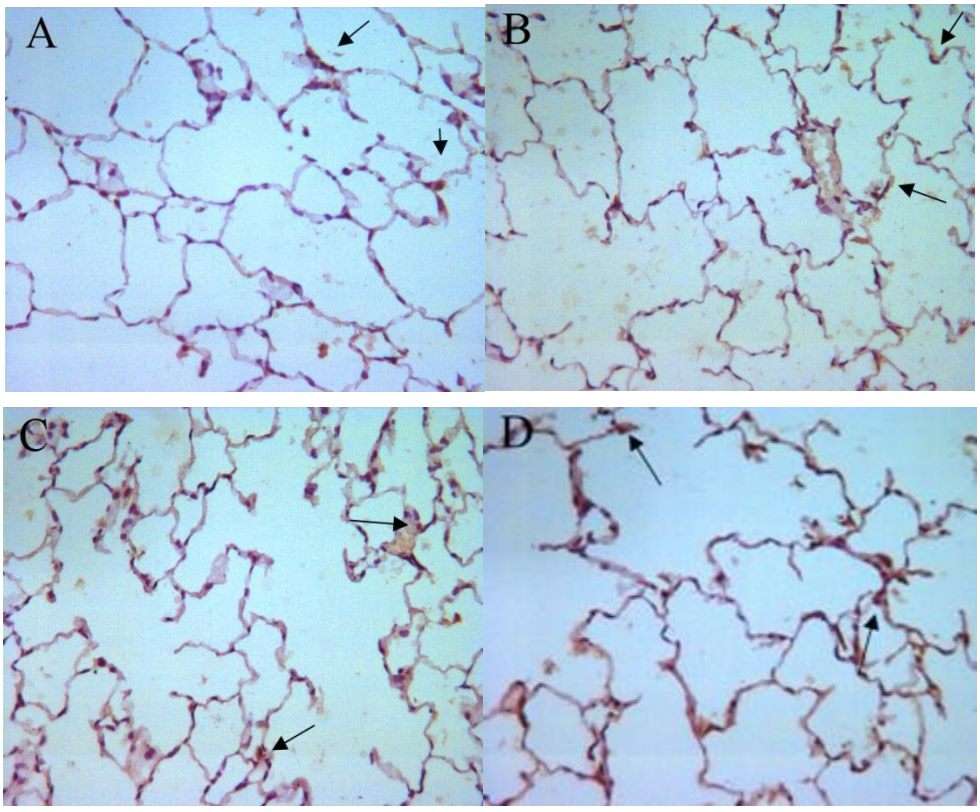

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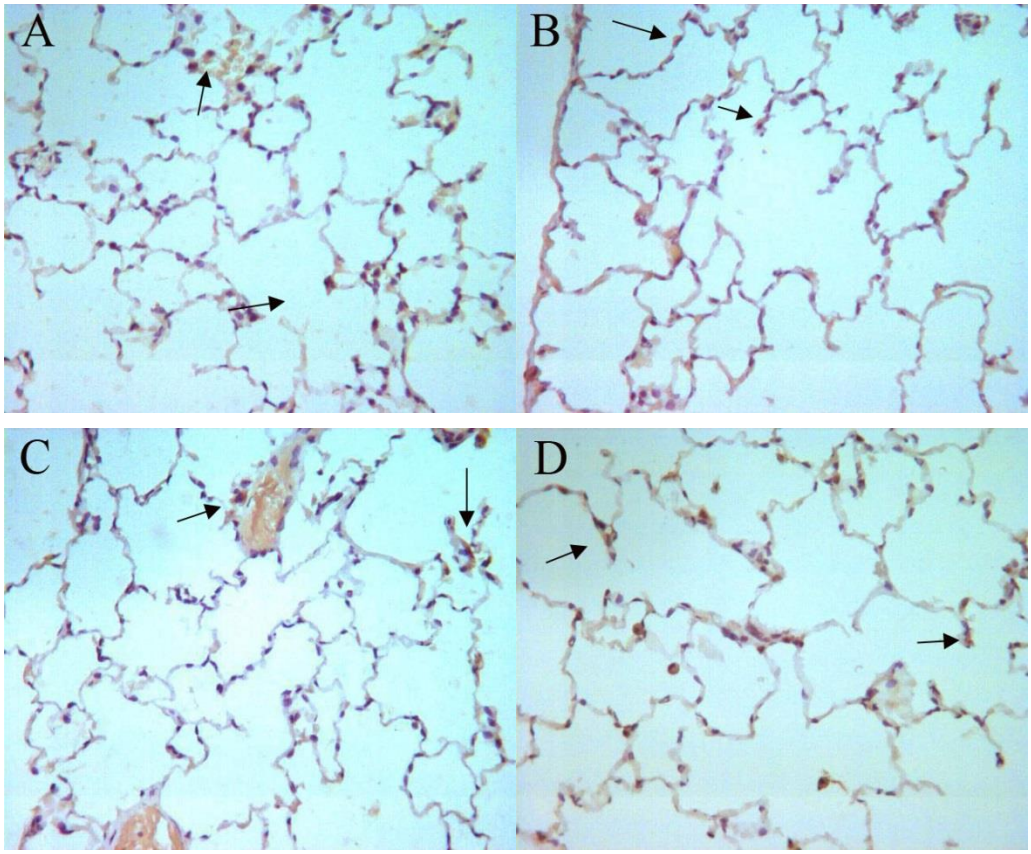

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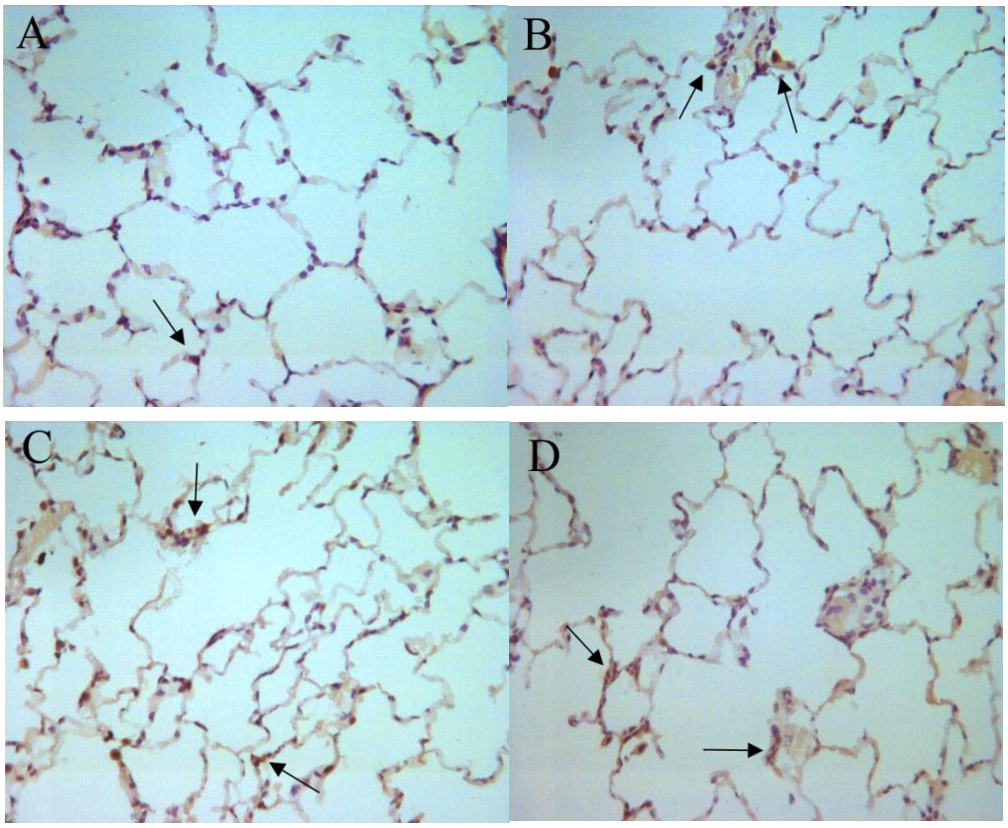

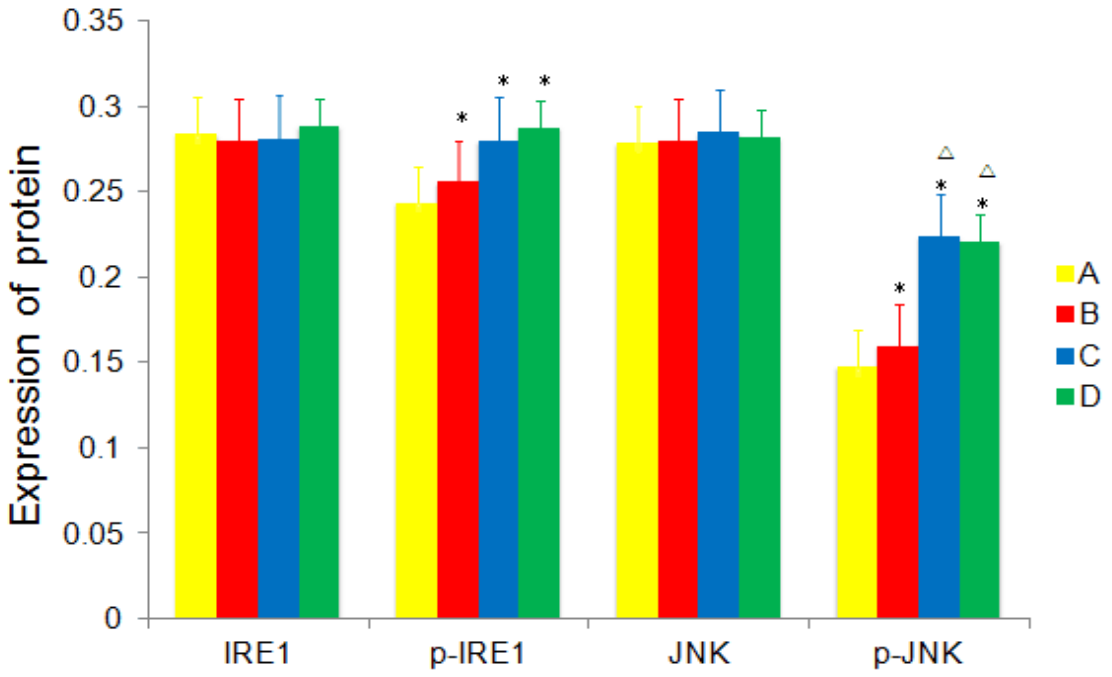

FIG. 3. **Immunohistochemistry assay was used to visualize the expression of IRE1, p-IRE1, JNK and p-JNK protein in lung tissues of each group .I. Rat's lung tissues were sectioned and subjected to Immunohistochemisty assay of IRE1 protein, the IRE1 protein mainly located in cytoplasm (×400). II. Immunohistochemisty assay detected p-IRE1 protein, the p-IRE1 protein was mainly located in cytoplasm (×400) .III. Immunohistochemisty assay detected JNK protein, the JNK protein was mainly located in cytoplasm (×400), and had a small amount of expression in the nucleus. IV. Immunohistochemisty assay detected p-JNK protein, after phosphorylation activation, JNK rapidly transferred to the nucleus, the p-JNK protein was mainly located in nucleus (×400). A: Control group; B: CS-2 group; C: CS-4 group; D: Ex-S group. *P<0.05 VS control group; △P<0.05 VS CS-2 group.**

Western blot results showed that there was no significant difference in IRE1 and JNK expression in each group. Compared with the control group, the expression of P-IRE1 and P-JNK in CS-2 group was significantly up-regulated, while the expression of P-IRE1 and P-JNK in CS-4 group and EX-S group were further increased. The expression of P-IRE1 and P-JNK protein in EX-S group and CS-4 group were not significantly different (FIG. 4).

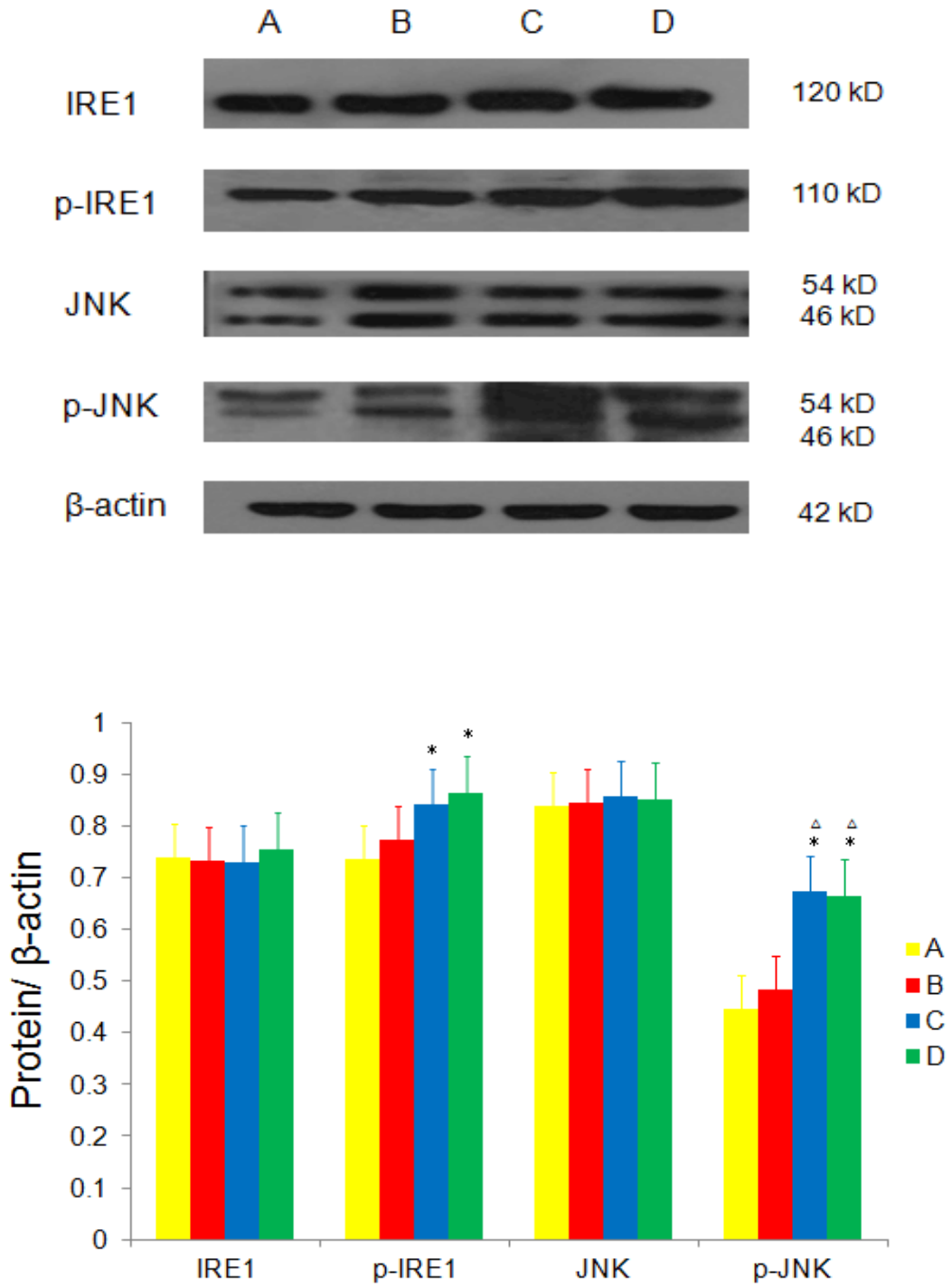

FIG. 4. **Western blot assay was used to detect the expression of IRE1, p-IRE1, JNK and p-JNK protein in lung tissues of each group. A: control group; B: CS-2 group; C: CS-4 group; D: Ex-S group. *P<0.05 VS control group; ▵P<0.05 VS CS-2 group.**

Linear correlation analysis between different parameters for COPD model rats

Correlation analysis was between P-IRE1 and P-JNK protein expression and lung function and structural cell apoptosis rate in lung tissue in rats. The results showed that P-IRE1 protein expression was positively correlated with P-JNK protein expression, P-IRE1 and P-JNK expression was positively correlated with apoptosis rate, P-IRE1 and P-JNK expression was negatively correlated with lung function (TABLE 2A and 2B).

TABLE 2A. **Linear correlation analysis between different parameters for COPD model rats.**

Parameters	Apoptosis rate	FEV0.3 (%)	P-JNK	P-IRE1
Apoptosis rate	—	-0.850*	0.456*	0.668*
FEV 0.3(%)	-0.850*	—	-0.384*	-0.613*
P-IRE1	0.668*	-0.613*	0.747*	—
P-JNK	0.456*	-0.384*	—	0.747*

Note: Correlation analysis was between p-IRE1 and p-JNK protein expression in Immunohistochemistry and lung function and structural cell apoptosis rate in lung tissue in rats. The results showed that p-IRE1 protein expression was positively correlated with p-JNK protein expression, p-IRE1 and p-JNK expression was positively correlated with apoptosis rate, p-IRE1 and p-JNK expression was negatively correlated with lung function. *P<0.01.

TABLE 2B. **Linear correlation analysis between different parameters for COPD model rats.**

Parameters	Apoptosis rate	FEV0.3 (%)	P-JNK	P-IRE1
Apoptosis rate	—	-0.850*	0.624*	0.664*
FEV	0.3(%)	-0.850*	-0.582*	-0.614*
P-IRE1	0.664*	-0.614*	0.779*	—
P-JNK	0.624*	-0.582*	—	0.779*

Note: Correlation analysis was between p-IRE1 and p-JNK protein expression in Western blot and lung function and structural cell apoptosis rate in lung tissue in rats. The results showed that p-IRE1 protein expression was positively correlated with p-JNK protein expression, p-IRE1 and p-JNK expression was positively correlated with apoptosis rate, p-IRE1 and p-JNK expression was negatively correlated with lung function. *P<0.01.

Discussion

Chronic obstructive pulmonary disease (COPD) has attracted more and more attention in recent years, because of its high morbidity and mortality, it has increasingly become a major burden of modern society [1]. In recent years, it has been found that apoptosis of pulmonary structural cells exists in COPD patients, which means that apoptosis of pulmonary structural cells is involved in the pathological process of COPD. This finding was also confirmed in COPD animal models.

The main pathological changes of COPD are chronic bronchitis and emphysema. The causes of emphysema include inflammation, oxidative stress and imbalance of protease-antiprotease [5]. Compared with the control group, the staining results showed that the alveolar structure was disordered, the alveolar wall became thinner, and finally fused into big alveoli. CS-4 group, the lung structure of the animals was significantly damaged, and the alveoli fused in large numbers.

Combined with lung function, it was suggested that COPD animal model was successfully established by passive smoking.

Endoplasmic reticulum (ER) is an organelle of eukaryotic cells. It is responsible for the folding and processing of membrane proteins and secreted proteins. Changes in the internal and external environment will lead to the accumulation of unfolded or misfolded proteins, and lead to endoplasmic reticulum stress (ERS) [6,7]. This process is mainly accomplished through three signaling pathways mediated by the three membrane proteins inositol requiring 1 (IRE1), activated transcription factor 6 (ATF6) and double strand RNA-dependent protein kinase-like ER kinase (PERK) in the endoplasmic reticulum. When stress persists or is too strong, cells will trigger apoptosis mechanisms and initiate apoptosis. Endoplasmic reticulum stress induced apoptosis is called ERS-induced apoptosis (ERSIA) [8-12]. There are three signaling pathways involved in the completion of ERSIA: CHOP signaling pathway, JNK signaling pathway and Caspase-12 (4) signaling pathway.

Smoking is the most important reason for the pathogenesis of COPD. There are more than 4000 substances in cigarette smoke, including carcinogens, oxides and aldehydes, which have the potential to cause cell damage and apoptosis [4]. Respiratory epithelial cells act as the first barrier to respiratory defense, and smoking can induce endoplasmic reticulum stress response in human respiratory epithelial cells [3,11]. In this study, both immunohistochemistry and Western blot assay confirmed that endoplasmic reticulum stress occurred in the lungs of passive smoking rats. IRE1 was activated in endoplasmic reticulum stress response, and the expression of P-IRE1 and P-JNK was upregulated. The expression of p-IRE1 and p-JNK was positively correlated with apoptosis rate and negatively correlated with lung function. The results suggest that the IRE1 pathway activates the JNK pathway and induces apoptosis in pulmonary endoplasmic reticulum stress induced by cigarette smoke. If we intervene in endoplasmic reticulum stress pathway, we can delay the development of COPD. If we look for appropriate genes to intervene in the endoplasmic reticulum stress pathway, COPD can be treated [2].

Conclusion

In summary, in addition to protease-antiprotease imbalance, inflammation and oxidative stress, apoptosis, as a possible mechanism of COPD, has attracted more and more attention in recent years. Our previous studies have confirmed that smoking promotes the activation of the PERK/CHOP signaling pathway and promotes the apoptosis of lung structural cells, thereby promoting the occurrence and development of COPD [2,12-15]. This study further confirmed that smoking also activates IRE1 signaling pathway and JNK pathway, and induces apoptosis of pulmonary structural cells, promoting the development of COPD. Further study of the detailed mechanism of IRE1 and JNK pathways in COPD may provide a new direction for the treatment of COPD.

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