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Adiponectin attenuates endoplasmic reticulum stress and alveolar epithelial apoptosis in COPD rats

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Abstract. – OBJECTIVE: The present study was designed to evaluate the effect of Adiponectin (APN) against alveolar epithelial apoptosis in chronic obstructive pulmonary disease (COPD) rat models.

MATERIALS AND METHODS: Thirty-six male Sprague-Dawley (SD) rats were randomly assigned to three groups: Sham group, COPD group, and COPD + APN group (2.5 ug/kg/day). To assess the effect of APN, histopathological evaluations, lung function, and the apoptotic index (AI) of alveolar septal cells, were performed. In addition, the levels of oxidative stress and endoplasmic reticulum stress were measured.

RESULTS: HE staining demonstrated that APN inhibited pathological injury in COPD rats. In addition, APN could restore the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) in serum. APN also inhibited the levels of endoplasmic reticulum stress pathway including CHOP, phospho-JNK and Caspase-12 in alveolar epithelial cell. Furthermore, APN significantly inhibited the protein levels of Caspase-3 and apoptosis in alveolar epithelial cell of COPD rats.

CONCLUSIONS: Our findings suggested that APN might effectively ameliorate the progression of COPD via inhibiting the endoplasmic reticulum stress-induced alveolar epithelial apoptosis in rats.

Key Words:
Adiponectin, CDPD, Endoplasmic reticulum stress, Alveolar epithelial cell apoptosis.

Introduction

Chronic obstructive pulmonary disease (COPD) is a kind of disease that can be prevented and treated, characterized by persistent airflow limitation in progressive development, which is related to the enhanced chronic inflammatory response of airways and lung tissues to tobacco smoke and other harmful gases or particles; the acute exacerbation and complications affect the course of disease. According to the large-sample survey in China in 2007, the prevalence rate of COPD was 8.2% in people aged above 40 years old[1]. COPD has a high disability and death rate and has become the third leading cause of death in the world[2]. The repeated acute exacerbation of COPD caused by virus or bacterial infection, etc.[3,4], accelerates the progression of disease, reduces the life quality of patients, and increases the death rate[5-7].

Major pathological changes of COPD include the bronchial inflammation and emphysema[8], and it is currently believed that COPD is related to the chronic inflammation, oxidative stress and protease-anti-protease system imbalance in airway and lungs. In recent years, studies have found that the increased apoptosis of alveolar epithelial cells, airway epithelial cells, and other lung tissue cells, are also an important pathogenesis of COPD[9,10], among which oxidative stress is an important pathogenesis of COPD[11,12]. The oxidant and antioxidant system in normal human body is in an equilibrium state, but oxidative stress will be caused in the body when the number of reactive oxygen species increases greatly far beyond the scavenging capacity of antioxidant in lung tissues. This will lead to oxidative stress in the body, thus resulting in airway injury, inflammatory response, protein carbonation, anti-protease inactivation, lipid peroxidation, apoptosis, etc., and further inducing the occurrence and development of COPD[13].

Endoplasmic reticulum is an important organelle in eukaryotes, as well as the main site for intracellular protein synthesis, processing and folding, transport and intracellular calcium storage, which feels the changes in intracellular

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environment in time to maintain the balance of intracellular environment[14]. Some stimulating factors, such as infection, hypoxia, starvation, oxidative stress, calcium balance disorder and physical and chemical stimulation, can induce the acute stress response in cells, increase the synthesis of structural protein or secretory protein, increase the protein synthesis load in endoplasmic reticulum, change the internal environment in endoplasmic reticulum, make the unfolded and misfolded protein gather in endoplasmic reticulum lumen, damage the normal function of endoplasmic reticulum and lead to endoplasmic reticulum stress (ERS)[15,16]. ERS is a self-protection response of cells to the outside harmful stimulation. However, when ERS is too serious or the harmful stimulation lasts too long, the expressions of apoptosis-related genes will be upregulated, the apoptotic signaling pathways will be initiated, and the damaged cells will be apoptotic eventually[17]. ERS can induce apoptosis through three ways: (1) activation and transcription of CCAAT/enhancer binding protein homologous protein (CHOP) gene; (2) activation of C-Jun N-terminal kinase (JNK) pathway; (3) activation of Caspase-12 specific in endoplasmic reticulum[18]. In recent years, studies have confirmed that ERS and its related apoptosis play important roles in the pathogenesis of COPD. Adiponectin (APN) has been found as a kind of protein mainly secreted by adipocytes in recent years, and its anti-inflammatory, anti-atherosclerosis, glucolipid metabolism-regulating and memory and cognitive disorder-regulating effects, have been reported in many studies[19-22]. At present, its biological characteristics have been attached increasingly more importance, and some studies have shown that APN can inhibit oxidative stress and protect bronchial epithelial cells and myocardial tissues[23,24]. However, there are few studies on the relationship between APN and COPD, so APN was used to intervene in COPD rats in this study.

Materials and Methods

Animal Model and Grouping

36 male SD rats (140-160 g) were provided by the First People's Hospital of Xuzhou Animal Center. They were arisen in polycarbonate cages and kept with 12-hour light-dark cycle and continuous access to food and water. Instillation of LPS together with cigarette smoke exposure were used to establish the COPD model in rats as previously described[25,26]. Briefly, after the rats were anesthetized with chloral hydrate (250 mg/kg), the LPS (1 mg/mL, 0.2 mL, Sigma-Aldrich, St. Louis, MO, USA) was dripped through intratracheal instillation on the 1st, 15th and 30th day during this experiment. All rats, except the sham group, were placed in a sealed box and exposed twice a day to smoke from 6 commercial unfiltered cigarettes (ChungHua Cigarettes, Shanghai, China). The treatment was sustained for 60 days. Rats in sham group were treated with air instead. Rats were divided into three groups: (1) Sham group: rats were intraperitoneally injected with normal saline (1 mL/day, ip) for 60 days; (2) COPD group: COPD rats were treated with normal saline (1 mL/day, ip) for 60 days (1 mL, ip); (3) COPD with APN group: COPD rats were treated with APN for 60 days (2.5 μg/kg/day dissolved in 1 mL normal saline, ip, Sigma-Aldrich, St. Louis, MO, USA). The study was approved by the Animal Ethics Committee of The First People's Hospital of Xuzhou Animal Center.

Measurement of Lung Function

6 rats from each group were randomly selected for measurements of lung function; after they were anesthetized with chloral hydrate, the trachea cannula was performed and connected with the small animal ventilator. The variables were observed by a spirometer, which is recommended for small animals; FEV0.3/FVC% and PEF were measured.

Measurement of SOD and MDA

Blood was collected and separated in a refrigerated centrifuge and the samples were centrifuged at 3000 rpm for 20 min at 4°C. The activities of SOD and the content of MDA were measured according to the manufacturer's instruction (Beyotime, Shanghai, China).

Histopathological Analysis

The middle or upper lobe of the right lung was excised from each rat and fixed in 4% formalin. The samples were cut into 5 μm sections followed with embedded in paraffin. Hematoxylin-Eosin (HE) was used to stain the paraffin sections. After that, a light microscope was used to visualize the pathological conditions in the lung tissues on randomized sections. Measuring the mean linear intercept (MLI) and mean alveoli number (MAN) was used to assess emphysema[27].

Tunel Assay

The apoptosis of alveolar epithelial was detected by tunel assay according to the manufacturer (Roche, Basel, Switzerland). Horseradish peroxidase (HP)-mediated diaminobenzidine reaction was used to visualize the TUNEL-positive cells, following the counterstain. Fields were photographed at a magnification of 200× and were randomly selected. The apoptosis index was used to measure the degree of apoptosis.

Western Blot

The lung tissues were frozen and stored at -70°C. Next, they were homogenized and added with lysis buffer in which phosphatase and protease inhibitors were included. Protein centration was measured by bicinchoninic acid (BCA) method, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the tissue lysates. After blotting onto polyvinylidene difluoride (PVDF) membrane, samples were incubated with specific antibodies against Chop, phospho-JNK, JNK, Caspase-12, Caspase-3 and β-actin (1:1000) (Cell Signaling Technology, Danvers, MA, USA). Next, the samples were incubated at 4°C overnight. The respective secondary antibodies conjugated to HRP were incubated for 1 h, followed by three-time washing. The membrane was then incubated with ECL (Millipore, Billerica, MA, USA) for luminescence generation. The proteins were visualized and detected, and the grey level of each protein was normalized against to the β-actin. The results were expressed as fold increase compared with the Sham.

Statistical Analysis

All results are presented as the means ± standard deviation (SD). Statistical analyses were performed using both GraphPad Prism 6.02 (GraphPad Software, La Jolla, CA, USA) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare differences among groups. An unpaired *t*-test was used for the comparison between 2 groups. A value of $p < 0.05$ was considered statistically significant.

Results

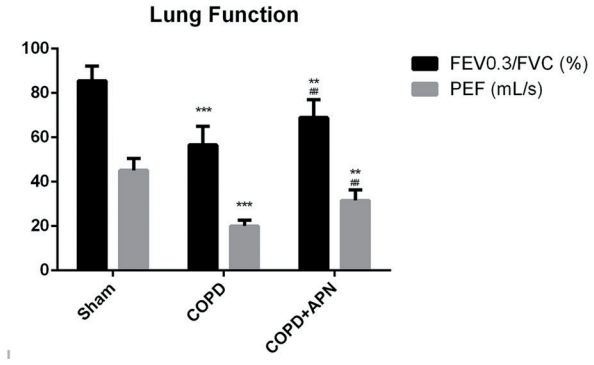
Effects of APN on Lung Function

The lung function was measured by animal spirometer and the result shows that compared with the Sham group, FEV0.3/FVC was decline in the COPD group and APN-treated group ($p < 0.01$) and PEF has the same outcome ($p < 0.01$). However, treatment with APN has a reversal effect ($p < 0.01$) (Figure 1 A).

A

Comparison of lung function in the three groups

Group	FEV0.3/FVC (%)	PEF (mL/s)
Sham	85.57±6.75	45.14±5.37
COPD	56.62±8.41	20.04±2.58
COPD+APN	68.92±8.03	31.53±4.76



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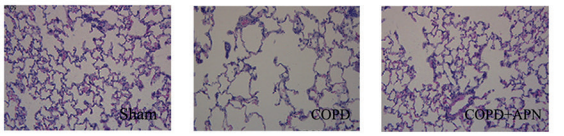

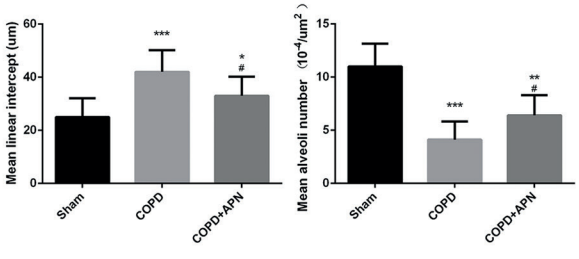

Figure 1. Effects of APN on the Lung Function and Histopathological examination in COPD rats. ***A,*** Effect of APN on the Lung Function in COPD. Compared with Sham group, the lung function was decreased in COPD groups. Compared with COPD group, the lung function was recover in COPD+APN groups (**$p < 0.01$ and ***$p < 0.001$ *vs*. Sham group; $^{\#\#}p < 0.01$ *vs*. COPD group). ***B,*** Effect of APN on the histopathological examination in COPD. The HE staining showed that compared with Sham group, the mean linear intercept (MLI) was increased in COPD groups, while the mean alveoli number (MAN) was decrease. Compared with COPD group, the MLI decreased and the MAN increased after the intervened with APN (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs*. Sham group; $^{\#}p < 0.05$ *vs*. COPD group).

Effects of APN on the Histopathological Examination

The histopathological examination was measured by HE staining; the result analyzed from lung tissue demonstrated that emphysema histologically advanced in COPD groups; in contrast, the histological markers of emphysema, such as MLI and PEF, were normal in Sham group. After intervening with APN, the section from COPD-APN group showed MLI and PEF were significantly lower than COPD group ($p < 0.05$). The result indicated APN has the effect of inhibiting pathological injury from COPD (Figure 1B).

Effects of APN on the Activities of SOD and the Content of MDA

The serum obtained from the COPD groups reveal that the activities of SOD were quite lower compared with the sham group ($p < 0.001$) (Figure 2A). In contrast, the content of MDA was found increased much higher than that in Sham group ($p < 0.01$) (Figure 2B). These results suggest oxidative occurred in COPD models. However, the results from COPD+APN group declare that APN could restore the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) in serum ($p < 0.01$) (Figure 2).

Effects of APN on the Endoplasmic Reticulum Stress

There were three important apoptosis ways involved in ERS: JNK, CHOP and Caspase-12. The protein expression in alveolar epithelial cells was measured by Western blot and the result analysis showed that the protein expression of p-JNK, CHOP and Caspase-12 in alveolar epithelial cells was quite lower in Sham group than COPD groups ($p < 0.001$). These results proved the COPD makes ERS happened in alveolar epithelial cells. The expression of CHOP, p-JNK and Caspase-12 could be decreased after treatment with APN ($p < 0.001$ for JNK and CHOP, $p < 0.05$ for Caspase-12); APN could suppress the ERS induced by COPD in alveolar epithelial cells (Figure 3).

Effects of APN on Alveolar Epithelial Cell Apoptosis

The apoptosis of alveolar epithelial cell was detected by TUNEL staining; the protein expression of Caspase-3 was considered the apoptotic executioner[28]. Both TUNEL staining and Caspase-3 testing demonstrated the apoptosis in alveolar epithelial cell in COPD groups: furthermore, the quantitative analysis of apoptosis index and the ratio of cleaved Caspase-3 against β-actin indicated that the apoptosis level in-

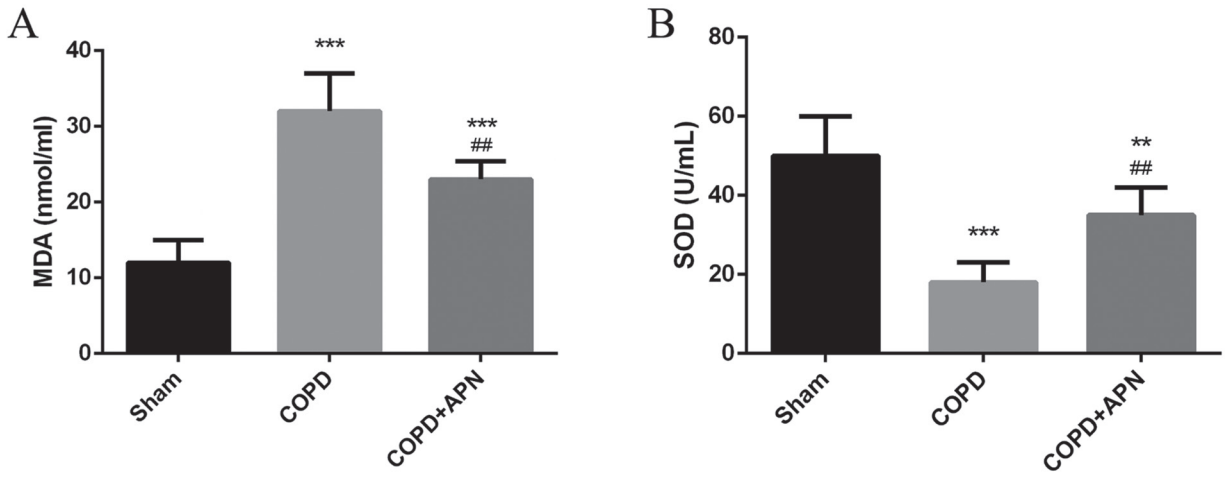

Figure 2. Effects of APN on the activities of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in COPD rats. ***A,*** Effect of APN on the activity of SOD. Compared with Sham group, activities of SOD were significantly decreased in COPD group and COPD+APN group. Compared with COPD group, the activity of SOD was increased in COPD+APN groups. (***$p < 0.001$ *vs*. Sham group; $^{\#\#}p < 0.05$ *vs*. COPD group). ***B,*** Effect of APN on the content of MDA. Compared with Sham group, the content of MDA was significantly increased in COPD groups. Compared with COPD group, the content of MDA was significantly decreased in COPD+APN group. (**$p < 0.01$ and ***$p < 0.001$ *vs*. Sham group; $^{\#\#}p < 0.01$ *vs*. COPD group).

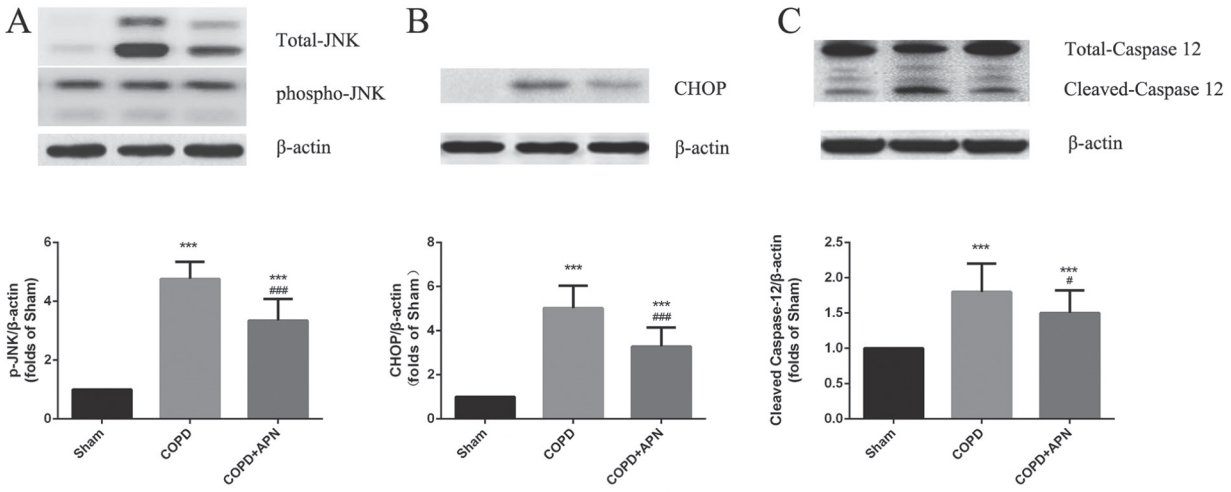

Figure 3. ***A,-C,*** Effects of APN on the endoplasmic reticulum stress (ERS) in alveolar epithelial cell in COPD. The ERS in alveolar epithelial cell was detected by protein expression of JNK, CHOP and Caspase-12. COPD stimulates the protein expression of JNK, CHOP and Caspase-12, while representatives Western blots and densitometry data for the levels of phospho-JNK/JNK, CHOP, and cleaved Caspase-12 in each group. APN administration reduces their increases. (***$p < 0.001$ *vs.* Sham group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ *vs.* COPD group).

crease much more in COPD groups than Sham group ($p < 0.001$), while the consequence could be attenuated by the treatment with APN ($p < 0.01$) (Figure 4).

Discussion

The major pathological changes of COPD are chronic bronchial inflammation and emphysema, and its pathological essence is the decreased elastic function of lung tissues, resulting in excessive expansion of lung tissues and progressive increase of residual volume. With the progression of disease, the lung function declines progressively. At present, the main objective criteria for determining incomplete airway limitation in COPD patients are mainly dependent on the pulmonary function test. The pulmonary function test has an important significance in COPD diagnosis, evalu-

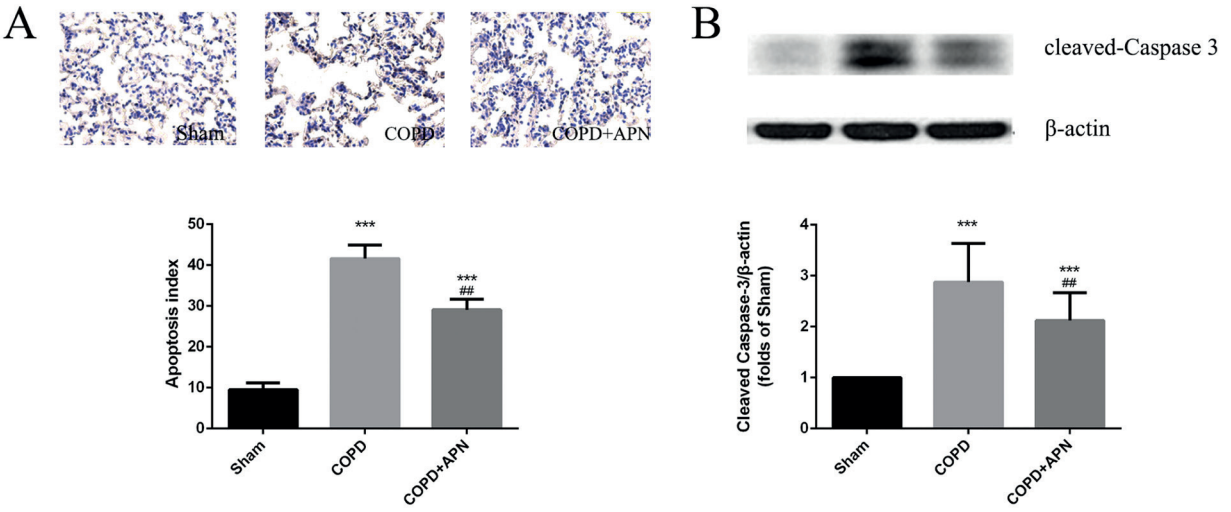

Figure 4. Effects of APN on the apoptosis of alveolar epithelial cell in COPD. The apoptosis of alveolar epithelial cell was detected by TUNEL assay and protein expression of caspase-3. Both TUNEL staining and the cleaved Caspase-3 expression analysis indicated that apoptosis level of alveolar epithelial cell increased in COPD groups than Sham groups. Compared with COPD groups, the apoptosis level reduced in COPD+APN group (***$p < 0.001$ *vs.* Sham group; $^{\#\#}p < 0.01$ *vs.* COPD group).

ation of severity, exacerbation, healing and treatment of disease, etc. Forced expiratory volume in 1 second/forced vital capacity (FEV1/FVC) is able to detect the mild airflow limitation, which is a sensitive index of COPD diagnosis.

Compared with those in normal Sham group, FEV0.3/FVC (%) of rats in model group was significantly decreased, but the forced vital capacity was significantly increased. The differences were statistically significant ($p < 0.05$), indicating that rats in model group had the airflow limitation and obstructive ventilation dysfunction, which was consistent with the pulmonary function changes of COPD rat model. In COPD model group, the alveolar cavity was enlarged, the alveolar wall became thinner and the number of alveoli was reduced. In the intervention group, the alveolar septum of lung tissues was damaged slightly; in sham group, the bronchial mucosa was normal, the alveolar structure of lung tissues was complete without damage to alveolar septum. The pathological features of lung tissues in rats in COPD groups revealed that the alveolar structure was damaged and the alveolar cavity was enlarged, showing the pulmonary emphysema-like changes, but the APN intervention could protect the lung structure and alleviate the emphysema in rats.

Biofuel, cigarettes smoke and air pollution, are the main causes of COPD occurrence and development, and they are the root causes of oxidative stress in the body at the same time[29,30]. Oxidative stress can lead to the progression of COPD through the enhanced gene expression of pro-inflammatory mediator, damage and apoptosis of lung tissue structural cells, protease-anti-protease imbalance, mucus hypersecretion and other mechanisms. The occurrence of oxidative stress is related not only to the increase in reactive oxygen species and oxides, but also to the decreased activity of antioxidant substance in the body[31]. The lungs are in the high-oxygen environment with a large area and abundant blood supply, so they are prone to oxidative stress-mediated tissue damage[32]; the effective assessment of oxidative stress is helpful in evaluating the severity of disease more accurately and in prediction of treatment response and prognosis. There are natural antioxidant systems in the human body, including antioxidant enzymes and non-enzyme substances, which can help to improve the damage of oxidative stress to human body. Superoxide dismutase (SOD) belongs to the antioxidant enzyme, as well as an important member in the endogenous antioxidant in the body, which can be used as a marker for the reserve of antioxidant substances in the body. It is proved in some experiments that in the early stage of COPD, SOD will increase transiently, which may be associated with the long-term exposure to the oxides and increased SOD gene expression[33]. With the progression and repeated acute exacerbation of disease, SOD shows a decreasing trend in the body[34], and it may be related to the great consumption of antioxidants in the body. Malondialdehyde (MDA) is the final product of cell membrane lipid peroxidation and can be detected in bronchoalveolar lavage APNid (BALF), sputum and blood. Studies[35,36] have revealed that the level of MDA in blood of COPD patients is significantly increased compared with that in normal people, which is negatively correlated with FEV1, while SOD is significantly decreased and positively correlated with FEV1[37].

The results of this experiment showed that the MDA level in rats in COPD groups was significantly higher than rats in Sham group. However, SOD activity showed a significant negative correlation, but there were some restitute trends after APN intervention. The above results suggested that oxidative stress occurs in COPD rats; the antioxidant and free radical scavenging capacities of rats are decreased, and APN can alleviate the degree of oxidative stress in rats.

With the deepening of research on COPD mechanism, more and more studies have shown that COPD has a correlation that cannot be ignored with ERS-induced alveolar epithelial cell apoptosis. Caspase-12 is mainly located in outer endoplasmic reticulum membrane in the form of zymogen, which is a key molecule that mediates the apoptotic-signaling pathway of endoplasmic reticulum[38]. ERS leads to the activation of Caspase-12, initiates Caspase cascade reaction and causes the apoptosis via activation of Caspase-3[39], but it is not related to another two typical pathways: death receptor pathway and mitochondrial apoptosis pathway[40,41]. Like Caspase-12, CHOP plays an important role in ERS-induced apoptosis and can promote the apoptosis. CHOP is almost undetectable in normal physiological status and significantly induced in ERS, thus participating in the regulation of apoptosis-related gene expressions, which is considered as a marker molecule of ERS in cells[42].

In this study, the expressions of CHOP, p-JNK, Caspase-12 and Caspase-3 in lung tis-

sues were detected via Western blot, and the apoptosis of alveolar epithelial cells was detected using the TUNEL method. The experimental results revealed that CHOP, p-JNK, and Caspase-12 were expressed in alveolar epithelial cells, and the gray level results present clear growth trends in COPD groups, while the expression of Caspase-3 and apoptosis index in COPD groups were also significantly increased by comparing with Sham group. Therefore, this experiment showed that ERS-induced cell apoptosis in lung tissues did occur in COPD rats, and APN can alleviate the degree of ERS-induced cell apoptosis in rats. APN, also known as adipocyte complement regulatory protein, is a kind of cytokine with the molecular weight of 28-30 kDa, mainly synthesized and secreted by adipocytes, which has anti-inflammatory and/or pro-inflammatory activity[43]. At present, studies on the exact relationship between APN and lung function in COPD are still controversial and further investigations are needed[44-46].

Conclusions

This study provided some references for the effect of APN in the process of COPD through the attenuation of endoplasmic reticulum stress and alveolar epithelial apoptosis.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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