ORIGINAL RESEARCH

Pingchuanning Decotion Alleviates Bronchial Asthma Airway Inflammation Through ROS/HMGB1/Beclin-1 Mediated Cell Autophagy

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ABSTRACT

Objective • Bronchial asthma is a prevalent respiratory disorder characterized by airway inflammation. This study aimed to investigate the protective effect of Pingchuanning decoction (PCN) on airway inflammation in bronchial asthma, focusing on the role of autophagy and its underlying molecular mechanism.

Methods • Using an in vitro lipopolysaccharide (LPS)-induced inflammatory damage model of human airway epithelial cells (16HBE), we assessed the effect of PCN. Various experiments were performed to evaluate the expression of autophagy-related genes, autophagosome and vesicle counts, and reactive oxygen species (ROS) levels.

Results • First, PCN reduced LPS-induced cellular inflammation. Second, PCN decreased the number of autophagosomes and autophagic vesicles. And third, PCN significantly reduced reactive oxygen species (ROS) levels. Most importantly, PCN also down-regulated LPS-induced expression of HMGB1, Beclin-1, and autophagy-related gene 5 (ATG5) while enhancing the expression of B-cell lymphoma 2 (Bcl-2), which further reduced the LC3II/I ratio.

Conclusion • PCN reduces the 16HBE inflammatory response by inhibiting the overexpression of ROS/HMGB1/Beclin-1 mediated cell autophagy. Therefore, it may serve as a potential drug for treating bronchial asthma. (*Altern Ther Health Med.* 2024;30(1):270-277).

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INTRODUCTION

Bronchial asthma, a chronic inflammatory airway disease affecting millions globally, has seen rising incidence rates, particularly due to factors like aging and environmental pollution. In the past 25 years, with the gradual intensification of global aging, the incidence of asthma has also grown annually, which means that asthma has become one of the most common chronic diseases in the world. The number of people with bronchial asthma worldwide has exceeded 358 million. China has significant asthma morbidity and mortality rates. There are around 30 million asthma sufferers, with a prevalence rate of 0.5% and a mortality rate of 1.6-36.7/100000, which significantly impacts how people live. Therefore, as a public health problem, asthma's huge medical and nursing costs have caused a serious economic burden for society and individuals. In the past few years, the asthma control rate has improved dramatically due to the increased awareness of asthma and the promotion of standardized asthma treatment. Inhaled corticosteroids can relieve the clinical symptoms of asthma but cannot cure asthma. Therefore, researchers need to deeply study the molecular mechanisms of aetiology and pathogenesis to provide new targets and ideas for the diagnosis and treatment of asthma.

Asthma is a chronic inflammatory airway illness involving eosinophils, neutrophils, airway epithelial cells, etc.

There are many causes of acute asthma attacks, such as viruses, poor patient compliance, pollution, etc. Its main characteristics include chronic airway inflammation, airway hyperresponsiveness, reversible airflow limitation, and airway remodeling. And there is no clear answer to how asthma is caused and how it works. Asthma is defined as a heterogeneous disease in the latest GINA guidelines. Some scholars have shown that the main mechanism of bronchial asthma is the imbalance of T-helper cell 1 (Th1) and T-helper cell 2 (Th2) expression, which leads to airway hyperresponsiveness, airway inflammation, excessive airway mucus secretion and irreversible airway remodeling. In recent years, the function of cell autophagy in the occurrence and progression of respiratory disorders has been the subject of increasing research. According to numerous investigations, cell autophagy has been implicated in the pathophysiology of chronic obstructive pulmonary disease, acute lung damage, asthma, cystic fibrosis, pneumonia, pulmonary hypertension, pulmonary tuberculosis, and other disorders.[1-3]

Protein of the High Mobility Group (HMG1) Nuclear protein related to chromatin and a common mucin linked to extracellular damage is HMGB1. It is an important autophagy regulator. It has been demonstrated that HMGB1 controls autophagy at the extracellular, cytoplasmic, and nuclear levels, indicating that it is directly implicated in this process. As a result of the activation of numerous defence systems, ROS build up in cells once oxidative stress is produced. In macromolecules and eukaryotic cells, autophagy is a process of organelle destruction and recovery that is linked to oxidative stress. Increased autophagy flux and release in eukaryotic cells are both facilitated by enhanced activation of reactive oxygen species. Reactive oxygen species (ROS) are a signal molecule to activate autophagy under stress and other conditions. These stimuli induce the ROS-dependent translocation of HMGB1 from the nucleus to the cytoplasm during autophagy. Oxidative stress controls HMGB1 release and consequent inflammation.[4-7] Under certain conditions, HMGB1 destroys the binding between Beclin-1 and Bcl-2 through competitive binding with Beclin-1 and dissociates Bcl-2. Nuclear HMGB1 can enter the cytoplasm and interact with the autophagy-related protein Beclin-1 to release it from the Beclin-1/Bcl-2 complex and induce autophagy.[8-10]

PCN is an asthma treatment that has been developed through many years of clinical practice and experimentation. It is composed of 11 herbs such as ephedra, Asarum and Dilong, which can warm lung and resolve phlegm, relieves cough, and asthma. In the last 10 years, we have done a lot of clinical and animal studies on asthma and looked closely at how PCN works to treat asthma. Previous studies found that PCN could reduce the expression levels of nitric oxide (NO), tumor necrosis factor (TNF-a), Interleukin- 5 (IL-5), platelet-activating factor (PAF), granulocyte colony-stimulating factor (GM-CSF) and Interleukin- 4 (IL-4) in bronchoalveolar lavage fluid (BALF) of asthmatic guinea pigs, thereby treating asthma, suppressing airway inflammation and remodeling the airways. Moreover, PCN inhibited the expression of extracellular regulated protein kinases (ERK2) mRNA and C-FOS mRNA in SD rats with asthmatic lung tissue, reducing airway inflammation and remodelling. Therefore, PCN may relieve the clinical symptoms of asthma by alleviating inflammatory injury and airway remodeling. On this premise, we hypothesize that PCN can reduce the formation of reactive oxygen species, diminish the release of HMGB1, change its competitive binding with Bcl-2 to Beclin-1, block autophagy, and so reduce airway inflammation.

Table 1. The herbal composition of Pingchuanning Decoction (PCN).

Chinses name	Scientific name	Family	Drug name
Ma huang	Ephedra sinica Stapf	Ephedraceae	Chinese Ephedra
Xing ren	Prunus armeniaca L.	Rosaceae	Apricot
Chen pi	Citrus reticulata Blanco	Rutaceae	Satsuma Orenge
Zhe beimu	Bulbus Fritillariae Thunbergii	Liliaceae	Bulb of Thunberg Fritillary
Xi xin	Asarum heterotropoides Fr. Schmidt var.	Aristolochiaceae	Manchur Wildginger
Su zi	Perilla frutescens (L.) Britt.	Lamiaceae	Common Perilla
Huang qi	Astragali Radix	Fabaceae	Milkvetch Root
Tai zi sheng	Radix Pseudoxtellariae	Caryophyllaceae	Heterophylly Falsestarwort Root
Di long	Pheretima	Megascolecidae	Earthworm
Ban xia	Pinelliae Rhizoma	Araceae	Pinellia Ternata
Fang feng	Saposhnikoviae Radix	Umbelliferae	Divaricate Saposhniovia Root

MATERIALS AND METHODS

Drug administration

Pingchuanning (PCN) is a traditional Chinese medicine compound formula containing 11 herbs. It is used for the treatment of coughing and spitting, chest tightness and shortness of breath, and inability to lie down. The PCN was bought from The First Affiliated Hospital of Anhui University of Chinese Medicine. Table 1 lists the components. All herbs were combined and cooked twice. Blend, filter, and concentrate the two decoctions to 150 mL, 0.84 g/mL. Cool and store in a 4°C brown bottle. Each group (n = 20) had 20 SD male rats. The administration group received intragastric administration of 1 mL/100 mg PCN decoctions, while the normal group received intragastric administration of the same volume of normal saline. Once a day for 10 days. After the last gavage and fasting for 12 h, SD rats were anesthetized with 2% sodium barbiturate (20 mg/100 g). Blood was drawn from the lower abdominal aorta, left at room temperature for 2 h, and then centrifuged at 3000 rpm for 15 min. The supernatant was filtered after inactivation at 56 °C for 30 min.

16HBE cell culture and grouping

Human bronchial epithelial cells (16HBE, BNCC338044) were purchased from BeiNa Biotechnology Co. (Heibei, China). 16HBE was cultured in DMEM medium (Hyclone, USA) containing 10% fetal bovine serum (Hyclone, USA) and 1% penicillin-streptomycin (Beyotime, China) and placed in 37°C, 5% CO_2. 16HBE adherent cells in logarithmic growth phase were selected and were divided into normal control group (Con), LPS (1200 ng/mL), 3-MA group (2.5 mM), RAPA group (0.1 μM), optimal PCN concentration group, and Glycyrrhizin (0.15 μM) group.

Detection of 16HBE cell viability by CCK8

Following digestion, the 16HBE cell concentration was adjusted to 2 105 cells/mL. Cells were plated in 96-well plates

with 100 L of cell suspension. In 16HBE cells, different concentrations of LPS working solution (200 ng/mL, 400 ng/mL, 800 ng/mL, 1000 ng/ml, 1600 ng/ml, 2000 ng/ml), drug-containing serum group (5%, 10%, 15%, 20%), and LPS optimal dose + drug-containing serum group (5%, 10%, 15%, 20%) were divided into groups of 100 L each. Time points for the LPS treatment were selected at 6, 12, 24, and 36 hours. The ideal time for screening was the optimal dose of LPS+ Cell counting kit 8 (CCK8) measuring time for the serum-containing group. CCK8 was used to monitor cell activity.

Detection of the ROS level released from each group by flow cytometry.

The mitochondrial respiratory chain is the primary source of ROS in the cell, and cellular ROS levels rise when mitochondria are triggered externally. The reactive oxygen species (ROS) were detected using a DCFH-DA kit, a fluorescent probe. 37°C, 0.25% trypsin digestion, and single-cell suspensions were obtained. For 5 minutes, cells were harvested using a centrifuge. Cells were treated with 10 mM DCFH-DA solution (final concentration). The cells were kept at 37°C and out of the light for 30 minutes while being shaken every 5 minutes. Cells were rinsed twice with serum-free media after incubation to eliminate non-DCFH-DA. Intracellular ROS release variations were detected by flow cytometry, with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Observation of ultrastructure of cells in each group by transmission electron microscope

Logarithmic growth stage 16HBE cells were obtained, and the cell concentration was adjusted to 2×10^5 cells/mL. The growth medium was discarded, fixed solution was added, and the supernatant was removed by centrifugation at 4°C for 2-4 h at low speed. Subsequently, cell clumps were fixed, dehydrated, embedded, and sliced in 60-80 nm ultrathin sections using an ultrathin sectioning machine. Sections were double stained with uranium and lead and looked at with a transmission electron microscope. Images were taken to analysis later.

Detection of the protein expression by Western blot

All proteins were extracted using protease and phosphatase inhibitors in a Radio Immunoprecipitation Assay (RIPA) (G2002, Servicebio, China). The protein sample was separated in 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Transferring protein to polyvinylidene difluoride (PVDF) membrane (IPVH00010, ISEQ00010, Millipore, Billerica, MA, USA). The membrane was treated with HMGB1, Beclin-1, Bcl-2, LC3, and ATG5 primary antibodies overnight at 4°C. ImageJ analyzed stripe data.

Detection of HMGB1, Beclin-1, Bcl-2 and ATG5 protein expression in cells by immunofluorescence

Cell clumps were deparaffinized and blocked with serum for 30 min. Slices were treated at 4°C overnight after being coated with an antibody. After cleaning the slices, the second antibody (ab150077, Abcam, Cambridge, MA, USA) was applied and incubated for 50 min without light. After washing the slices, we stained the nucleus with 4',6-diamidino-2-phenylindole (DAPI) (G1012, Service bio, Wuhan, China) for 10 min at room temperature without light. We sealed the slices with an anti-fluorescence sealer (G1401, Service bio, Wuhan, China). Fluorescence microscopy produced images of the slices. Excitation wavelengths of DAPI and Fluorescein isothiocyanate isomer (FITC) are 330-380nm and 465-495 nm.

Detection of the levels of inflammatory cytokines by ELISA

ELISA kit identified IL-6, IL-8, and TNF-a cellular levels. Sample extraction and analysis procedures were carried out as specified in the kit manual. Using ELISA, the absorbance was measured at 450nm, and the concentration of the sample was determined using the standard curve.

Statistical Analysis

Data was analyzed using Statistical Product and Service Solutions (SPSS) 22 (IBM, Armonk, NY, USA). All data were presented as mean ± SD ($\bar{x} \pm s$). Multiple groups of random measurement data were tested by one-way analysis of variance (ANOVA). The difference was considered statistically significant when $P < .05$.

RESULTS

The appropriate concentration of LPS and PCN in serum-containing medicines was determined.

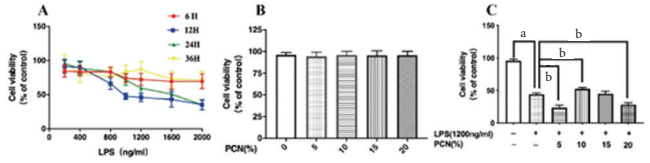
In this study, we used the classic LPS-induced asthma airway inflammation in vitro model to screen the concentration of LPS. The results of CCK8 showed a certain dose-effect and time-dependent relationship in LPS-induced cell damage (Figure 1A). With the increase of LPS dose and the co-culture time, the cell survival rate was 45.9 ± 6.0% after 1200ng/ml LPS action for 12 h, which was close to the IC50. Therefore, 1200 ng/ml LPS and 12 h were selected as the best treatment concentration and time for 16HBE cells.

There wasn't a big difference in comparing the survival of 16HBE cells treated with PCN-containing serum in the range of 5% to 20% to that of the control group. (Figure 1B). This indicates that PCN-containing serum did not significantly affect the activity of 16HBE in this dose range. Cells were pre-treated with PCN drug-containing serum for 1 h, and then the cell survival rate was detected by 12 h LPS with 1200 ng/ml. The results showed that 10% PCN drug-containing serum could significantly inhibit LPS-induced cell death, and the cell survival rate was 58.95% ±4.55%, which was significantly different from that of the LPS group (Figure 1C).

LPS generated an increase in ROS and autophagic vesicles, which was decreased by PCN.

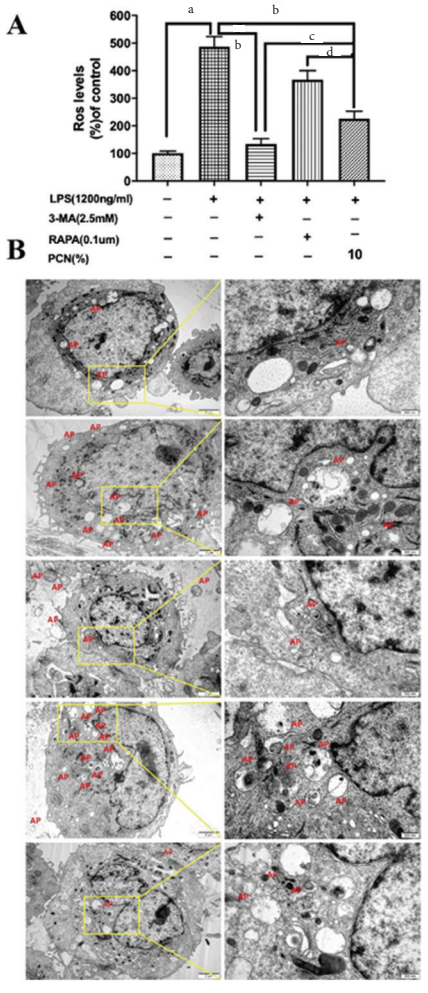
Flow cytometry was utilized to determine the amount of ROS each group's cells generated. The test groups had

Figure 1. Screening of the optimal concentration of LPS and PCN in serum containing drugs (n = 6, $\bar{x} \pm s$). CCK8 reagent was used to assess cell viability. The screening of drug concentration includes LPS (A), PCN drug-containing serum (B), LPS + PCN (C).



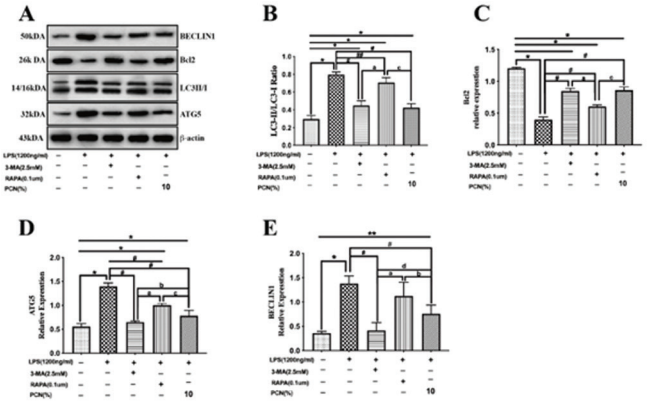
[a]$P < .05$, compared with the blank group
[b]$P < .05$, compared with the LPS model group

Figure 2. ROS levels and ultrastructure of cells in each group (n=3-6). The reactive oxygen species was detected by fluorescent probe DCFH-DA (A). Ultrastructure of cells in each group was observed by transmission electron microscope (B). Experimental grouping information: A1-A2: normal group; B1-B2: LPS group; C1-C2:3-MA group; D1-D2: RAPA group; E1-E2: PCN group.



[a]Compared with normal group, $P < .01$
[b]Compared with LPS group, $P < .01$
[c]Compared with 3-MA group, $P < .01$
[d]Compared with RAPA group, $P < .01$

Figure 3. PCN inhibited the expression of the Beclin-1, LC3, and ATG5 proteins while promoting the expression of the Bcl-2 protein (n = 3). Western blot was used to identify Beclin-1, Bcl-2, ATG5, and LC3 (A). Cells' autophagy-related protein expression was indicated by Beclin-1 (B), Bcl-2 (C), ATG5 (D), and the ratio of LC3-II to LC3-I (E).



Note: Compared with normal group, $^{*}P < .01$, $^{**}P < .05$; Compared with the LPS group, $^{\#}P < .01$, $^{\#\#}P < 0.05$; Compared with 3-MA group, $^{a}P < .01$, $^{b}P < .05$; Compared with RAPA group, $^{c}P < .01$, $^{d}P < .05$.

increased ROS levels. Each treatment group's ROS level was lower. ROS levels in the PCN and RAPA groups were substantially higher than in the 3-MA group. In the PCN group, the amount of ROS was much lower than in the RAPA group (Figure 2A).

Since ROS produced under stress can accelerate the creation of autophagosomes and autophagic vesicles, we observed the cell structure of each group using projection electron microscopy (Figure 2B). As in the normal group, the cells had normal structure and morphology, and the nuclei had enough chromatin distributed evenly. Endoplasmic reticulum and mitochondria were seen in the endoplasmic reticulum. Autophagosome and autophagic vesicles, the signature structure of autophagy, was observed in the cells after 1200 ng/ml LPS modeling for 12 h. The RAPA group showed an increase in autophagosome and autophagic vesicles, and a small number of mitochondria showed bacteria-induced dissolution and fracture. The morphology and ultrastructure of the cells were improved after the intervention of 3-MA and PCN groups. The basic morphology of the cells was better; autophagosome and autophagic vesicles were seen, among which mitochondria were more complete.

PCN increased the expression of Bcl-2 while decreasing the expression of Beclin-1, LC3, and ATG5 proteins.

Next, we determined the molecular expression of autophagy-related proteins (Figure 3). Compared to the normal group, the protein expression of Beclin-1, ATG5, and the LC3II/LC3 ratio were elevated in the LPS, RAPA, and PCN groups. Bcl-2 protein expression was reduced. In the 3-MA group, Bcl-2 protein expression dropped, and the LC3II/LC3I ratio increased. Beclin-1 and ATG5 protein

expression did not differ significantly from the normal group. Compared to the group exposed to LPS, the protein expressions of Beclin-1, ATG5, and LC3II/LC3I ratio were considerably lowered in the 3-MA, RAPA, and PCN groups, but the level of Bcl-2 was dramatically elevated. PCN has much higher Beclin-1 and ATG5 protein expression than 3-MA, although Bcl-2 and LC3II/LC3I ratio protein expressions were not statistically different. Beclin-1, ATG5, and the LC3II/LC3I ratio were reduced in the PCN group, although Bcl-2 was increased.

To confirm further that PCN inhibits autophagy, we employed immunofluorescence to verify the protein expression of Beclin-1, Bcl-2, and ATG5 in each group of cells (Figure 4). In the LPS group and the RAPA group, the expression of ATG5 and Beclin-1 protein increased, whereas the expression of Bcl-2 protein decreased. In contrast to the LPS group, ATG5, Beclin-1, and Bcl-2 protein expression reduced in the 3-MA and PCN groups but rose in the RAPA group. The expression of ATG5, Beclin-1, and Bcl-2 proteins in the PCN group did not change significantly from that of the 3-MA group. Compared to the RAPA group, ATG5, and Beclin-1 protein expression dropped in the PCN group, whereas Bcl-2 protein expression rose. In conclusion, we can demonstrate that PCN significantly inhibits autophagy.

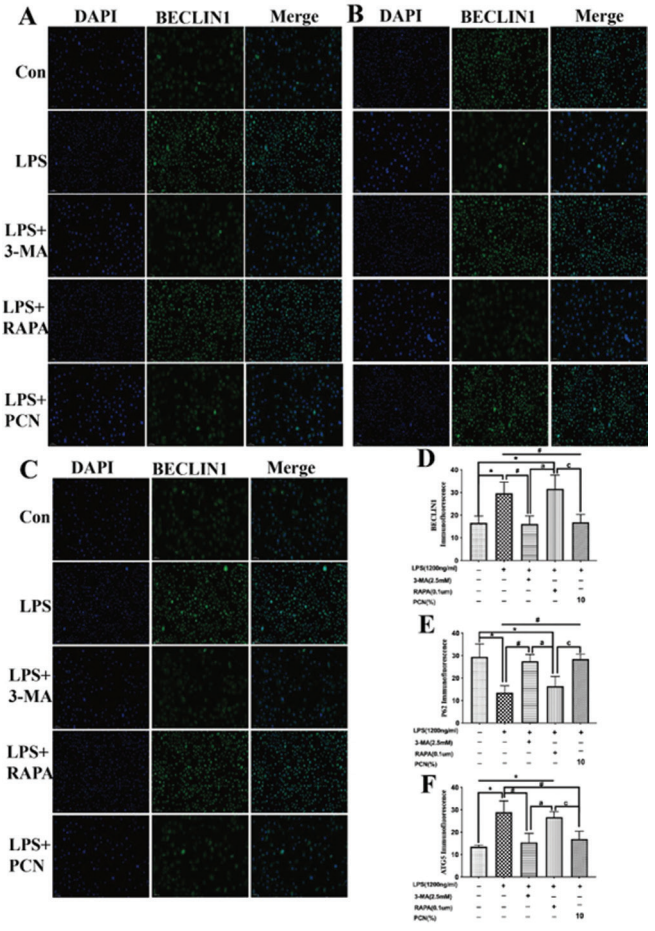
PCN can inhibit the levels of inflammatory cytokines in LPS induced cells.

To explore whether PCN can alleviate LPS-induced cellular inflammation, we measured the levels of inflammatory cytokines in 16HBE cells (Figure 5). LPS and RAPA groups expressed more IL-6, IL-8, and TNF-α than the normal group. The 3-MA, PCN, and RAPA groups had lower IL-6, IL-8, and TNF-α levels than the LPS group, whereas the RAPA group showed no change. RAPA group showed greater levels of IL-6, IL-8, and TNF-α than PCN group.

PCN reduces LPS induced ROS and autophagic vesicles by inhibiting the HMGB1/Beclin-1 pathway.

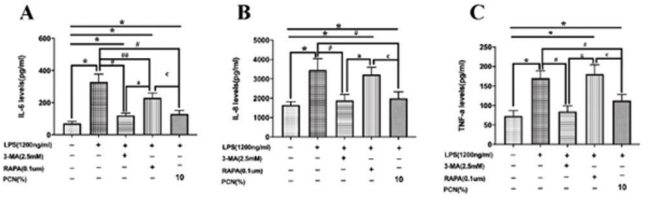
As we know, HMGB1 can compete with Bcl-2 for Beclin-1, so we used the HMGB1 inhibitor glycyrrhizin to determine whether PCN works through the HMGB1/Beclin-1 pathway. We first observed the ROS level of cells in each group (Figure 6). Under the stress of LPS, the ROS of cells increased significantly, and GLY and PCN could reverse this phenomenon. Subsequently, we observed the ultrastructure of the cells in each group. As demonstrated in Figure 6B, the basic structure and morphology of the cells in the normal control group were preserved, and the nuclear chromatin was abundant and equally distributed; the endoplasmic reticulum and mitochondria were seen intact. The landmark structure of autophagosome and autophagy vesicles could be seen in the cells made by 1200 ng/ml LPS for 12 h. The morphology and ultrastructure of autophagy were improved after intervention in GLY and PCN groups, and autophagy vesicles and mitochondria could be seen.

Figure 4. PCN inhibited the expression of Beclin-1 and ATG5 proteins and promoted the expression of Bcl-2 (n = 3). Cells of each group were fixed. Paraffin-embedded and sectioned. Beclin-1, Bcl-2, and ATG5 were stained on the sections (A-C, 400X). The Mean gray value of Beclin-1, Bcl-2 and ATG5 were shown in (D-F).



Note: $^{*}P < .01$ versus the normal group; $^{\#}P < .01$ versus the LPS group; $^{a}P < .01$ versus the 3-MA group; $^{c}P < .01$ versus the RAPA group.

Figure 5. PCN decreased (n = 6) LPS-induced levels of the cytokines. IL-6 (A), IL-8 (B), and TNF- a (C) were identified in cells using an ELISA kit.

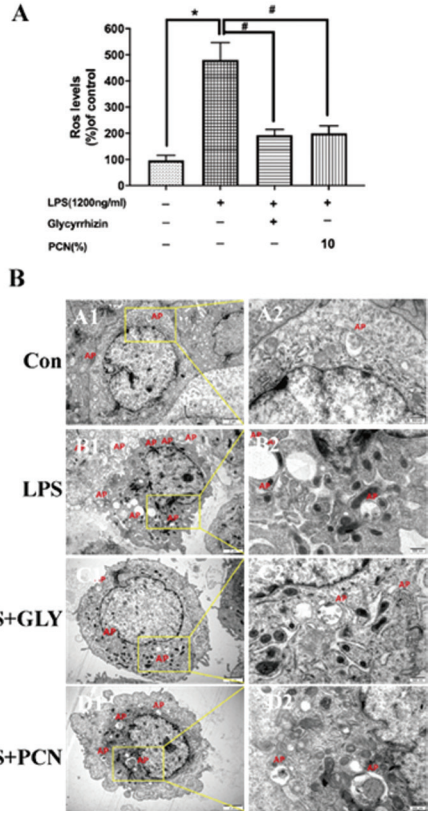


Note: Compared with normal group, $^{*}P < .01$ and $^{**}P < .05$. Compared with LPS group, $^{\#}P < .01$, $^{\#\#}P < .05$. Compared with 3-MA group, $^{a}P < .01$ and $^{b}P < .05$. Compared with RAPA group, $^{c}P < .01$ and $^{d}P < .05$.

PCN inhibits autophagy in 16HBE cells by inhibiting LPS induced HMGB1/Beclin-1 pathway.

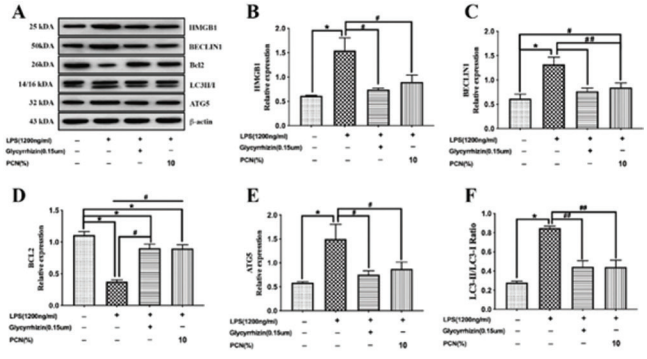
To further corroborate the PCN mechanism, we measured the expression level of cell-related proteins in each group using Western blot (Figure 7) and immunofluorescence

Figure 6. PCN reduced LPS-induced ROS and autophagy vesicles by inhibiting HMGB1/Beclin-1 pathway (n=3-6). The reactive oxygen species was detected by fluorescent probe DCFH-DA (A). Ultrastructure of cells in each group was observed by transmission electron microscope (B). Experimental grouping information: A1-A2: normal group; B1-B2: LPS group; C1-C2:3-MA group; D1-D2: RAPA group; E1-E2: PCN group.



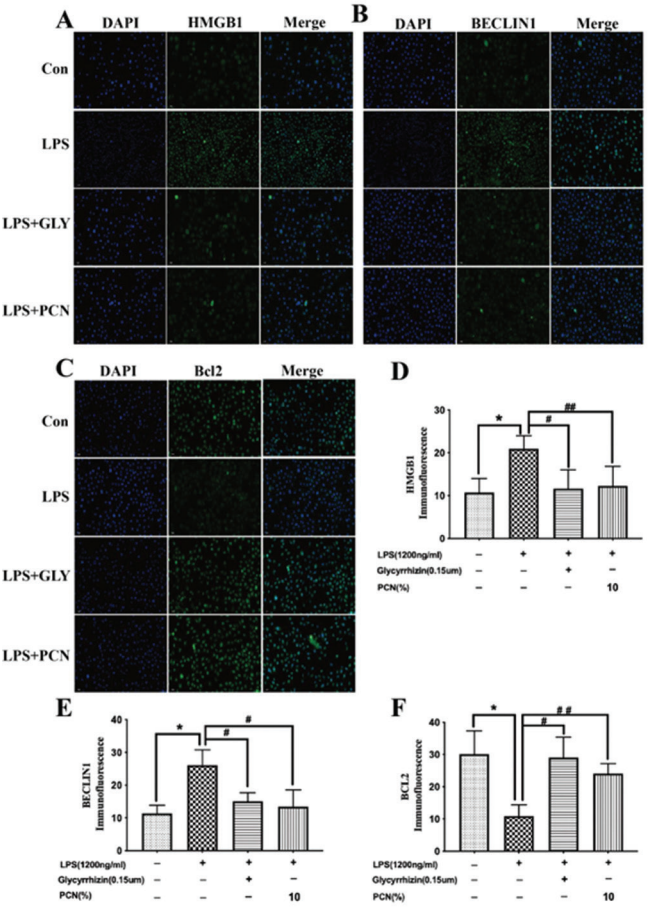
Note: Compared with the normal group, $^{*}P < .01$; Compared with the LPS group, $^{\#}P < .01$; Compared with the 3-MA group, $^{a}P < .01$; Compared with the RAPA group, $^{c}P < .01$.

Figure 7. PCN inhibited autophagy of 16HBE cells by inhibiting LPS-induced HMGB1/Beclin-1 pathway (n=3). HMGB1, Beclin-1, Bcl-2, ATG5 and LC3 were determined by Western blot (A). HMGB1 (B), Beclin-1 (C), Bcl-2 (D), ATG5 (E) and the ratio of LC3-II to LC3I (F) reflected the level of the autophagy-related protein in cells.



Note: Compared with the normal group, $^{*}P < .01$; Compared with the LPS group, $^{\#}P < .01$, $^{\#\#}P < .05$.

Figure 8. PCN inhibited autophagy of 16HBE cells by inhibiting LPS-induced HMGB1/Beclin-1 pathway (n = 3). Each group's cells were fixed. It was paraffin-embedded and sectioned. The slices were then immunostained with Beclin-1, Bcl-2, and ATG5 antibodies (A-C, 400×). The mean gray value for Beclin-1, Bcl-2, and ATG5 was illustrated in (D-F).



Note: As compared to the normal group, $^{*}P < .01$; Compared with the LPS group, $^{\#}P < .01$, $^{\#\#}P < .05$.

(Figure 8). In contrast to the normal group, the level of HMGB1, Beclin-1, and ATG5 proteins rose in the LPS and PCN groups, while Bcl-2 protein expression reduced and the ratio of LC3II/LC3I increased. GLY group and normal group expression of Beclin-1, ATG5, HMGB1 protein, and LC3II/LC3I ratio were not significantly different. Compared to the LPS group, the protein expression of HMGB1, Beclin-1, and ATG5 was decreased. In both the GLY and PCN groups, the expression of Bcl-2 protein went up, and the ratio of LC3II/LC3I went down. However, the expression of HMGB1, Beclin-1, ATG5, Bcl-2, and the LC3II/LC3I ratio did not change between the PCN and GLY groups.

In immunofluorescence, we found consistent outcomes. In the LPS group, the expression of HMGB1, Beclin-1, and Bcl-2 proteins increased relative to the normal group, but Bcl-2 protein expression decreased. GLY group and PCN group didn't differ significantly. GLY and PCN reduced HMGB1 and Beclin-1 protein expression while increasing Bcl-2.

DISCUSSION

Asthma is a diverse disease with a growing prevalence rate that has become one of the world's most frequent chronic diseases. Asthma's occurrence and progression pose a major hazard to human health and have a negative impact on sufferers' quality of life. China's asthma rate has climbed in recent years, and the country now has one of the highest populations of asthma patients in the world. Asthma prevalence in China is 0.5%, with approximately 30 million people suffering from the disease. Furthermore, its direct and indirect expenditures imposed a significant human and socioeconomic strain.

We discovered that LPS-induced cellular inflammation, ROS levels, the quantity of autophagy vesicles, and autophagy vesicles were reversed with similar effects to the 3-MA autophagy inhibitor group after interfering with PCN-containing medicines in serum. At the protein level, we identified autophagy-related proteins and discovered that PCN lowered the ratio of LC3II/I and downregulated LPS-induced Beclin-1 and ATG5 expression. Inhibiting ROS and autophagy in the 16HBE inflammatory damage model cells, PCN can therefore reduce airway inflammation. PCN reduced the expression levels of nitric oxide, tumor necrosis factor, IL-5, IGE, platelet-activating factor, granulocyte colony-stimulating factor, IL-4 and Txb2 in BALF of asthmatic guinea pigs, to treat asthma and inhibit airway inflammation and airway remodeling. Moreover, PCN inhibited the expression of GMCSF mRNA and eosinophil chemokine in the lung tissue of asthmatic guinea pigs. Therefore, PCN may relieve the clinical symptoms of asthma by alleviating inflammatory injury and airway remodeling.

In recent years, numerous findings have elucidated the function of autophagy in a range of lung disorders. Lung illnesses have diverse autophagy roles.[12,13] There is a link between autophagy and asthma, though.[14-17] Cell autophagy is now being looked at as a new way to treat asthma. More research needs to be done on how asthma starts. Cell autophagy affects the occurrence and development of asthma from various angles, involving immune response and inflammatory response.[18] Asthmatics have increased autophagy, according to numerous studies. Autophagosomes in patients with severe asthma were significantly higher than those in patients with mild asthma and healthy people.[19] Electron microscope showed that the autophagosome formation of airway epithelial cells increased significantly in patients with severe asthma. Neutrophils autophagy increases and exacerbates asthma through airway epithelial cell damage.[15] Induce an inflammatory response by activating bronchial epithelial cells and peripheral blood eosinophils.[20] The expression level of autophagy-related genes was higher in sputum and peripheral blood of patients with severe asthma than in normal people. The autophagy inhibitor 3-MA impeded the expression of LC3-II protein. To better study the mechanism of PCN, we intend to explore the relationship between autophagy and inflammation further.

We first established an in vitro inflammatory model by stimulating 16HBE cells with LPS. The LPS-induced cellular inflammatory injury model showed ROS accumulation, increased inflammatory cytokines, and increased autophagosomes. ROS is a general term for a class of active oxidative compounds produced by aerobic metabolism, including superoxide anions, free radicals, and hydrogen peroxide. Under normal circumstances, low levels of ROS are degraded by several antioxidant enzymes or substances, and the body's oxidation and antioxidant systems maintain in balance. When intracellular antioxidants cannot effectively degrade ROS, the accumulation of ROS will cause oxidative stress. Due to susceptibility to environmental factors, asthma patients will stimulate the excessive production of endogenous reactive oxygen species, leading to airway epithelial cell damage. A large number of cytokines and excessive ROS aggravate the oxidative stress of cells, which is increased in asthma patients.[21-23] Mathew found that ROS can induce autophagy and play an important role in autophagy.[24,25] ROS produced by cells is involved in autophagy as a signal molecule.[26,27] The production of ROS in bronchial epithelial cells increased the production of autophagy. This indicates a close link between reactive oxygen species and autophagy.

Th2-mediated airway inflammation is one of the basic characteristics of bronchial asthma.[28] Autophagy plays an important role in Th2 immune response in a large number of asthma animal models and patient tissues.[29] Normally, Th1/Th2 is in equilibrium, swerves abnormal Th2 increases the incidence of asthma and produces many inflammatory cytokines. Therefore, Th1/Th2 is closely related to autophagy.[30,31]

We discovered that LPS-induced cellular inflammation, ROS levels, the quantity of autophagy vesicles, and autophagy vesicles were reversed with similar effects to the 3-MA autophagy inhibitor group after interfering with PCN-containing medicines in serum. At the protein level, we identified autophagy-related proteins and discovered that PCN lowered the ratio of LC3II/I and downregulated LPS-induced Beclin-1 and ATG5 expression. Inhibiting ROS and autophagy in the 16HBE inflammatory damage model cells, PCN can therefore reduce airway inflammation.

HMGB1 has a crucial role in the nuclear, cytoplasmic, and extracellular regulation of autophagy and is a major regulator of autophagy. Stimulation of ROS can promote the cytoplasmic transport of HMGB1, thereby enhancing autophagy flux and extensive release from eukaryotic cells. ROS produced under stress and other conditions is a signal molecule to initiates autophagy. These stimuli cause HMGB1 nucleus-to-cytoplasm translocation, which is ROS-dependent. Oxidative stress controls HMGB1 and inflammation.[4-6] Under certain conditions, HMGB1 destroys the interaction between Beclin-1 and Bcl-2 through competitive binding with Bcl-2. HMGB1's oxidation-sensitive C106, adjacent C23 and C45, regulates localization and autophagy. The C106S mutant has more cytoplasmic HMGB1 and binds better to Beclin-1, separating Bcl-2 and Beclin-1. In other words, under stress conditions, nuclear HMGB1 can

access the cytoplasm and interact with autophagy-related protein Beclin-1 to release it from the Beclin-1/Bc12 complex and induce autophagy.[8-10] HMGB1 is a highly conserved nuclear protein that is essential for DNA creation. It can be recruited to the DNA target by specific binding proteins to promote the local bending of the double strand, thus affecting the structure of the DNA target sequence, participating in the recombination, repair, replication, transcription, gene expression, and cell differentiation of DNA.

Therefore, to further clarify whether HMGB1 mediates the mechanism of PCN to alleviate airway inflammation, we selected HMGB1 inhibitor GLY as a reference. The results demonstrated that PCN-containing serum reduced the level of HMGB1, Beclin-1, and ATG5 protein in 16HBE inflammatory injury model, up-regulate Bcl-2 protein expression, reduced LC3II/I ratio, and reduced the number of autophagy bodies and autophagy vesicles in cells. After Gly was utilized to block HMGB1 expression, Beclin-1, and ATG5 were down-regulated, Bcl-2 was up-regulated, the ratio of LC3II/I reduced, and autophagosomes and vesicles decreased. This indicates that PCN can regulate the ROS/HMGB1/Beclin-1 pathway to inhibit the autophagy in 16HBE inflammatory injury model.

CONCLUSIONS

In summary, PCN has a protective effect on the inflammatory model of 16HBE cells. The mechanism may be that the cellular autophagy pathway mediated by ROS/HMGB1/Beclin-1 pathway reduces airway inflammatory response. PCN has a protective effect against LPS-induced inflammatory damage in 16HBE. It may be related to the inhibition of ROS/HMGB1/Beclin-1 overexpression and suppression of autophagy and inflammatory response.

DATA AVAILABILITY

The authors will give any qualified researcher the raw data supporting this article's results.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

XW, YG, XF and ZL designed the study and performed the experiments, RW and QY collected the data, LZ and WY analyzed the data, XW, YG, XF and ZL prepared the manuscript. All authors read and approved the final manuscript. XW and YG contributed to the manuscript equally.

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