

Bu-Fei Yi-Shen Formula Alleviates Oxidative Stress and Improves Airway Epithelial Barrier Dysfunction in COPD

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Objective: This study aims to investigate the potential of Bu-Fei Yi-Shen Formula (BYF) in ameliorating airway epithelial barrier dysfunction in chronic obstructive pulmonary disease (COPD) and to elucidate the underlying mechanisms.

Methods: By establishing both in vivo and in vitro models of COPD, this study examined lung function, lung tissue pathology, inflammatory cytokines levels, cellular apoptosis rate, oxidative stress intensity, and TEER. Concurrently, it quantified the expression levels of proteins relevant to apical junctions, apoptosis, and the Nrf2 signaling pathway. These exhaustive analyses were undertaken to decipher the intricate mechanisms by which BYF ameliorates the disruption of airway epithelial barrier integrity in COPD.

Results: BYF significantly improved lung function, attenuated lung tissue pathological damage, reduced inflammatory cytokines levels, inhibited cellular apoptosis, upregulated the expression of apical junctional proteins, and alleviated oxidative stress injury in a COPD rat model. In vitro, BYF restored the CSE-induced decreases in TEER values and apical junctional protein expression in BEAS-2B cells, while reducing airway epithelial cell apoptosis and oxidative stress injury. Furthermore, BYF counteracted the inhibitory effects of CSE on Nrf2, facilitating the expression of HO-1, a downstream protein of Nrf2. The addition of ML385 exacerbated the apoptosis, oxidative stress, and barrier dysfunction induced by CSE; however, the co-administration of ML385 and BYF reversed the inhibitory effects of ML385.

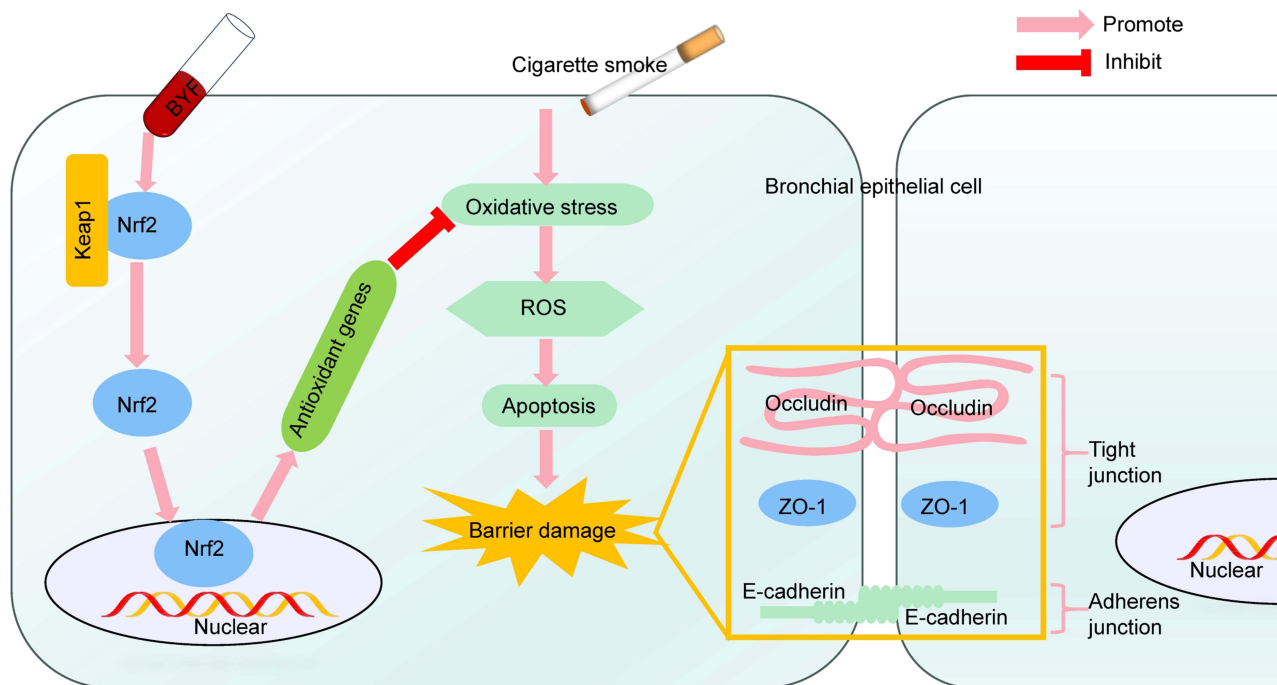
Conclusion: These findings underscore that BYF ameliorates airway epithelial barrier dysfunction in COPD by activating the Nrf2/HO-1 signaling pathway.

Keywords: chronic obstructive pulmonary disease, oxidative stress, epithelial barrier, Bu-Fei Yi-Shen formula

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a respiratory disease characterized by persistent and irreversible airflow limitation, intricately linked to inflammatory responses in the airways due to inhalation of noxious gaseous substances.¹ With a high incidence, mortality, and morbidity rate, COPD presently stands as one of the top three causes of death globally.² Projections estimate that the annual mortality rate due to COPD and its associated conditions will surpass 5.4 million by the year 2060, positioning it as a formidable global public health challenge.³ Smoking is identified as the principal etiological factor for COPD, with cigarette smoke (CS)-induced oxidative stress precipitating damage to the respiratory epithelium and compromising the integrity of the epithelial barrier, thereby heightening susceptibility to bacterial and viral infections.^{4,5} Current therapeutic regimens for COPD predominantly encompass the administration of bronchodilators, long-acting β_2 -agonists, antimuscarinic agents, and anti-inflammatory drugs, each of which is not

Graphical Abstract



devoid of associated adverse effects.⁶⁻⁸ This underscores the imperative need for developing and implementing safe and efficacious treatment modalities for COPD.

The respiratory epithelium, an essential component of pulmonary innate immunity, constitutes the primary defense against injurious particulate matter and pathogens. It is crucial in orchestrating innate barrier immunity and maintaining homeostatic balance.⁹ The apical junctional complex maintains the respiratory epithelial barrier function, consisting of tight junctions (TJ) and adherens junctions (AJ), where TJ (such as Zonula occludens-1, Occludin) and AJ (such as E-cadherin) proteins are crucial for the formation of the epithelial barrier.^{10,11} Research has shown that CS can alter the permeability of the respiratory epithelium and reduce the expression of Zonula occludens-1 (ZO-1), Occludin (OCC), and E-cadherin (E-cad) in the bronchial epithelium and pulmonary tissues of individuals with COPD, leading to structural compromise of the respiratory epithelial barrier.¹² Abnormalities in the barrier's structure can result in respiratory inflammatory infections, decreased ciliary clearance, increased mucus secretion, and destruction of pulmonary tissue, all of which are significant pathological changes within the progression of COPD.^{13,14} Therefore, enhancing the function of the respiratory epithelial barrier holds considerable importance for the treatment of COPD.

Oxidative stress is one of the main pathogenic mechanisms in COPD.¹⁵ CS exposure leads to an increase in oxidative stress within respiratory epithelial cells. Excessive oxidants not only penetrate the secretory layer of the respiratory mucosa to directly damage epithelial cells but also induce cellular apoptosis, which in turn further triggers apoptotic processes.¹⁶ Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor, plays a vital role in suppressing oxidative damage.¹⁷ It has been discovered that CS can inhibit the activity of Nrf2, indirectly escalating the oxidative stress seen in COPD, thus exacerbating pulmonary tissue damage and expediting the progression of the disease.¹⁸ Therefore, antioxidant therapy has received widespread attention in the field of COPD research.

Traditional Chinese Medicine has distinct advantages in the management of stable COPD, effectively ameliorating symptoms, reducing the frequency of acute exacerbations, and improving life quality.¹⁹ The BYF, comprising twelve Chinese herbs, shows promising efficacy in COPD patients. The combination of ginsenoside Rh1, paeonol, astragaloside,

icariin, and nobletin alleviates lung tissue injury, regulates the Nrf2 pathway to ameliorate oxidative stress, and protects the pulmonary air-blood barrier in COPD rats.²⁰ Loganin, peimine, and perilla fruit extract reduce oxidative stress and the expression of inflammatory factors.^{21–23} Lycium barbarum polysaccharide 3 and schisandrin A activate Nrf2, decrease ROS and MDA levels, and restore SOD activity.^{24,25} Earthworm and *Ardisiae japonicae herba* extracts exert anti-inflammatory and antioxidant properties by activating the Nrf2 pathway, thereby alleviating oxidative stress-related renal injury.^{26,27} In summary, these natural active components exert protective effects against various tissue injuries primarily by activating the Nrf2 pathway, reducing oxidative stress and inflammatory responses. Clinical studies indicate that BYF enhances lung function and exercise capacity, and diminishes the frequency and duration of acute exacerbations.^{28–30} Animal studies suggest that BYF reduces pulmonary and systemic inflammation in COPD rats, eases oxidative stress and airway mucus hypersecretion, and corrects the protease-antiprotease imbalance as well as collagen deposition.^{31–33}

Nonetheless, the effects of the BYF on airway epithelial barrier dysfunction in COPD and its underlying mechanisms remain elusive. This study investigated the role of BYF in ameliorating airway epithelial barrier dysfunction by establishing both in vivo and in vitro COPD models.

Materials and Methods

Materials

The BYF (Patent number: ZL201110117578.1) was provided by the School of Pharmacy at Henan University of Traditional Chinese Medicine. It consists of Ginseng Root, Astragali root, Asiatic cornelian Cherry fruit, *Paeoniae rubra* root, *Ardisiae japonicae herba*, *Citri reticulatae* pericarpium, Barbary wolfberry fruit, *Schisandra chinensis* fruit, *Epimedii folium*, *Thunbergii fritillary bulb*, *Perillae* fruit, and Earthworm. Tobacco were provided by Henan Tobacco Industry (Hongqi Canal® filter cigarette; tar content: 10 mg; nicotine content: 1.0 mg; carbon monoxide content: 12 mg, Zhengzhou, China). *Klebsiella pneumoniae* (*Kp*) was supplied by the National Center for Medical Culture Collections with a strain number of 46114.

Establishment of the COPD Model in Rats

Sixty 8-week-old male Sprague-Dawley rats (180–200 g) were supplied by SPF Biotechnology Co., Ltd (SCXK Beijing 2024–0001, Beijing, China). Following 7 days of adaptive feeding, the rats were randomly divided into five groups: the Control group, the COPD group, the low-dose Bufei Yishen Formula (BYFL) group, the high-dose Bufei Yishen Formula (BYFH) group, and the carbocysteine (S-CMC) group. A COPD rat model was established using a combination of CS exposure and repeated *Kp* infection. Except for the Control group, the remaining groups of Rats were exposed to CS (3000 ± 500 ppm) twice daily for 8 weeks, with each exposure lasting 30 minutes and an interval of more than 3 hours between the two exposures. The *KP* was resuspended in physiological saline, and the bacterial concentration was adjusted to 6 × 10⁸ CFU/mL. The bacterial suspension was administered via nasal instillation, alternating between the left and right nasal cavities. Each rat received 0.1 mL of the suspension once every 5 days. Rats in the Control group were housed in a clean air environment and received an equal volume of normal saline via nasal instillation.²⁹ This study was conducted in strict adherence to the “Guidelines for Ethical Review of Laboratory Animal Welfare” (GB/T 35892–2018). Animal experiments were approved by the animal care committee of the First Affiliated Hospital, Henan University of Chinese Medicine (No. YFYDW2024018).

Drugs and Treatment

Starting from the 9th week, rats in the BYFL group were intragastrically administered with 5.8 g/(kg·d) of BYF suspension. Rats in the BYFH group received 11.6 g/(kg·d) of BYF suspension by gavage. The S-CMC group was given 0.14 g/(kg·d) of carbocysteine tablet suspension (0.1 g/tablet, Baiyunshan, China) suspension via intragastric gavage. Rats in the Control and COPD groups were intragastrically administered with 2 mL of normal saline daily. The conversion of equivalent drug doses is calculated as follows (D: Dose; HI: Body shape coefficient; W: Body weight):

$$D_{\text{rat}} = D_{\text{human}} \times (HI_{\text{rat}}/HI_{\text{human}}) \times (W_{\text{rat}}/W_{\text{human}})^{2/3}$$

At end of week 16, the rats were sacrificed by intraperitoneal injection of pentobarbital sodium at a dose of 30 mg/kg, followed by subsequent processing.

Pulmonary Function Testing

At weeks 0, 4, 8, 12, and 16 of the experiment, tidal volume (TV) and minute volume (MV) were measured using a whole-body plethysmography system. Following the completion of drug intervention at week 16, the rats were anesthetized, their tracheas were exposed, and connected to a pulmonary function testing system to assess forced vital capacity (FVC) and forced expiratory volume in 0.1 seconds (FEV_{0.1}).

Hematoxylin-Eosin (HE) Staining

The left lung tissues of the rats were harvested, fixed, embedded, and sectioned, followed by HE staining. Light microscopy was used to observe the integrity of lung tissue structure, the presence of tracheal wall shrinkage and thickening, and the occurrence of massive inflammatory cell infiltration or extensive alveolar wall rupture in each group of rats. Mean linear intercept of alveoli mean alveolar number was calculated using ImageJ 1.43u software to evaluate the extent of alveolar rupture.

Detection of Inflammatory Cytokines IL-6, TNF- α , IL-1 β , and TGF- β 1

The levels of IL-6 (Elabscience, E-EL-R0015c, China), TNF- α (Elabscience, E-EL-R2856, China), and TGF- β 1 (Elabscience, E-EL-R0012c, China) in serum and IL-6 (Elabscience, E-EL-R0015c, China), IL-1 β (Elabscience, E-EL-R0012, China), and TGF- β 1 (Elabscience, E-EL-R0012c, China) in bronchoalveolar lavage fluid (BALF) were detected by enzyme-linked immunosorbent assay following the kit instructions.

TUNEL Assay

Paraffin sections were dewaxed and rehydrated, followed by antigen retrieval with proteinase K, membrane permeabilization, and buffer equilibration at room temperature. Subsequently, terminal deoxynucleotidyl transferase enzyme reaction solution was added dropwise, and the sections were incubated at 37°C for 1 hour. After washing with phosphate-buffered saline, the sections were counterstained with 4',6-diamidino-2-phenylindole for 10 minutes in the dark. Finally, the sections were mounted with anti-fade mounting medium, and apoptotic cells were observed and imaged using a fluorescence microscope.

BYF Preparation of Medicinal Serum

8-week-old Male Sprague Dawley rats (180–200 g) were supplied by SPF Biotechnology Co., Ltd. Thirty rats were randomly divided into two groups (15 rats per group): Control and BYF groups. The Control group was given 2 mL of physiological saline by gavage, and the BYF group was given BYF (18 g/(kg·d)) by gavage,³⁴ both for 7 consecutive days. One hour after the last administration, abdominal aortic blood was collected, centrifuged for 15 minutes, filtered through a 0.22 μ m filter, aliquoted, and kept at –80°C for future use.

Preparation of CS Extract

A filter-less Hongqi Canal cigarette was lit, and smoke was drawn into a serum-free DMEM culture medium using a 20 mL syringe, then filtered through a 0.22 μ m filter. The absorbance at 320 nm was measured to ensure consistency, with the absorbance value (A value) of different batches of the CSE solution maintained at approximately 2.0, defining this as the 100% CSE solution.³⁵

Cell Culture

The BEAS-2B cell line was acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences. BEAS-2B cells were cultured in DMEM medium (supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, provided

by Beijing Solarbio Science & Technology Co., Ltd). at 37°C in a 5% CO₂ incubator. Cells were passaged at a 1:3 ratio upon reaching 80%-90% confluence, with cells in the logarithmic growth phase of the third generation selected for subsequent experiments. The BEAS-2B cells were divided into Control group, CSE group, and BYF groups at 5%, 10%, and 20% concentrations, ML385 group, and ML385+BYF (10%) group. All groups except the Control group were treated with 10% CSE to establish the COPD model. The ML385 group received ML385 administration 2 h prior to CSE intervention, while the BYF intervention group was treated with BYF 2 h after CSE intervention.

Cell Viability Assay

BEAS-2B cells were seeded at 5,000 cells per well in a 96-well plate and cultured for 24 hours before intervention with varying concentrations of CSE, BYF, and ML385 (1 mmol/L solution with dimethyl sulfoxide, MedChemExpress, 24429, USA), alongside a Control group, with six replicates per group. After 24 hours of intervention, 10 µL of CCK-8 solution was added and incubated for an appropriate duration before measuring the optical density at 450 nm using a spectrophotometer (SpectraMax i3x, Molecular Devices, USA).

Oxidative Stress Marker Detection

GSH (Beyotime, S0057S, China), SOD (Beyotime, S0101S, China), and MDA (Beyotime, S0131S, China) levels were measured in lung tissue homogenates and cell lysates using assay kits per manufacturer instructions.

Measurement of Reactive Oxygen Species (ROS)

After cultured in 6-well plates, the BEAS-2B cells were digested with 0.25% trypsin. The cell suspension was collected and centrifuged at 1000 rpm for 5 min. The cells were then resuspended in PBS, counted, and adjusted to 1×10^6 cells. Next, the cells were incubated with 10 µmol/L DCFH-DA (Elabscience, E-BC-K138-F, China) at 37 °C for 20 min. After being washed three times, the cells were resuspended in 200 µL PBS and detected by flow cytometry (Powclin, SFLO, China) at 488/525 nm. The intracellular ROS levels were analyzed using FlowJo software.

Western Blotting

Proteins related to the Nrf2 pathway, apoptosis, and apical junctional complexes including Bcl-2 (1:1000, CST, 2764S, USA), Caspase-3 (1:1000, CST, 9662S, USA), Nrf2 (1:1000, Affinity, BF8017, China), HO-1 (1:1000, Affinity, AF5393, China), E-cadherin (1:20000, Proteintech, 20874-1-AP, China), Occludin (1:5000, Proteintech, 27260-1-AP, China), ZO-1 (1:5000, Proteintech, 21773-1-AP, China), Bax (1:20000, Proteintech, 50599-2-Ig, China), GAPDH (1:50000, Proteintech, 60004-1-Ig, China), and β-Tubulin (1:5000, Proteintech, 80713-1-RR, China) were detected. Proteins were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. After blocking, membranes were incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 1 hour (Goat Anti-Mouse IgG (H+L) HRP (1:20,000, HA1001, HA1006, China) and Goat Anti-Rabbit IgG (H+L) HRP (1:50000, Huaan, HA1001, China)). Protein bands were visualized using the ChemiDoc MP imaging system (BioRad, USA) and quantified optical density values using ImageJ software.

Immunofluorescence

BEAS-2B cells were seeded at 2×10^4 cells per well on coverslips in 6-well plates, supplemented with 1 mL of culture medium after 4 hours, and incubated overnight at 37°C in a 5% CO₂ incubator. After 24 hours of intervention, cells were fixed with PFA for 15 minutes, washed with PBS, and then blocked with 3% BSA on the coverslips for 30 minutes at room temperature. Primary antibodies were applied and incubated overnight at 4°C (E-cadherin (1:200, Proteintech, 20874-1-AP, China), Occludin (1:500, Proteintech, 27260-1-AP, China), and ZO-1 (1:100, Proteintech, 21773-1-AP, China)). Secondary antibodies were then applied for 50 minutes at room temperature; DAPI was added to stain for 10 minutes in the dark; and an anti-fade mounting medium was used before visualization under an upright fluorescence microscope (Nikon Eclipse C1, Nikon, Japan).

Apoptosis Rate Assessment

After 24 hours of treatment, 2×10^5 BEAS-2B cells from each group were collected into centrifuge tubes, resuspended in 500 μL of Annexin V Binding Buffer, then 5 μL of Annexin V and 5 μL of PE-Cy5.5 stain were added, mixed, and incubated for 15 minutes. Following incubation, an additional 400 μL of Annexin V Binding Buffer was added, and the rate of apoptosis was measured within 30 minutes using a flow cytometer (BD FACSCelesta, BD Biosciences, USA).

Transepithelial Epithelial Electrical Resistance (TEER)

Cells were seeded at a density of 7×10^5 per well on the apical side of Transwell inserts (Corning, 3379, USA) and cultured for at least 72 h to establish a functional epithelial monolayer. After corresponding drug interventions, the long electrode tip of the ESR meter (Millicell-ERS, Millipore, USA) was inserted into the basolateral chamber and the short electrode tip into the apical chamber. TEER was measured in triplicate for each well, and the mean value was calculated. $\text{TEER } (\Omega/\text{cm}^2) = (\text{Cell resistance value} - \text{Baseline resistance value}) \times \text{Bottom area of the Transwell upper chamber filter } (\text{cm}^2)$.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 21.0). Comparisons between two groups were conducted with the *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was used; if variances were equal, the least significant difference (LSD) test was applied, whereas Dunnett's T3 method was used for unequal variances. Graphs were generated using GraphPad Prism 7.0 software. A *P*-value < 0.05 was considered statistically significant. Quantitative data are presented as mean $\pm$ standard deviation (SD).

Results

BYF Improves Lung Function and Alleviates Lung Tissue Pathological Damage in COPD Rats

Pulmonary pathological morphology and lung function are core indicators reflecting the severity of COPD. To clarify the interventional effect of BYF on disease progression in COPD rats, this study systematically evaluated the pulmonary pathological characteristics and lung function parameters of rats in each group. HE staining results showed that, compared to the COPD group, BYF intervention significantly alleviated the typical lung tissue pathological damage in COPD rats. The specific manifestations included reduced destruction of alveolar structure, alleviated thickening and shrinkage deformation of bronchial walls, and a marked improvement in the dense infiltration of local inflammatory cells (Figure 1A–D). COPD is not only characterized by local chronic inflammation of the airways and lung tissues but also presents as a systemic inflammatory disease. Based on this, this study detected the levels of inflammatory cytokines in the serum of rats in each group. The results showed that BYF intervention significantly reduced the levels of IL-6, TGF- β 1, and TNF- α in serum and levels of IL-6, TGF- β 1, and IL-1 β in BALF ($P < 0.01$, Figure 1E–J). Additionally, the BYFH group could significantly increase the body weight of rats ($P < 0.01$, Figure 2A and B). Lung function test results further confirmed that BYF could effectively improve the respiratory function of COPD rats and significantly increase key lung function indicators such as TV, MV, FVC, FEV_{0.1}, and FEV_{0.1}/FVC ratio ($P < 0.05$ or $P < 0.01$, Figure 2C–I). In summary, BYF can improve lung function by alleviating lung tissue damage and systemic inflammatory response in COPD rats, thereby effectively reducing disease severity.

BYF Attenuates Airway Epithelial Cell Apoptosis and Barrier Function Impairment in COPD Rats

Cell apoptosis is one of the important pathogenic mechanisms of COPD. When airway epithelial cells undergo apoptosis, it leads to the destruction of epithelial structural integrity, increased epithelial permeability, and accumulation of inflammatory cells, which in turn impairs the respiratory epithelial barrier function and accelerates the initiation and progression of COPD.³⁶ To investigate the effect of BYF on airway epithelial cell apoptosis in COPD rats, Western blot analysis was used to detect the expression levels of apoptosis-related proteins in lung tissue. The results showed that,

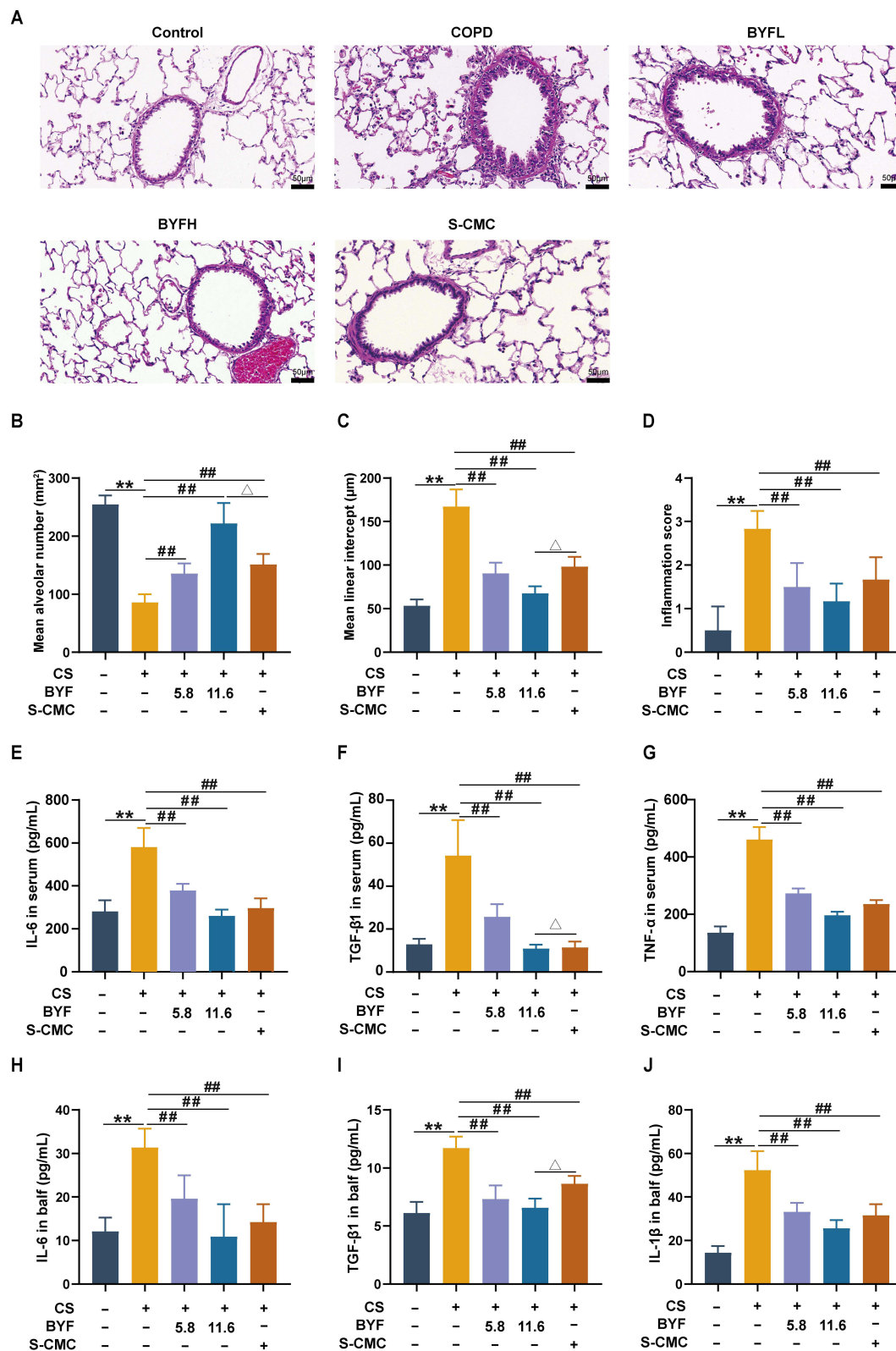


Figure 1 The effect of BYF on Lung Tissue Pathological Damage in COPD Rats. **(A)** Photographs of pulmonary airway histopathology in Rats (HE, $\times 300$, $n=6$). Scale bar, 50 μm . **(B)** Mean alveolar number (MAN). **(C)** Mean linear intercept (MLI). **(D)** Inflammation score. **(E–G)** The levels of IL-6, TNF- α , and TGF- $\beta 1$ in Serum ($n=6$). **(H–J)** The levels of IL-6, IL-1 β , and TGF- $\beta 1$ in BALF ($n=6$). ** $P<0.01$, vs. Control group. ### $P<0.01$, vs. COPD group. $\Delta P<0.05$, vs. S-CMC group.

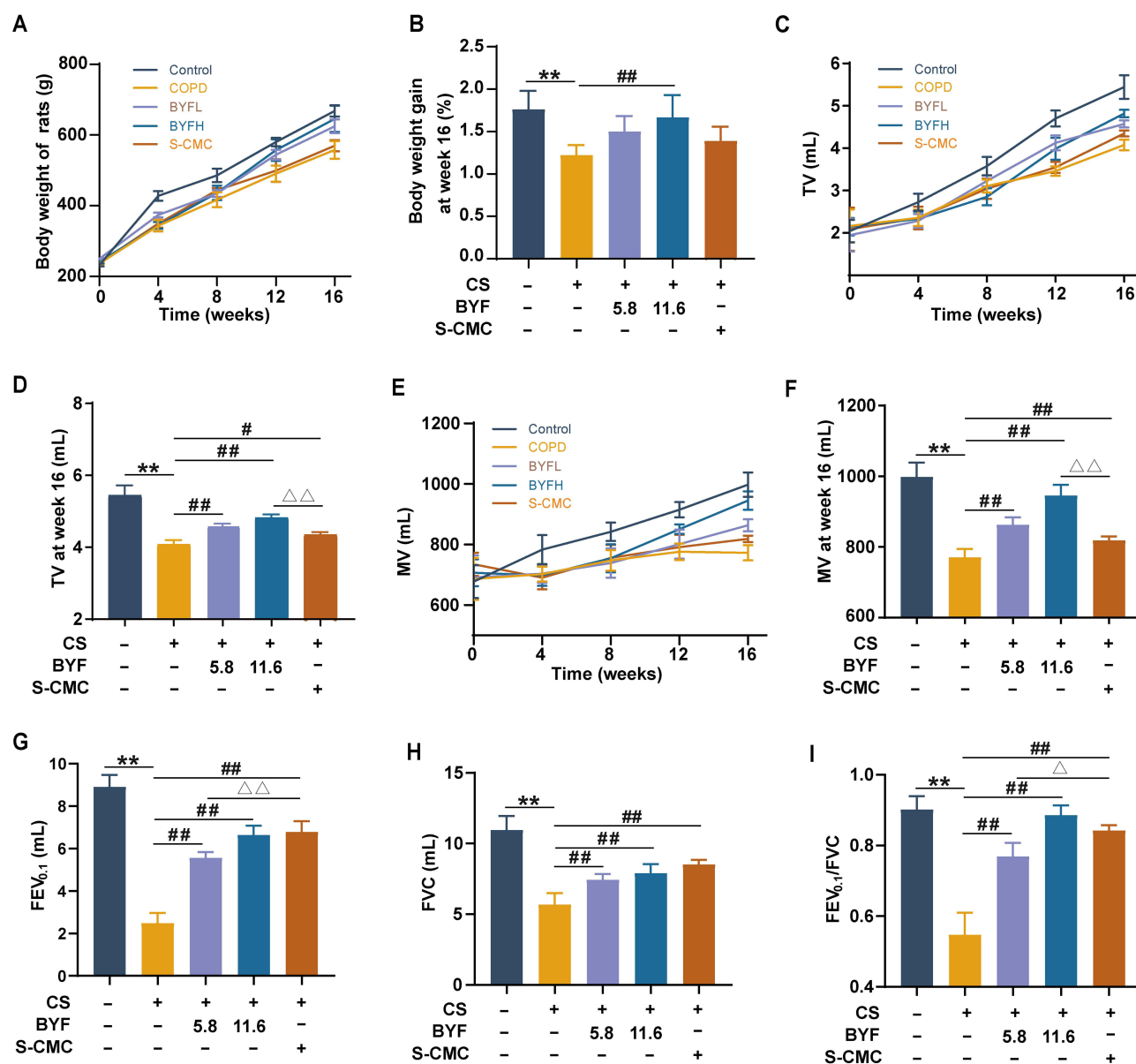


Figure 2 The effect of BYF on Lung Function in COPD Rats. (A) Body weight of rats from 0–16 week. (B) Body weight gain at week 16. (C) Tidal volume (TV). (D) TV at week 16. (E) Minute volume (MV). (F) MV at week 16. (G) forced expiratory volume in 0.1 seconds (FEV_{0.1}). (H) forced vital capacity (FVC). (I) FEV_{0.1}/FVC. ***P*<0.01, vs. Control group. #*P*<0.05, ###*P*<0.01, vs. COPD group. Δ*P*<0.05, ΔΔ*P*<0.01, vs. S-CMC group.

compared to the control group, the expression of pro-apoptotic proteins Bax and Caspase-3 was significantly upregulated, while the expression of anti-apoptotic protein Bcl-2 was significantly downregulated in the lung tissue of rats in the COPD model group. BYF intervention could significantly reverse this abnormal expression trend (*P*<0.01, Figure 3A–D). TUNEL assay results further confirmed that BYF could significantly reduce the number of apoptotic airway epithelial cells in COPD rats (*P*<0.01, Figure 3E and F).

In addition, this study also detected the expression of epithelial barrier junction-related proteins (E-cad, ZO-1, and OCC) in airway epithelium. The results showed that BYF intervention significantly increased the expression levels of the aforementioned barrier junction proteins (*P*<0.01, Figure 3G–J). Collectively, these data indicate that BYF exerts a significant protective effect against the apoptotic process and barrier function impairment of airway epithelial cells in COPD rats.

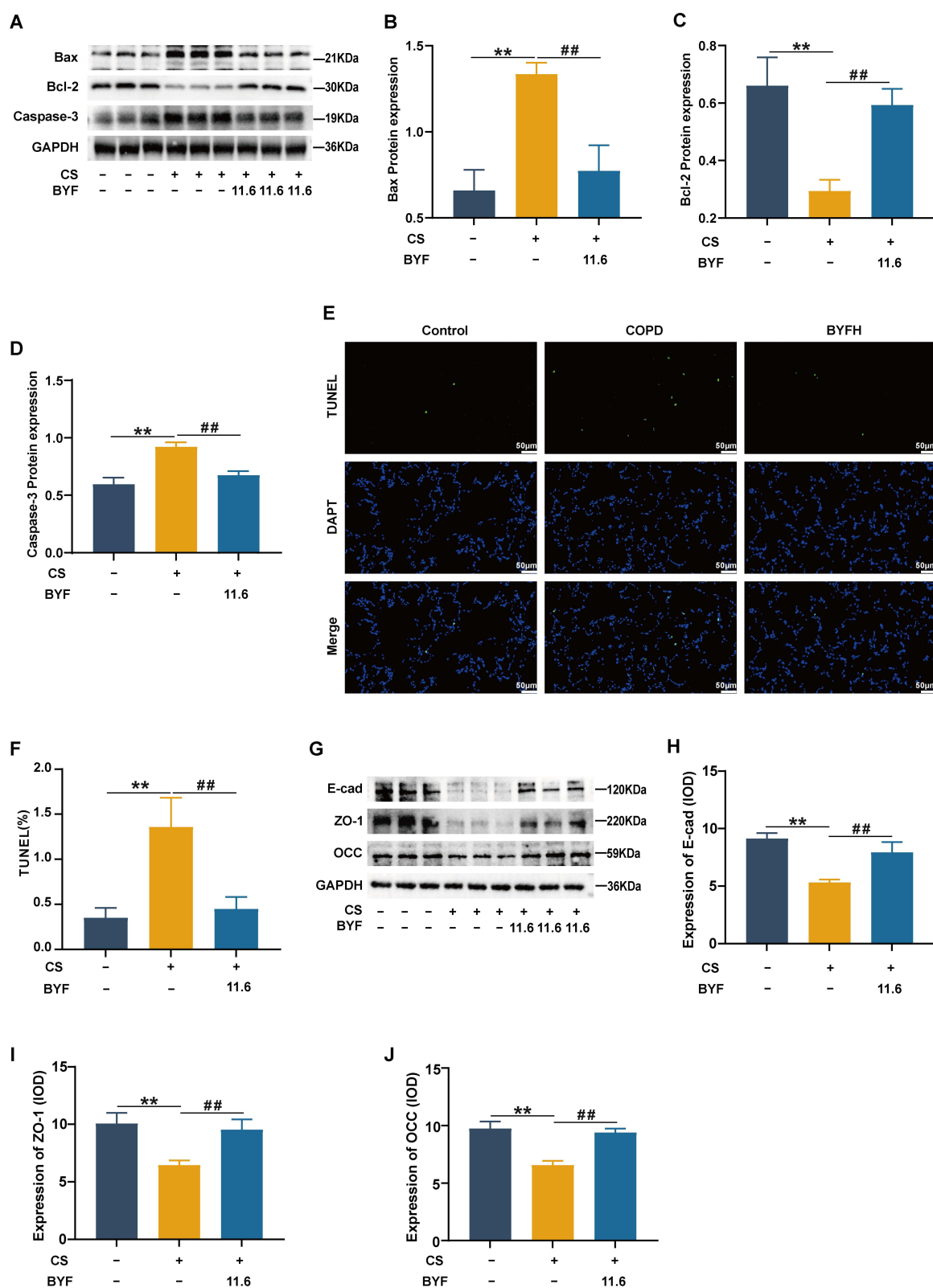


Figure 3 The effect of BYF on Airway Epithelial Cell Apoptosis and Barrier Function Impairment in COPD Rats. (A–D) The expression of Bax, Bcl-2, and caspase-3 were detected by Western blot analysis (n=3). (E and F) TUNEL Assay for Airway Epithelial Cell Apoptosis Detection (n=6). Scale bar, 50 μm. (G–J) The expression of E-cad, ZO-1, and OCC were detected by Western blot analysis (n=3). **P<0.01, vs. Control group. ##P<0.01, vs. COPD group.

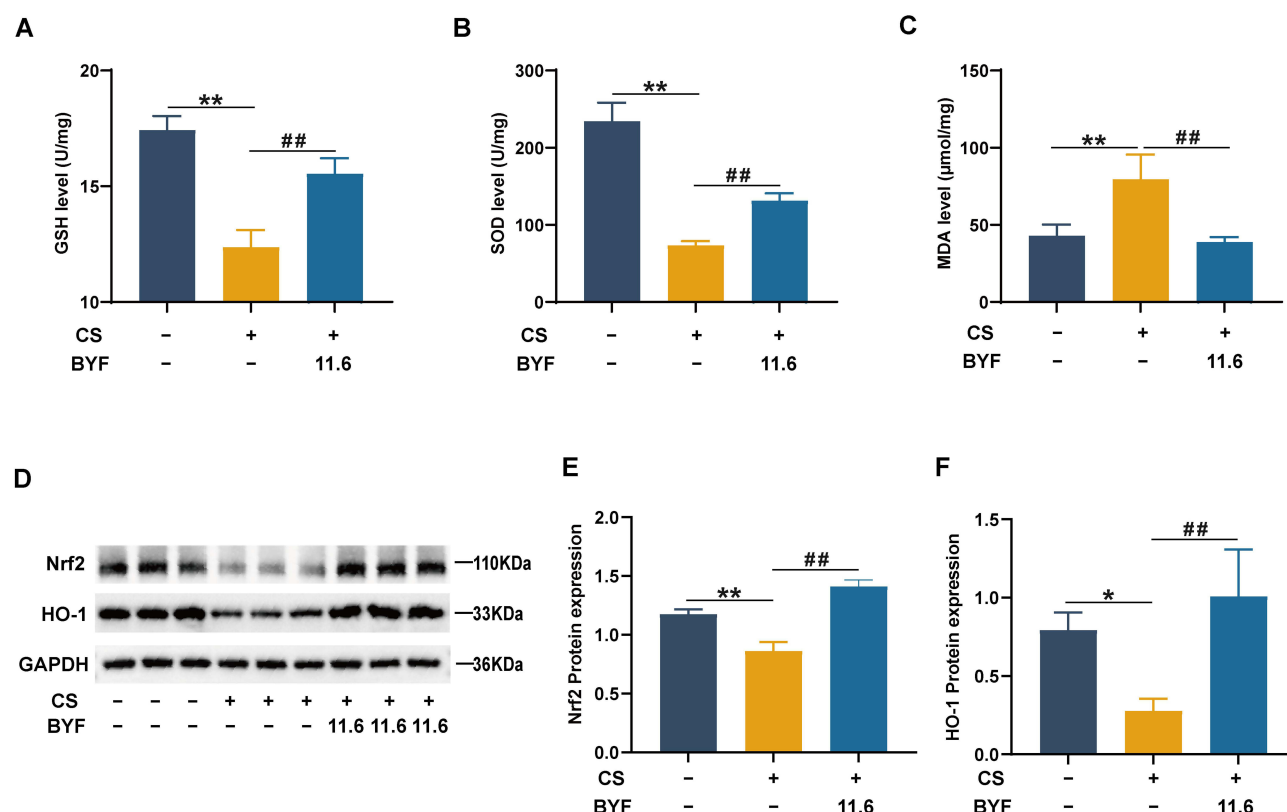


Figure 4 The effect of BYF on the expression of oxidative stress and Nrf2 signaling pathway-related proteins in COPD Rats. (A–C) The expression levels of GSH, SOD, and MDA levels were examined in Lung Tissue (n=6). (D–F) The expression of Nrf2 and HO-1 were detected by Western blot analysis in Lung Tissue (n=3). * $P<0.05$, ** $P<0.01$, vs. Control group. ### $P<0.01$, vs. COPD group.

BYF Reduces Lung Tissue Oxidative Damage in COPD Rats

Oxidative stress is one of the key driving factors in the occurrence and development of COPD.³⁷ It can not only directly cause damage to body tissues but also exacerbate organelle and DNA destruction, and induce programmed cell death processes such as apoptosis.³⁸ To clarify the antioxidant activity of BYF in COPD rats, the levels of oxidative stress-related indicators in lung tissue homogenates were detected. The results showed that BYF could increase the activities of GSH and SOD while decreasing the expression of MDA ($P<0.01$, Figure 4A–C). Given the central role of oxidative stress in COPD progression, and considering that heme oxygenase-1 (HO-1) expression is regulated by the Nrf2 signaling pathway—a crucial multi-organ protective pathway demonstrated to exert core regulatory effects on the pathological processes of inflammatory diseases (eg., cardiovascular diseases, pulmonary diseases, kidney diseases, and metabolic disorders) across various experimental animal models—this study further detected the expression levels of Nrf2/HO-1 pathway-related proteins via Western blot analysis.³⁹ The results showed that BYF intervention significantly upregulated the protein expression levels of Nrf2 and HO-1 in the lung tissue of COPD rats ($P<0.05$ or $P<0.01$, Figure 4D–F). It is thus speculated that BYF may exert its antioxidant protective effect on the lung tissue of COPD rats by activating the redox-sensitive Nrf2/HO-1 signaling pathway.

BYF Alleviates CSE-Induced Impairment of BEAS-2B Cellular Epithelial Barrier Function

To further clarify the specific role and molecular mechanism of BYF in regulating airway epithelial barrier function, this study successfully established an in vitro cell model simulating COPD-related epithelial cell damage using the human bronchial epithelial cell line BEAS-2B. The CCK-8 assay was used to evaluate the impact of different CSE and BYF medicinal serum concentrations on the proliferative activity of BEAS-2B cells. After a 24-hour intervention with various

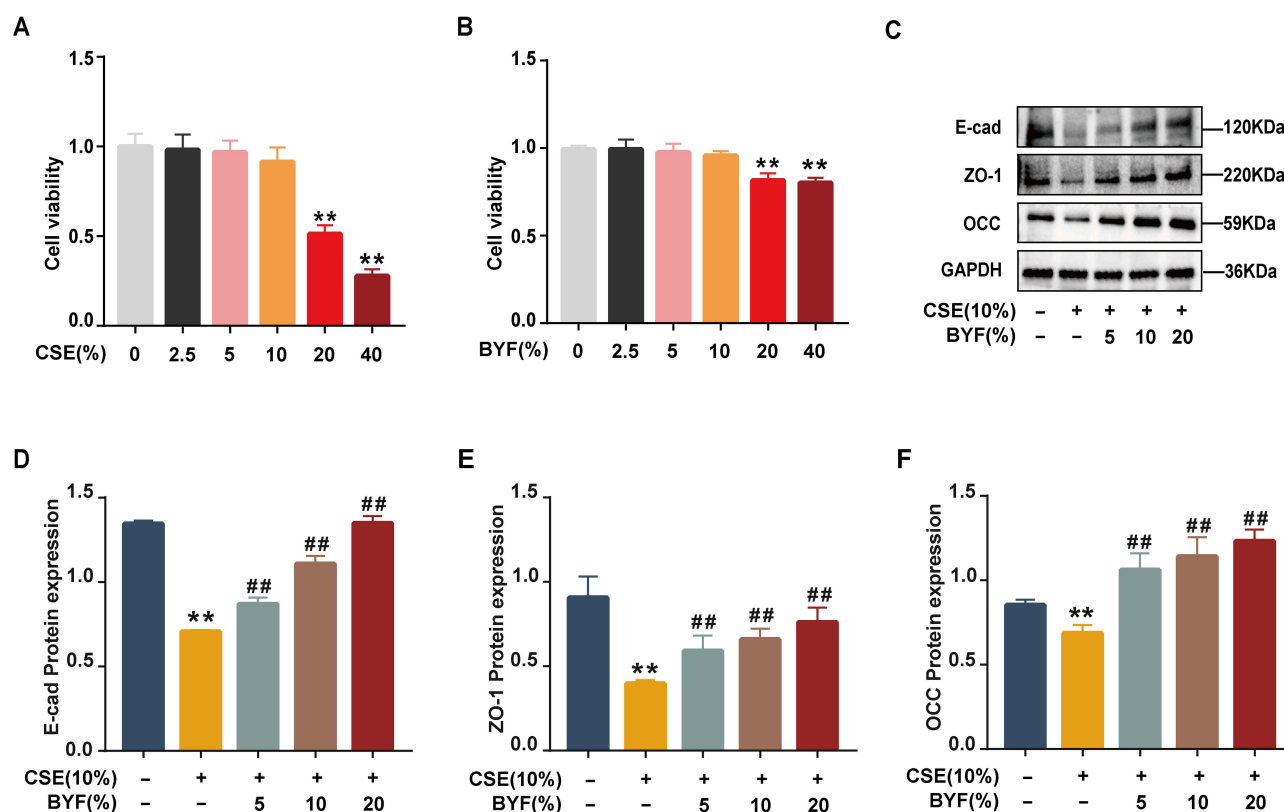


Figure 5 The effect of BYF on tight junction protein expression in BEAS-2B cells. (A and B) CCK-8 assays were used to measure cell viability (n=6). (C–F) The expression of E-cad, ZO-1, and OCC were detected by Western blot analysis (n=3). ** $P<0.01$, vs. Control group. ## $P<0.01$, vs. CSE group.

concentrations of CSE and BYF, the results indicated that compared to the control group, concentrations of 2.5%, 5%, and 10% CSE and BYF did not significantly affect the viability of BEAS-2B cells; however, the cell viability at 20% and 40% concentrations was significantly lower than that of the control group ($P<0.01$, Figure 5A and B). Consequently, a 10% concentration was deemed optimal for intervention.

The respiratory epithelium serves not only as a physical barrier, employing ciliary motion to clear mucus-entrapped particles but also mobilizes the immune system to protect the respiratory tract.⁴⁰ Western blot results demonstrated that compared to the control group, the expression levels of E-cad, OCC, and ZO-1 were reduced in the CSE group, indicating that the integrity of the cellular barrier was compromised. In contrast, the BYF group exhibited a concentration-dependent increase in the expression of E-cad, OCC, and ZO-1 ($P<0.01$, Figure 5C–F). Immunofluorescence assay results were consistent with Western blot findings, showing a concentration-dependent reversal by BYF of the decrease in E-cad, OCC, and ZO-1 expression induced by CSE ($P<0.01$, Figure 6A–F). TEER, a quantitative method, assesses the dynamics of tight junction integrity in cell culture models and is a critical indicator of epithelial barrier function.⁴¹ TEER results revealed that compared to the control group, the TEER of the CSE group decreased gradually over time with the intervention, with the most significant reduction observed at 24 hours post-intervention; compared to the CSE group, the BYF group enhanced the TEER of BEAS-2B cells at various time points ($P<0.01$, Figure 7A).

BYF Attenuates CSE-Induced Apoptosis in BEAS-2B Cells

To investigate the effect of BYF on apoptosis in BEAS-2B cells, we examined the expression of apoptosis-related proteins via Western blot. The results showed that compared to the control group, the CSE group exhibited significantly upregulated Bax and caspase-3 protein expression and downregulated Bcl-2 expression. In contrast, the BYF group demonstrated a concentration-dependent reduction in Bax and caspase-3 expression, with the 10% and 20% BYF

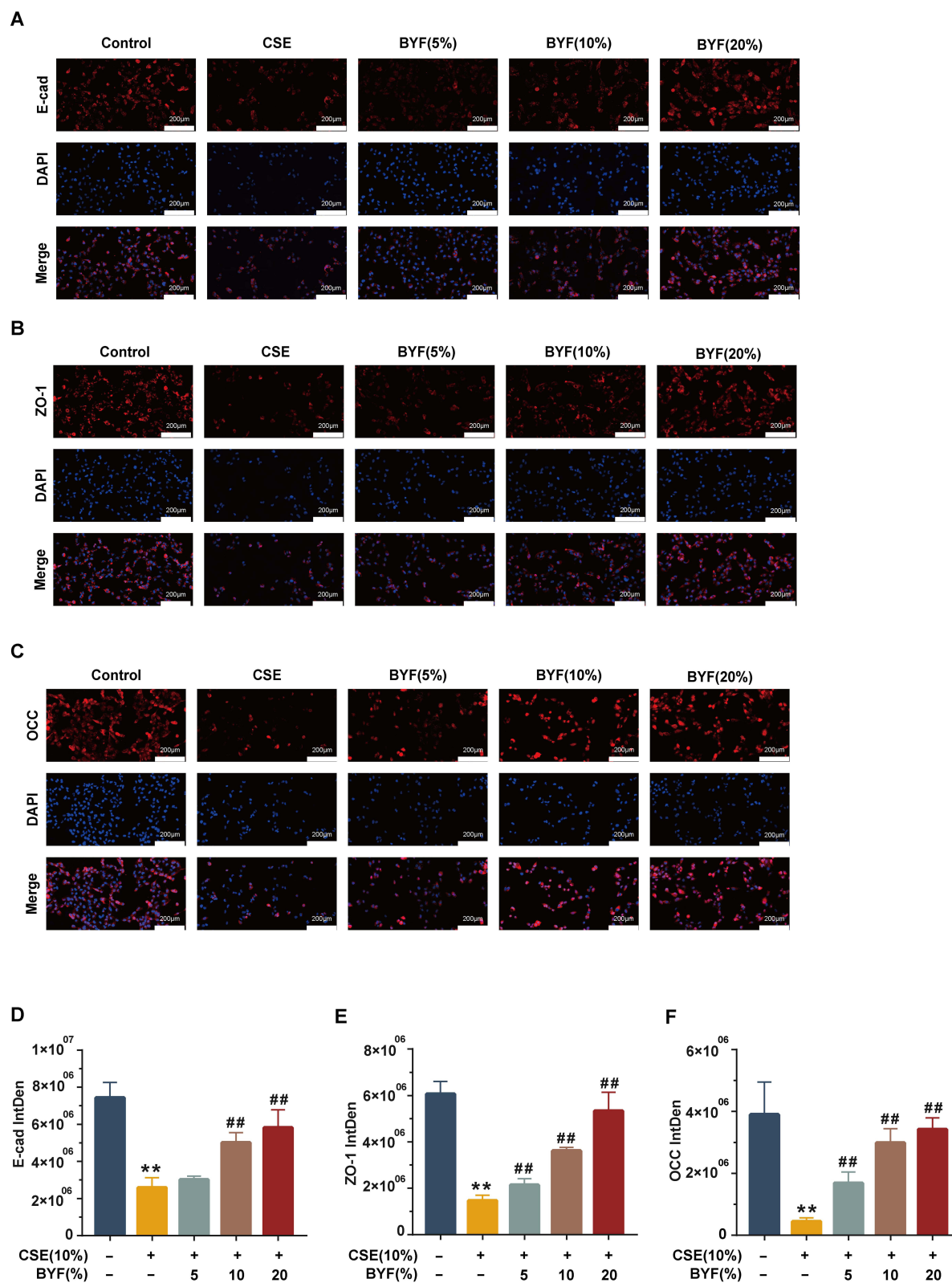


Figure 6 Immunofluorescence analysis of E-cad, ZO-1, and OCC expression in BEAS-2B cells. (**A–F**) The expressions of E-cad, ZO-1, and OCC in BEAS-2B were detected by immunofluorescence staining (n=6). Scale bar, 200 μ m. ** P <0.01, vs. Control group. ### P <0.01, vs. CSE group.

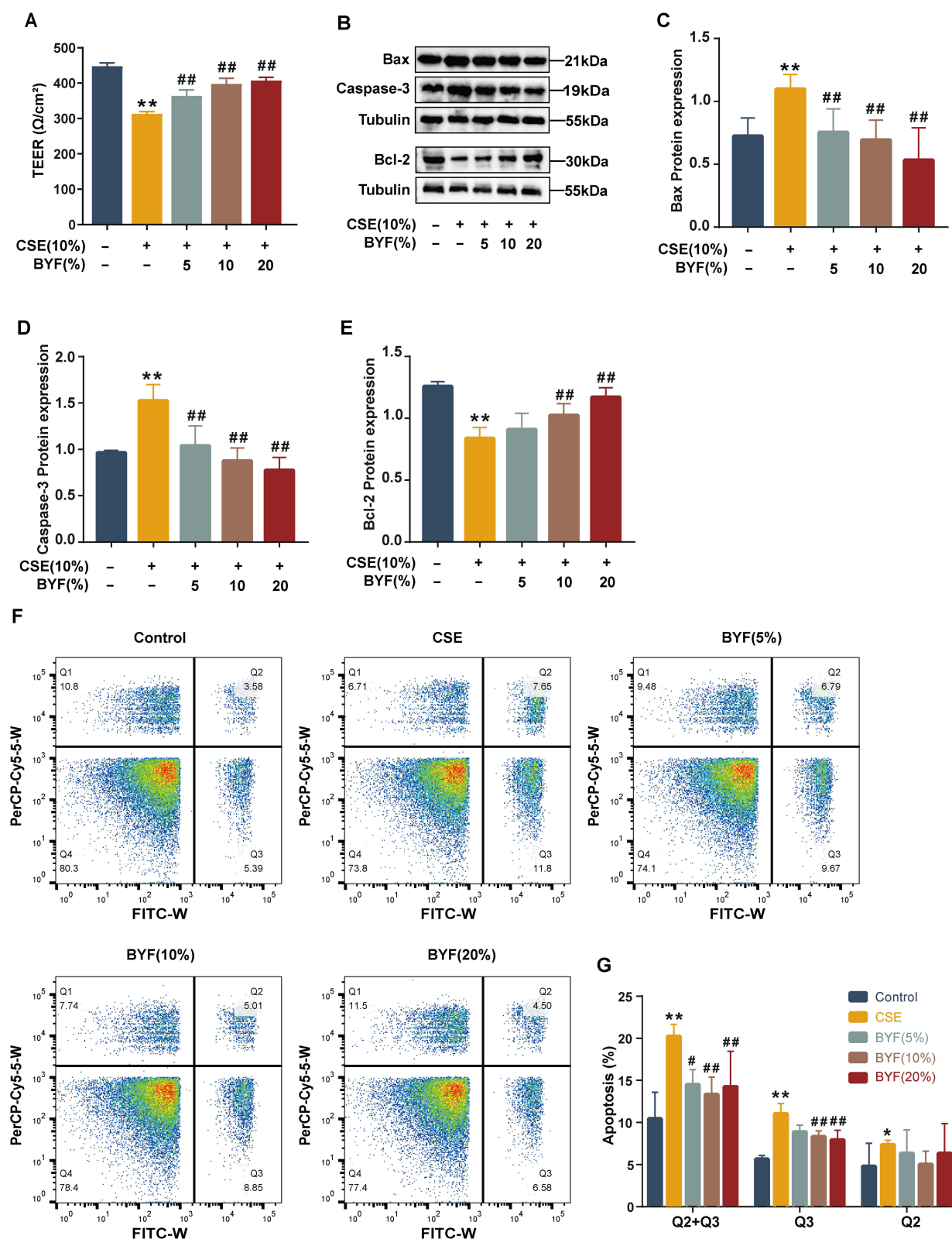


Figure 7 The effect of BYF on TEER and apoptosis in BEAS-2B cells. **(A)** Changes in TEER were measured after 24 h of intervention. (n=3). **(B–E)** The expression of Bax, caspase-3, and Bcl-2 were detected by Western blot analysis (n=3). **(F and G)** Flow cytometry assay was used to detect the apoptosis of BEAS-2B (n=3). Q3: early apoptosis rates, Q2: late apoptosis rates, Q2+Q3: overall apoptosis rates * $P < 0.05$, ** $P < 0.01$, vs. Control group. # $P < 0.05$, ## $P < 0.01$, vs. CSE group.

concentrations significantly increasing Bcl-2 expression ($P<0.01$, Figure 7B–E). Additionally, Flow cytometry determined early, late, and overall apoptosis rates; in comparison to the control group, the apoptosis rate was significantly increased in the CSE group; BYF significantly reduced both early and total apoptosis rates of the cells. ($P<0.05$ or $P<0.01$, Figure 7F and G).

BYF Attenuates CSE-Induced Oxidative Damage in BEAS-2B Cells

To explore the antioxidant activity of BYF in CSE-induced oxidative stress, we evaluated the activity of GSH, SOD, and MDA in cell lysates: In contrast to the control group, the CSE group showed significantly reduced GSH and SOD expression, and increased MDA expression; compared to the CSE group, the BYF group increased expression in a concentration-dependent manner, while also reducing MDA expression ($P<0.01$, Figure 8A–C). ROS are the key mediators of oxidative stress. Flow cytometric results demonstrated that BYF reduced the fluorescence intensity of ROS in a dose-dependent manner ($P<0.01$, Figure 8D and E). Next, to explore the role of Nrf2 in the improvement of airway epithelial barrier function by BYF, we assessed the expression levels of Nrf2 protein via Western blot. Relative to the control group, the CSE group showed significantly downregulated Nrf2 expression; in contrast, the 10% and 20% BYF concentrations significantly increased these expression levels. This study also analyzed the expression of HO-1 protein, which is regulated by Nrf2, and found that compared to the CSE group, the BYF group showed significantly upregulated HO-1 protein expression ($P<0.01$, Figure 8F–H). This suggests that the improving effect of BYF on the airway epithelial barrier function may be related to the antioxidant mechanism mediated by the Nrf2/HO-1 pathway.

The Effect of BYF on BEAS-2B Cell Barrier Impairment Under CSE Intervention with Nrf2 Inhibitor ML385

After 24 hours of intervention, results indicated that compared to the Control group, 2.5 mM and 5 mM ML385 concentrations had no significant effect on BEAS-2B cell viability; however, cell viability was significantly reduced in the 10 mM, 20 mM, and 40 mM ML385 groups ($P<0.01$, Figure 9A). Therefore, 5 mM ML385 was identified as the optimal intervention concentration.

To further confirm the role of Nrf2 in the improvement of respiratory epithelial barrier function by BYF, we used ML385 to inhibit the expression of Nrf2 in BEAS-2B cells. Western blot findings indicated that, as opposed to the BYF group, the ML385+BYF group had reduced Nrf2 and HO-1 expression, confirming that BYF exerts its pharmacological effects through the Nrf2/HO-1 signaling pathway. We then explored the effects of BYF on oxidative stress, cell apoptosis, and TEER in the BEAS-2B cell model under CS intervention after ML385 treatment. Western blot results showed that compared to the BYF group, the ML385+BYF group had significantly upregulated Bax and caspase-3 protein expression and downregulated Bcl-2 expression ($P<0.05$ or $P<0.01$, Figure 9B–G). TEER results indicated that compared to the BYF group, TEER in the ML385+BYF group cells was significantly reduced ($P<0.01$, Figure 9H). Oxidative stress results demonstrated compared to the BYF group, the ML385+BYF group had significantly upregulated MDA expression and significantly downregulated GSH and SOD expression ($P<0.01$, Figure 10A–C). Similarly, flow cytometric results demonstrated that ML385+BYF treatment also increased the fluorescence intensity of ROS ($P<0.05$, or $P<0.01$, Figure 10D and E). The above results further confirmed that BYF Ameliorates Cigarette Smoke-Induced Barrier Dysfunction in BEAS-2B Cells via Activation of the Nrf2/HO-1 Signaling Pathway.

Discussion

COPD is a prevalent, preventable, and treatable disease that has become a major global public health challenge.⁴² Cigarette smoking is the most common trigger for chronic obstructive pulmonary disease (COPD). Prolonged exposure to cigarette smoke leads to impaired airway epithelial barrier function and inflammatory responses, which further aggravate clinical symptoms.⁴³ Damage to the airway epithelial barrier is closely associated with the progressive exacerbation of COPD and the decline in quality of life. Therefore, effective strategies to ameliorate airway epithelial barrier injury are urgently needed. Chinese herbal medicines exhibit multi-component and multi-target characteristics with remarkable therapeutic efficacy, which have been widely recognized. By integrating transcriptomics, proteomics,

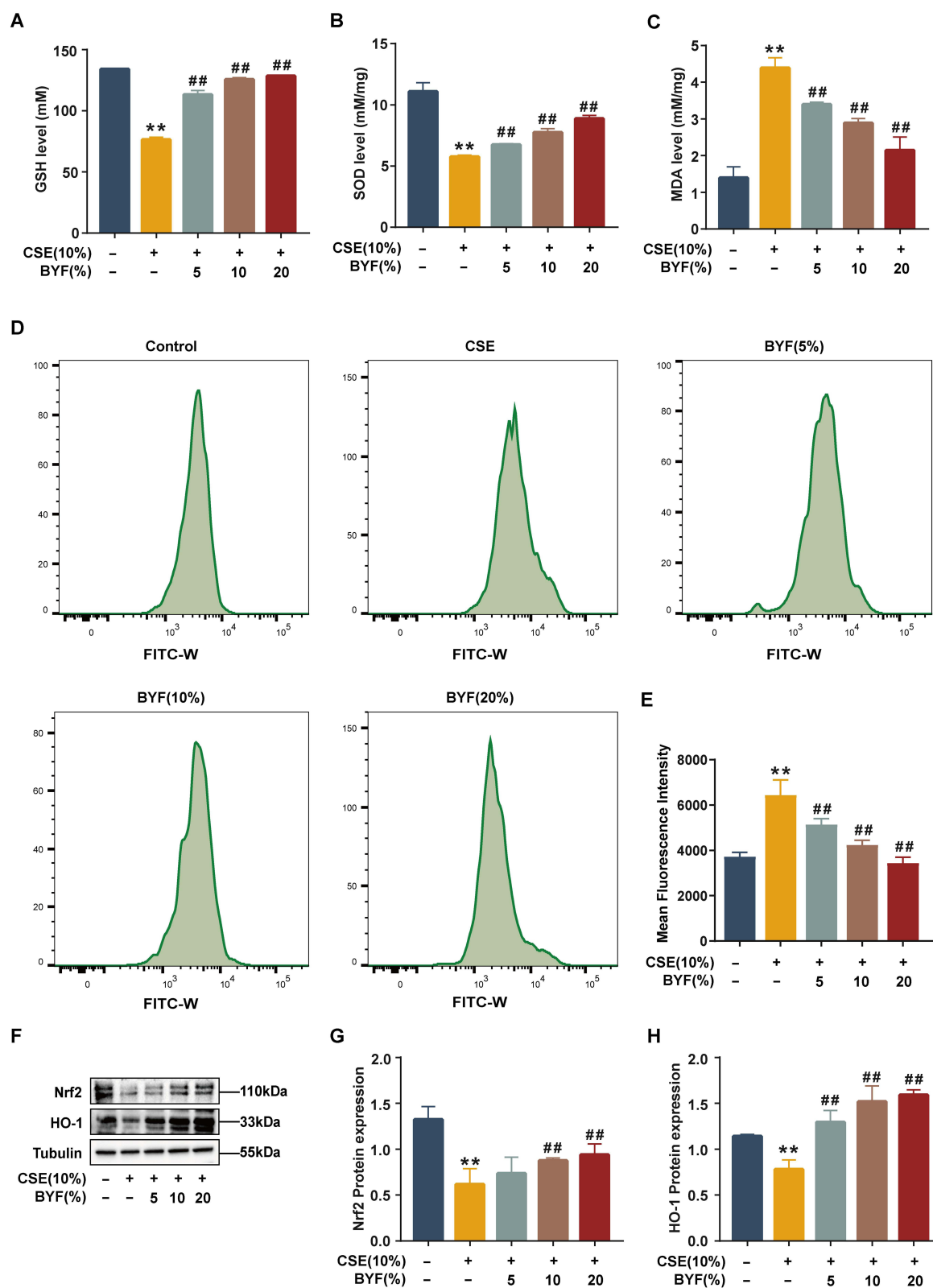


Figure 8 The effect of BYF on the expression of oxidative stress and Nrf2 signaling pathway-related proteins in BEAS-2B cells. (A–C) The expression levels of GSH, SOD, and MDA levels were examined in BEAS-2B (n=6). (D and E) The levels of ROS were determined by flow cytometry. (F–H) The expression of Nrf2 and HO-1 were detected by Western blot analysis (n=3). ** $P < 0.01$, vs. Control group. ## $P < 0.01$, vs. CSE group.

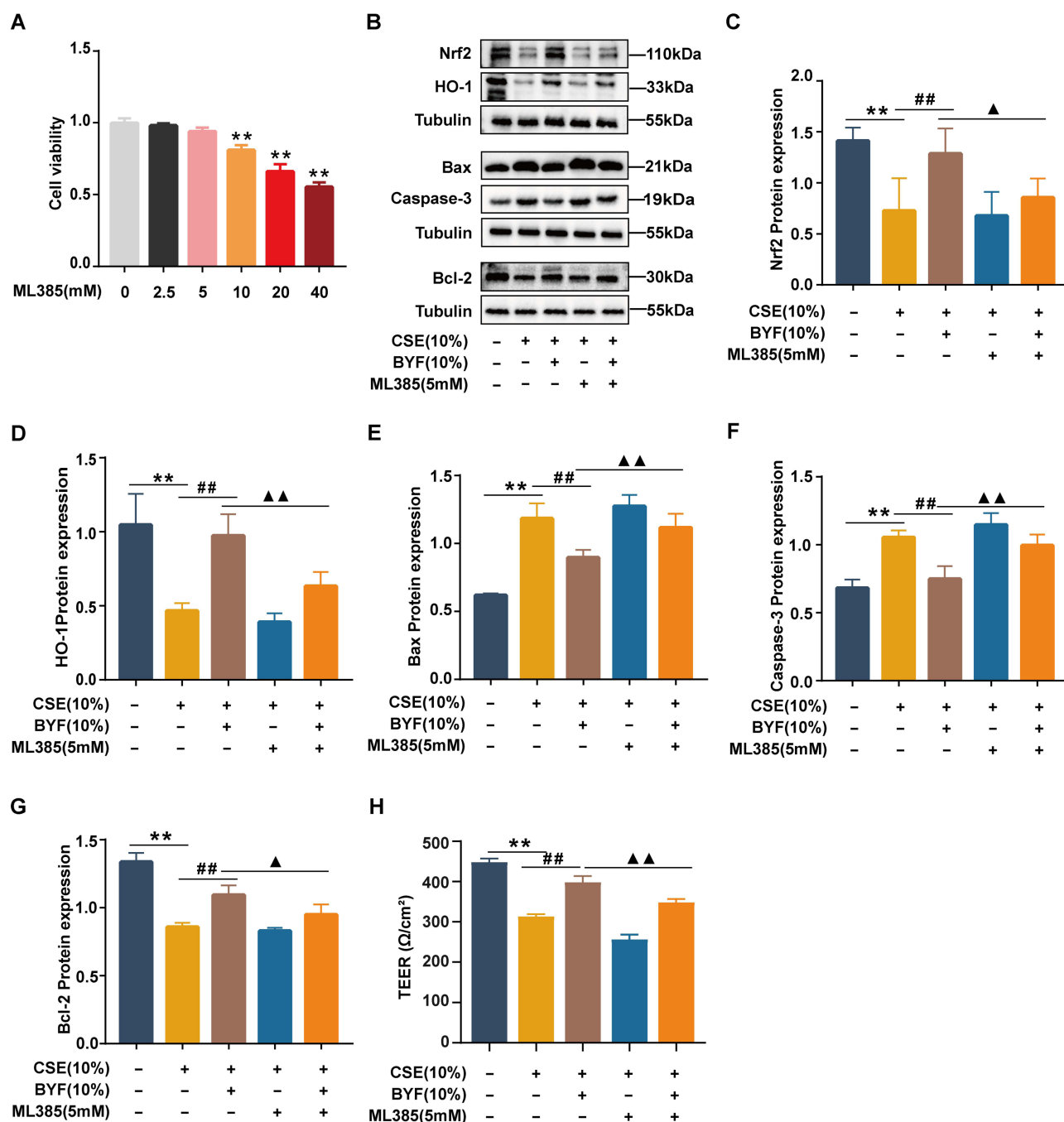


Figure 9 The effect of BYF on BEAS-2B apoptosis and Nrf2 signaling pathway-related proteins under CSE intervention with Nrf2 inhibitor ML385. **(A)** CCK-8 assays were used to measure cell viability (n=6). **(B–G)** The expression of Nrf2, HO-1, Bax, caspase-3, and Bcl-2 were detected by Western blot analysis (n=3). **(H)** Changes in TEER were measured after 24 h of intervention (n=3). ***P*<0.01, vs. Control group. ##*P*<0.01, vs. CSE group. ▲*P*<0.05, ▲▲*P*<0.01, vs. BYF group.

metabolomics and systems pharmacology, we revealed that BYF exerts therapeutic effects on COPD via regulating lipid metabolism, inflammation, oxidative stress and cell junction pathways at a systemic level. Furthermore, we employed UPLC-QE-Orbitrap-MS to identify the blood-absorbed components of the drug, and ultimately confirmed 10 blood-absorbed components that were significantly associated with the treatment of COPD: Ginsenoside Rb1, Ginsenoside Rg1, Astragaloside IV, Peimine, Schisandrin B, Icarin, Hesperidin, Nobiletin and Paeoniflorin.⁴⁴ Initial findings demonstrated BYF's capability to stabilize alveolar structure, alleviate pulmonary tissue pathology, reduce the thickness of pulmonary

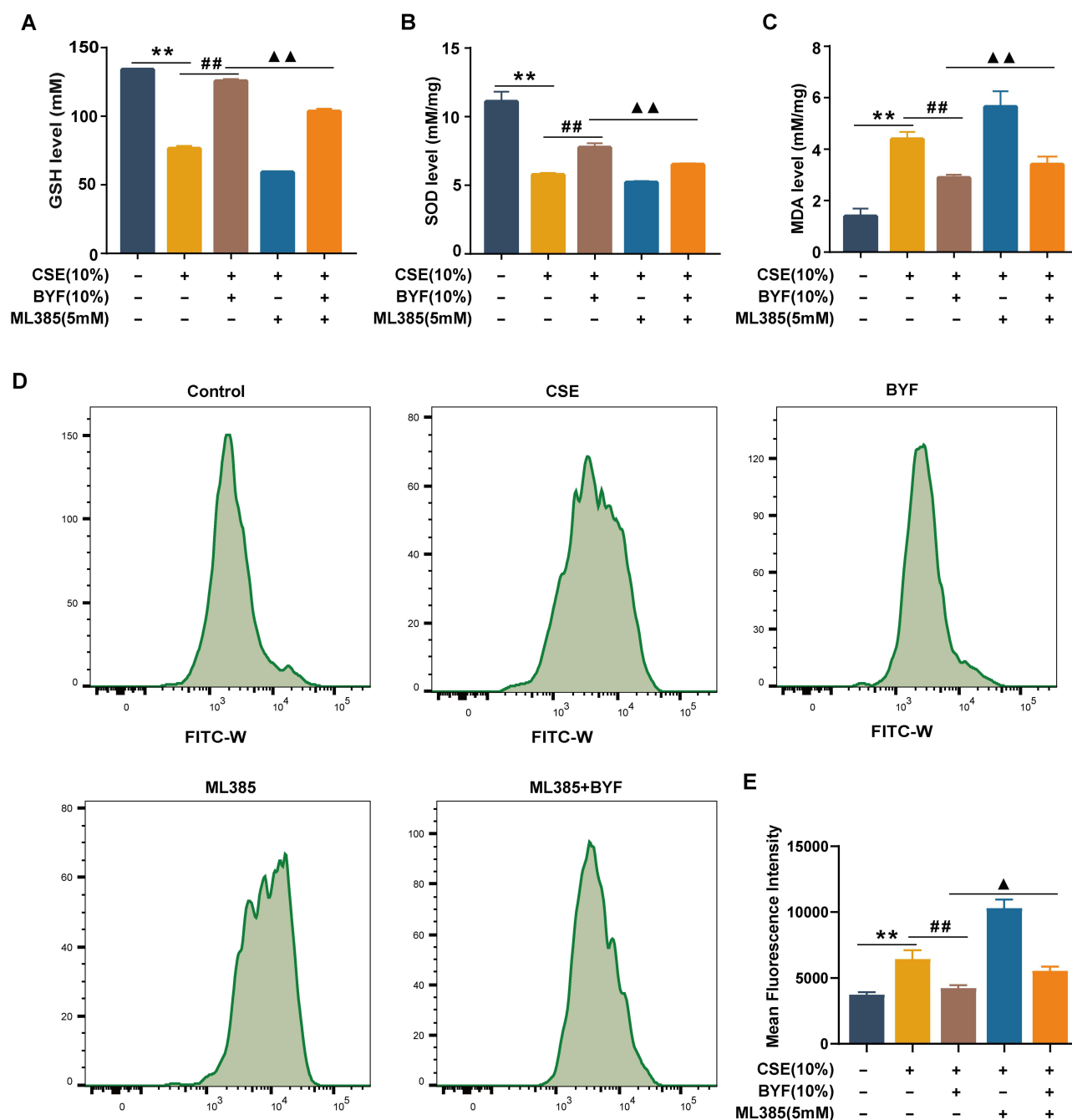


Figure 10 The effect of BYF on oxidative stress under CSE intervention with Nrf2 inhibitor ML385. (A–C) GSH, SOD, and MDA levels were examined in BEAS-2B (n=6). (D and E) The levels of ROS were determined by flow cytometry. ** $P < 0.01$, vs. Control group. ### $P < 0.01$, vs. CSE group. ▲ $P < 0.05$, ▲▲ $P < 0.01$, vs. BYF group.

arteriole walls in COPD rats, inhibit the proliferation of pulmonary artery smooth muscle cells, enhance skeletal muscle structure and function, and regulate immune function.^{45–47}

Cigarette smoking can lead to the deposition of a large number of toxic particles in the lungs, inducing the massive aggregation of inflammatory cells.⁴⁸ These inflammatory cells further release pro-inflammatory cytokines such as IL-6, TNF- α , and TGF- β 1, which not only exacerbate the inflammatory response in the lungs and cause damage to lung tissue epithelial cells but also stimulate the activation of fibroblasts and their participation in airway structural remodeling. This ultimately results in a reduction in the diameter of the airway lumen, leading to airflow limitation.⁴⁹ In addition, the

increased number of inflammatory cells promotes the release of a large number of proteases, accelerating the degradation of the alveolar wall and thereby inducing emphysema.⁵⁰

Pulmonary function tests are of great reference value for clarifying the type, lesion location, and severity of airflow limitation in COPD, as well as for evaluating the therapeutic effect and prognosis of COPD. Therefore, they are regarded as the “gold standard” for the clinical diagnosis of COPD.⁵¹ Among the parameters, TV and MV are key indicators for assessing airway patency, reflecting lung tissue elasticity, and respiratory muscle strength.^{52,53} FEV_{0.1}, FVC, and the FEV_{0.1}/FVC ratio are the most commonly used clinical parameters for evaluating ventilatory function. They can effectively reflect large airway resistance and are core indicators for the diagnosis of COPD and the grading of airflow limitation severity.^{54,55} Consistent with these clinical relevance, the results of this study demonstrate that BYF can significantly alleviate the degree of pulmonary function decline in COPD rats, improve the state of airflow limitation, relieve the pathological damage of lung tissue, and exert a significant inhibitory effect on both local lung tissue inflammation and systemic inflammatory responses.

The respiratory epithelium, a complex system comprising diverse cell types, collaborates with the immune system to maintain homeostasis of the respiratory tract environment and constitutes a complete respiratory barrier to prevent foreign particles and pathogens from infiltrating the submucosal interstices.¹⁴ Disruption of the integrity of the respiratory barrier leads to deep airway stimulation by inhaled foreign substances, triggering respiratory infections and immune responses, indicating a close relationship between respiratory epithelial barrier dysfunction and respiratory diseases.⁴⁰ Studies have shown that eight weeks of CS exposure in mice significantly diminishes the levels of barrier junction proteins,⁵⁶ CSE exposure reduces the TEER in cultured human lung adenocarcinoma cells at the air-liquid interface and suppresses the gene expression levels of claudin, OCC, E-cad and ZO-1.⁵⁷ Similarly, our experimental results demonstrate that in vivo CS exposure leads to reduced expression of ZO-1, OCC, and E-cad in the lung tissue of COPD rats. In vitro, CSE exposure causes the degradation of ZO-1, OCC, and E-cad between BEAS-2B cells, followed by a decline in cellular TEER, culminating in respiratory epithelial barrier damage.

Cell apoptosis is essential for the dynamic equilibrium of respiratory epithelial cells. Under normal conditions, apoptotic mechanisms regulate the proliferation of epithelial cells and remove damaged cells without inducing a chronic inflammatory response.⁵⁸ However, in COPD, sustained cellular damage caused by CS leads to an apoptotic response that is a key factor in the pathogenesis of COPD. Prolonged CS exposure, resulting in reduced integrity of intercellular connections, elevates pro-inflammatory cytokines and immune cells, thereby inducing apoptosis.^{16,18} As the disease severity increases, the apoptosis of epithelial cells rises, further disrupting the epithelium and creating a vicious cycle of epithelial barrier structural and functional disorders and chronic respiratory infections in COPD patients. The B-cell lymphoma-2 (Bcl-2) protein family plays a significant role in regulating apoptosis-related gene proteins, with Bcl-2 associated Xprotein (Bax) and Bcl-2 proteins respectively promoting and inhibiting apoptosis.⁵⁹ Cysteiny aspartate specific proteinase-3 (caspase-3) is a central protease in the Caspases family that mediates apoptosis, playing a decisive regulatory role in the process.⁶⁰ Heightened expression of the pro-apoptotic proteins Bax and caspase-3, alongside a reduced expression of the anti-apoptotic protein Bcl-2 in mouse lung tissue exposed to CS.⁶¹ Flow cytometry results in vitro demonstrate that CS elevates the proportion of apoptosis in HBEC cells.⁶² Our experimental results show that, as detected by Western blot, TUNEL staining, and flow cytometry, CSE can significantly increase the apoptotic rate of BEAS-2B cells in vitro. In vivo experiments, airway epithelial cell apoptosis also occurred in COPD model rats. These results are consistent with previous research reports.

Smoking introduces a substantial influx of ROS into the pulmonary matrix, instigating oxidative stress and inflicting direct injury to lung tissue, evidenced by the diminution of oxidative stress biomarkers glutathione (GSH) and superoxide dismutase (SOD), alongside an elevation of the lipid peroxidation marker malondialdehyde (MDA).^{57,63} GSH, a principal antioxidant, orchestrates the direct or indirect scavenging of free radicals and reactive nitrogen species via enzymatic pathways.⁶⁴ SOD catalyzes the dismutation of superoxide anions into hydrogen peroxide and dioxygen, forestalling the genesis of toxic hydroxyl radicals.⁶⁵ MDA quantification typically mirrors the extent of lipid peroxidation within tissues, offering an indirect metric of cellular impairment.⁶⁶ In the context of COPD, CS exposure precipitates an oxidative/antioxidative disequilibrium, catalyzing premature senescence of epithelial cells, compromising barrier junction integrity, and disrupting the epithelial barrier, culminating in impaired barrier functionality.¹⁶ Thus, elucidating the oxidative stress

mechanisms in COPD is pivotal for clinical intervention. Our research findings indicate that BYF can not only upregulate the expression levels of GSH and SOD while reducing the expression level of MDA, but also decrease ROS expression in a dose-dependent manner, suggesting that BYF can alleviate oxidative stress damage. In summary, BYF is capable of reversing the decreases in TEER and the expression of E-cad, ZO-1, and OCC in the COPD model, and can simultaneously inhibit oxidative stress responses and the process of cell apoptosis. However, the underlying molecular mechanisms through which it exerts these effects remain unclear. Nonetheless, the mechanisms underpinning these observations remain to be fully elucidated.

Nrf2, a transcription factor with pronounced expression in respiratory epithelial cells, has been shown to participate in the pathogenesis and development of COPD by regulating inflammation, macrophage function, and apoptotic autophagy, among other mechanisms.⁶⁷ It is typically targeted for ubiquitination and subsequent proteasomal degradation while remaining sequestered in a complex with Keap1. Upon oxidative challenge, the degradation pathway of Nrf2 is obstructed, releasing Nrf2 from the Keap1 complex to translocate to the nucleus and activate downstream antioxidant response elements, thereby orchestrating the expression of genes pivotal for antioxidative defense and cellular protection, thus safeguarding the respiratory epithelium.^{17,68} When oxidative products such as ROS exceed the body's clearance capacity, the resulting overload leads to an imbalance in Nrf2-mediated oxidation/antioxidation homeostasis and dysregulated expression of downstream antioxidant enzymes, thereby inducing oxidative stress in the body.⁶⁹ Heme oxygenase-1 (HO-1), a cytoprotective enzyme and downstream effector of Nrf2, is implicated in cellular defenses against apoptosis and oxidative stress, catalyzing heme degradation to yield carbon monoxide, biliverdin, and ferrous ions—agents that modulate apoptosis, inflammation, and oxidative stress, which is essential in the intracellular mechanism for counteracting oxidative stress.^{70,71} The Nrf2/HO-1 axis comprises a protective signaling cascade that fortifies pulmonary tissues against oxidative insult and damage throughout COPD progression. The attenuation of Nrf2 can precipitate a disruption in the cellular redox homeostasis, curtail reparative processes, accelerate senescence, and potentiate mortality, thereby elevating COPD susceptibility.^{68,72} Confirmatory studies have demonstrated a downregulation of Nrf2 expression in the pulmonary tissue of COPD patients. Cumulative research underscores the criticality of modulating the Nrf2 axis in the therapeutic landscape of COPD. For example, Allyl isothiocyanate, likewise, mitigates CS-induced apoptotic and oxidative phenomena in BEAS-2B via the Nrf2 pathway.⁷³ Similarly, our animal experiments demonstrated that CS downregulated the expression of Nrf2 in the COPD model, whereas BYF upregulated the expression of Nrf2 and HO-1, thereby ameliorating pathological injury and oxidative stress in the lung tissues of COPD rats.

To further investigate the association between the antioxidant mechanism of BYF and Nrf2, we employed CSE to stimulate BEAS-2B cells and establish an *in vitro* model. Concordant with our projections, our data reveal that CSE exposure can precipitate barrier dysfunction in BEAS-2B cells, characterized by attenuated expression of junctional proteins, a decrement in TEER, and an escalation in apoptotic and oxidative stress markers. BYF reversed the CSE-induced abnormalities in a concentration-dependent manner. These findings intimate that BYF confers a restorative effect on the compromised barrier function of BEAS-2B cells. Further, employing the Nrf2 inhibitor ML385 substantiated the role of Nrf2, and we found that ML385 markedly suppressed the antioxidant effects of BYF, suggesting that activation of Nrf2 is a key antioxidant pathway responsible for the effects of BYF.

Conclusions

Our study established *in vivo* and *in vitro* COPD models to elucidate the mechanism by which BYF ameliorates airway epithelial barrier injury in COPD. Experimental data demonstrated that *in vivo*, BYF significantly reversed the decline in pulmonary function and pathological damage to lung tissue in COPD rats. It also effectively inhibited the release of inflammatory cytokines, apoptosis of airway epithelial cells, and oxidative stress-induced injury. In parallel, *in vitro* experiments showed that BYF alleviated CSE-induced oxidative stress injury and apoptosis in human bronchial epithelial cells, while reducing epithelial cell permeability. These effects collectively contributed to the protection of airway epithelial barrier function. Furthermore, this study identified that activation of the Nrf2 signaling pathway could reverse the aforementioned aberrant changes induced by CSE.

Abbreviation

AJ, Adherent junction; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated Xprotein; BEAS-2B, Bronchial epithelial cells; BYF, Bu-Fei Yi-Shen Formula; Caspase-3, Cysteineyl aspartate specific proteinase-3; CCK-8, Cell Counting Kit-8; COPD, Chronic Obstructive Pulmonary Disease; CS, Cigarette Smoking; CSE, Cigarette Smoking Extract; E-cad, E-cadherin; FEV_{0.1}, Forced expiratory volume in 0.1 second; FVC, Forced vital capacity; GSH, Glutathione; HO-1, Heme oxygenase-1; Kp, *Klebsiella pneumoniae*; MDA, Malondialdehyde; MV, Minute Volume; Nrf2, Nuclear factor erythroid2-related factor2; OCC, Occludin; ROS, Reactive Oxygen Species; SOD, Super oxide dismutase; TEER, Transepithelial epithelial electrical resistance; TJ, Tight junction; TV, Tidal Volume; ZO-1, Zonula Occludens-1.

Data Sharing Statement

Data is provided within the manuscript.

Ethics Approval

This study was conducted in strict adherence to the “Guidelines for Ethical Review of Laboratory Animal Welfare” (GB/T 35892-2018). Animal experiments were approved by the animal care committee of the First Affiliated Hospital, Henan University of Chinese Medicine (No. YFYDW2024018).

Author Contributions

All authors have made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas.; have participated in the drafting, writing, revision or review of the article; have reached a consensus on the journal to which the article is submitted; have accepted the final published version of the article; agree to be responsible for the content of the article.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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