

Bu-Fei Formula Ameliorates Inflammation in a Preclinical COPD-Like Model by Targeting Mitochondrial Hyperactivity to Inhibit the NLRP3 Inflammasome

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Background: Chronic Obstructive Pulmonary Disease (COPD) is characterized by persistent airflow limitation and chronic airway inflammation. While the traditional Chinese medicine Bu-Fei Formula (BFF) effectively ameliorates COPD symptoms clinically, however, the precise molecular mechanisms underlying its therapeutic effects remain a knowledge gap.

Methods: The therapeutic effects and anti-inflammatory mechanisms of BFF in COPD were investigated using cigarette smoke + LPS-induced COPD-like rats and CSE-stimulated macrophages. BFF treatment was administered in both models for 8 weeks in a dose-dependent manner. Lung injury, collagen deposition, and macrophage ultrastructure were assessed by H&E, Masson's staining, and TEM. The evaluation of IL-1 β , IL-18, NLRP3, ASC, and Caspase-1 p20 expression was performed via ELISA, IHC, IF, and Western blot. Oxidative stress (MDA, SOD) and mitochondrial function (complexes I–V, ATP, ROS) were also evaluated.

Results: BFF treatment produced a pronounced improvement in lung pathology of COPD-like rats, characterized by alleviation of tissue damage, suppression of inflammatory and alveolar degeneration, and reduction of collagen accumulation in the interstitium. It also ameliorated inflammatory injury at the cellular level by inhibiting macrophage pyroptosis. Mechanistically, BFF suppressed the overexpression of mitochondrial respiratory chain complexes I–V. It reduced the levels of ROS, ATP, MDA, NLRP3, ASC, Caspase-1 p20, IL-1 β , and IL-18, while enhancing SOD activity. These findings suggest that BFF mitigates the inflammatory damage in COPD by suppressing the hyperactivation of mitochondrial energy metabolism, enhancing the efficiency of the oxidative respiratory chain, and reducing ROS production, and suppressing the NLRP3 inflammasome response.

Keywords: Bu-Fei formula, inflammation, NLRP3 Inflammasome, mitochondrial respiratory chain complexes, chronic obstructive pulmonary disease

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease of the lungs, marked by sustained airway inflammation and partially reversible airflow obstruction.¹ With the global aging population, environmental pollution, and smoking, COPD holds the position as the third most prevalent,² with over 100 million patients in China alone, accounting for approximately one-quarter of the global burden.^{3,4} Current treatments primarily rely on palliative care, including antibiotics, inhaled corticosteroids, and bronchodilators during acute exacerbations, but these guideline-recommended therapies have limitations in halting disease progression. However, these medications carry considerable

side effects, such as osteoporosis, immunosuppression, cardiac arrhythmias, and visual impairment.^{5,6} Superior therapeutic efficacy of traditional Chinese medicine (TCM) decoctions for COPD has been confirmed in clinical studies, warranting further investigation into their pharmacological mechanisms.

COPD pathogenesis, which is complex and multifactorial, has been linked to inflammatory responses, oxidative injury, disruption of protease–antiprotease homeostasis, premature cellular aging, and abnormal airway responsiveness.⁷ Among these, the inflammatory mechanism is currently considered the primary pathogenic driver.⁸ Upon exposure to smoke or infection, pattern recognition receptors trigger the assembly of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, enhancing pyroptotic cell death while simultaneously driving the liberation of pro-inflammatory cytokines.⁹ Reactive oxygen species (ROS), particularly those derived from mitochondria (mtROS), serve as upstream signals for NLRP3 activation.¹⁰ Excessive ROS accumulation impairs the electron transport chain (ETC). Unlike classical mitochondrial dysfunction characterized by energy failure, this can lead to a compensatory mitochondrial hyperactivity, reducing overall respiratory efficiency while amplifying mtROS and abnormal ATP production, thereby establishing a positive feedback loop that exacerbates NLRP3 inflammasome activation.^{11,12} Persistent inflammation leads to airway infiltration, parenchymal destruction, emphysema formation, and ultimately pulmonary function failure.^{13,14} Although clinical research and practice have demonstrated the therapeutic efficacy of Bu-Fei Formula (BFF) in COPD management, the mechanistic pathways responsible for its pharmacological actions remain incompletely understood. It remains necessary to conduct additional studies to delineate the precise molecular mechanisms through which BFF exerts its therapeutic benefits in COPD.

BFF is composed of six herbal ingredients: *Radix Astragali*, *Radix Ginseng*, *Radix Salviae Miltiorrhizae*, *Fructus Psoraleae*, *Cortex Mori*, and *Radix Stemonae*. These herbs contain bioactive compounds with anti-inflammatory, antioxidant, and antiasthmatic properties. *Radix Astragali* contains astragaloside, which ameliorates airway remodeling by scavenging free radicals.¹⁵ *Radix Ginseng* improves COPD through ginsenosides,¹⁶ while *Radix Salviae Miltiorrhizae* provides tanshinones, particularly tanshinone IIA, which inhibit inflammation and oxidative stress.¹⁷ *Fructus Psoraleae* exhibits anti-inflammatory and antioxidant effects via bavachin,¹⁸ and *Cortex Mori* contains morusin B and D, which downregulate inflammatory mediators such as NO, TNF- α , and IL-6.¹⁹ *Radix Stemonae* provides alkaloids with anti-inflammatory and antitussive activities.^{20,21} Clinical studies and clinical practice have substantiated the therapeutic efficacy of BFF in the management of COPD. Specifically, studies have demonstrated that the addition of BFF to conventional COPD treatment improves lung function, reduces inflammation, reduces the frequency of acute exacerbations, and enhances quality of life in patients with stable COPD.^{22,23} However, the underlying mechanisms remain inadequately explored. Therefore, further wet laboratory research is needed to elucidate the precise molecular mechanisms underlying the therapeutic effects of BFF in COPD.

We hypothesized that BFF ameliorates COPD by modulating mitochondrial hyperactivity and the NLRP3 inflammasome. Using both animal and cell models of COPD, this study systematically investigated the mechanisms underlying BFF therapy. Notably, BFF treatment significantly improved lung histopathology, decreased inflammatory infiltration and alveolar structural injury, and inhibited pyroptotic activity in macrophages. Mechanistically, BFF suppressed the over-expression of mitochondrial respiratory chain complexes I–V, enhanced oxidative phosphorylation efficiency, and reduced excessive ROS production (Figure 1). These results provide compelling evidence that BFF exerts its therapeutic effects by modulating mitochondrial energy metabolism, suppressing ROS production, and inhibiting the activation of the NLRP3 inflammasome, which in turn disrupts the feedback loop of persistent inflammation in COPD.

Materials and Methods

Main Reagents, Drugs, and Materials

Main Reagents and Drugs

Lipopolysaccharide (LPS, IL2020) and mitochondrial respiratory chain complex assay kits (complexes I–V: BC0510, BC3230, BC3240, BC0940, BC1445) were obtained from Solarbio (Beijing, China). Hongqi Canal[®] filter-tipped cigarettes (11 mg tar, 0.9 mg nicotine, 11 mg CO) were supplied by Henan China Tobacco Company (Zhengzhou, China). ELISA kits for IL-1 β (GEM0002-96T), IL-18 (GEH0010-96T), MDA (A003-1), and SOD (A001-1) were

NLRP3-mediated pyroptosis

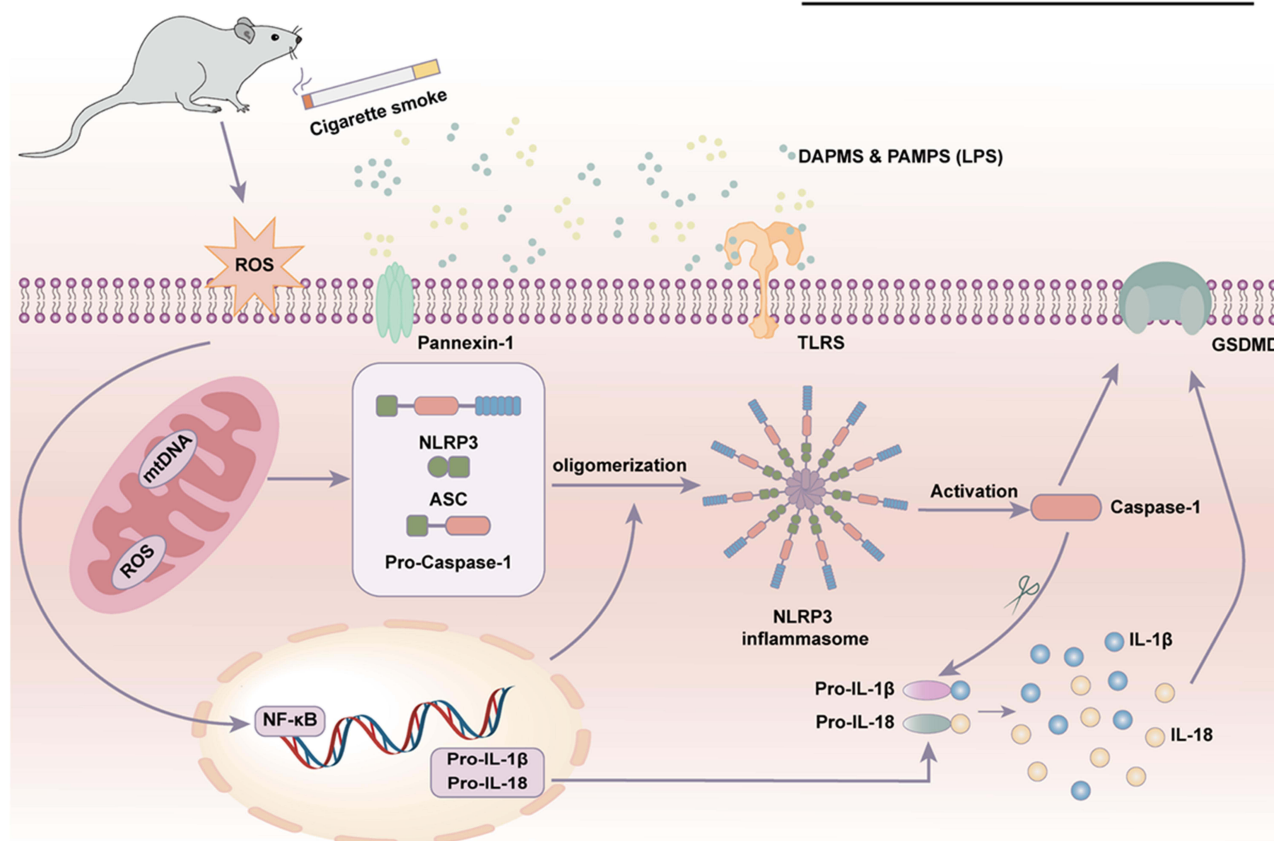


Figure 1 Activation of the NLRP3 inflammasome and the process of release of related inflammatory factors. Initial stimuli, such as cigarette smoke, induce ROS production and activate the NF- κ B pathway, leading to the transcriptional priming of pro-IL-1 β and pro-IL-18 (Signal 1). Subsequently, mitochondrial-derived danger signals (mtROS, mtDNA) trigger the oligomerization of NLRP3, ASC, and pro-Caspase-1 to form the active NLRP3 inflammasome. This complex activates Caspase-1, which cleaves pro-IL-1 β and pro-IL-18 into their mature, secreted forms, and cleaves GSDMD, resulting in pore formation in the cell membrane and lytic cell death (pyroptosis).

purchased from Servicebio (Wuhan, China) and Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively. ROS (KTB1910) and ATP (KTB1016) assay kits were provided by Abbkine Scientific (Wuhan, China). Primary antibodies against NLRP3 (GB114320-100), ASC (GB115270-100), Caspase-1 p20 (AF4005), and β -Actin (AF7018) were obtained from Servicebio (Wuhan, China) and Affinity Biosciences (Jiangsu, China).

Preparation of Bu-Fei Formula (BFF)

The herbal ingredients of Bu-Fei Formula (BFF), including *Radix Astragali* (Huangqi), *Radix Codonopsis* (Dangshen), *Fructus Psoraleae* (Buguzhi), *Radix Stemonae* (Baibu), *Cortex Mori* (Sangbaipi), and *Radix Salviae Miltiorrhizae* (Danshen), were provided by the Pharmacy Department of the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine according to the prescription ratio of 2:1:1.5:1:1:1.5. The herbs were soaked in an appropriate amount of water until fully saturated, then decocted and concentrated to prepare three dosage levels: low dose (4.2 g/kg), medium dose (8.4 g/kg), and high dose (16.8 g/kg). The dosages were calculated based on the body surface area conversion coefficient between humans and rats (conversion formula = standard dose / human body weight $\times$ 6.3). Decoctions were kept at 4°C prior to use.

Experimental Animals

50 specific pathogen-free (SPF) male Sprague-Dawley (SD) rats (250 $\pm$ 20 g) were used for the experimental procedures to avoid hormonal fluctuations. An additional 20 female SD rats were used for the preparation of drug-containing serum to prevent sex hormone cross-interference with the male modeling rats.²⁴ Sample sizes were determined based on

previous similar experimental studies to ensure adequate statistical power. All animals were obtained from Guizhou Huijiu Biotechnology Co., Ltd. (Guiyang, China). All animal study protocols received approval from the Animal Ethics Committee of Guizhou University of Traditional Chinese Medicine (Approval No. 2025101004). Animals were provided ad libitum access to food and water throughout the experimental period.

Cell Line

NR8383 rat alveolar macrophages (SCC-320411) were obtained from Solarbio (Beijing, China), grown in F-12K medium with 15% fetal bovine serum (FBS) and incubated at 37°C under 5% CO₂, using a suspension–adherent mixed culture method.

Establishment of COPD Rat Model and Drug Intervention

Based on a random number table, 50 healthy male SD rats were allocated into five groups ($n = 10$): Control group, Model group, and BFF groups at three dosage levels (4.2 g/kg, 8.4 g/kg, 16.8 g/kg). After a 7-day acclimatization period, a COPD-like rat model was established through a combination of cigarette smoke exposure and repeated LPS instillation.²⁵ Intratracheal instillation of 200 μ L LPS (1 g/L) was administered on days 0 and 14 to all rats except the Control group. Excluding day 14, rats were passively exposed to cigarette smoke in a custom-made exposure chamber twice daily (morning and afternoon) for 30 min each session, with an interval of at least 5 h between sessions, 7 days per week, for 12 consecutive weeks. General behavior and physical signs of the rats were monitored throughout the modeling period.

Starting from week 13, rats were administered treatments by gavage at a fixed time each morning. The BFF groups received the corresponding dosages of BFF decoction, while the Control and Model groups were given an isovolumetric amount of normal saline. All gavage administrations were precisely adjusted according to body weight at a rate of 1 mL per 100 g. The intervention continued for 8 consecutive weeks. On the day following the final administration, rats were weighed and anesthetized with isoflurane gas. Following collection from the abdominal aorta, blood samples were transferred into anticoagulant tubes and centrifuged at 3000 $\times$ g for 15 min at 4°C to obtain plasma. The plasma was immediately divided into 0.5 mL in cryovials and preserved at –80°C to avoid repeated freeze-thaw cycles. After euthanasia by overdose anesthesia, the lungs were harvested. The right upper lobe was immersed in 4% paraformaldehyde (PFA) for at least 24 h for histopathological analysis, while the remaining lung tissues were immediately immersed in liquid nitrogen for rapid freezing and preserved at –80°C for molecular experiments.

Preparation of Cigarette Smoke Extract (CSE) and Determination of Optimal Stimulation Conditions

A single cigarette was ignited, and the smoke was drawn through the filter tip using a 50 mL syringe and bubbled through 10 mL of basal culture medium with gentle shaking to ensure complete dissolution. The smoke extract from one cigarette dissolved in 10 mL of basal medium was defined as 100% CSE, which appeared pale yellow, clear, and free of precipitates. After filtration through a 0.22 μ m membrane, the CSE was diluted with serum-free medium to various concentrations (1%, 2%, 5%, 8%, 10%) for subsequent experiments.²⁶ NR8383 macrophages were plated at 4×10^4 cells/well in 96-well plates and cultured overnight for attachment. Following a 2-h synchronization in serum-free F-12K medium, cells were subjected to treatment with graded concentrations of CSE for exposure periods of 12, 24, or 48 hours. The assessment of cell viability, conducted via the CCK-8 assay, and cell survival rates were calculated for each concentration and time point. Simultaneously, IL-1 β and IL-18 levels in the supernatants were analyzed at the respective time points. The optimal CSE concentration was selected based on the following criteria: cell survival rate of 70–90% and significant elevation of inflammatory cytokines. Based on the experimental results, 5% CSE exposure for 24 h was selected as the modeling condition for subsequent cell experiments.

Preparation of Drug-Containing Serum and Determination of Optimal Dosage

Twenty healthy female SD rats (body weight: 200 $\pm$ 20 g) were acclimatized and then administered BFF decoction by gavage once daily for 7 consecutive days. The dosage was calculated according to the body surface area equivalence

principle (8.4 g/kg), and the gavage volume was adjusted based on body weight (1 mL/100 g). Rats assigned to the blank group were administered an equivalent volume of physiological saline. After the last treatment, blood was drawn from the abdominal aorta under anesthesia, incubated in a 37°C water bath for 15 min, and then centrifuged at 3000 r/min for 15 min at room temperature (RT) to obtain drug-containing serum. Blank serum was collected from untreated rats of the same batch as a control. All sera were preserved at -80°C until required.

To determine the appropriate concentration of BFF-containing serum for in vitro experiments, NR8383 macrophages were exposed to 5% CSE for 24 hours to establish a COPD-like cellular model. The cells were then exposed to a range of BFF-containing serum concentrations (0%, 1%, 2%, 4%, 6%, 8%, and 10%) or equal volumes of blank serum. Following a 24-hour incubation period at 37°C with 5% CO₂, cellular viability was determined via the CCK-8 method. Treatment with 4% drug-containing serum maintained cell viability above 70% and markedly suppressed IL-1 β and IL-18 concentrations relative to the model control group. Therefore, 4% was designated as the high-dose group (BFF-H), while 2% (half of the high dose) and 1% (one-fourth of the high dose) were designated as the medium-dose (BFF-M) and low-dose (BFF-L) groups, respectively, for subsequent experiments.

Chemical Characterization of BFF by UPLC-Q-Exactive-HRMS

The chemical constituents of BFF were systematically analyzed using (UPLC-Q-Exactive-HRMS) on a Thermo Scientific Q-Exactive system. The samples were separated on a Venusil MP C18 column (100 mm $\times$ 2.1 mm, 2.6 μ m), with its temperature controlled at 35°C. A binary mobile phase system was utilized, comprising 0.1% formic acid in water (B) and acetonitrile (A). The elution gradient was programmed as follows: 5% A was held for 5 minutes, increased to 15% over 10 minutes, raised further to 38% across 25 minutes, elevated to 65% over 20 minutes, climbed to 90% within 10 minutes, and finally maintained at 90% for 15 minutes. A constant flow rate of 0.3 mL/min was used, and 5 μ L of sample was injected.

The mass spectrometric detection was operated utilizing both ESI positive and negative polarities. Key ion source conditions included spray voltages of 3.5 kV (positive) and 3.2 kV (negative), a capillary temperature of 320°C, sheath and auxiliary gas flows set at 40 and 10 arbitrary units, respectively, and a probe heater maintained at 350°C. Subsequently, full scan spectra (m/z 100–1500) were collected at a resolution of 70,000 (FWHM). Data-dependent MS/MS analysis was then triggered at a resolving power of 17,500, employing a normalized collision energy of 30 eV.

Hematoxylin and Eosin (H&E) Staining

The right upper lobe of the lung was subjected to H&E staining to evaluate lung tissue injury. Lung tissues were fixed in 4% PFA for 24 h, rinsed with running tap water, and dehydrated by passing through a sequence of ethanol solutions (from low to high concentrations, with a 1-hour incubation time per step). The tissues were cleared in xylene, infiltrated with paraffin, and embedded. These blocks were sectioned at 5 μ m after cooling. The sections were then dried, deparaffinized, and rehydrated through a descending ethanol gradient (from high to low concentrations). After a water rinse, they were stained with hematoxylin, differentiated in acid alcohol, and counterstained with eosin. The final stages involved dehydration via an ascending ethanol series (from low to high concentrations), a final xylene clearance step, and mounting using neutral balsam. Histopathological changes, including alveolar structure, inflammatory cell infiltration, emphysema formation, and congestion, were observed under a light microscope by two independent pathologists blinded to the group allocation. Alveolar destruction was assessed by calculating MLI as previously described.²⁷

Masson's Trichrome Staining (Collagen Deposition Analysis)

Following deparaffinization with two 15-minute xylene washes, lung sections were progressively rehydrated through a graded ethanol series (from high to low concentrations), stained with Weigert's iron hematoxylin for nuclei, stained with acid fuchsin-ponceau for cytoplasm and collagen, differentiated with 1% phosphomolybdic acid, and counterstained with aniline blue for collagen. Subsequently, sections were gently rinsed in 1% acetic acid, passed through an ascending ethanol gradient for dehydration, cleared in xylene, and mounted with neutral balsam. Collagen was visualized as blue under light microscopy, while muscle fibers and cytoplasm appeared red. The extent of collagen deposition was quantified using Image J software by calculating the ratio of blue-stained area to total tissue area.²⁸

Transmission Electron Microscopy (TEM) Analysis

To examine ultrastructural changes, lung samples were trimmed into 1 mm³ cubes, fixed in pre-chilled 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide (4°C), and dehydrated through a graded acetone series (30% to 100%, with three final changes). After embedding, 60–90 nm sections were cut, transferred to copper grids, and doubly stained with uranyl acetate (10–15 min) and lead citrate (1–2 min), and examined under a transmission electron microscope to observe ultrastructural changes in lung tissue cells. The same procedure was applied to cell samples.

Immunohistochemistry (IHC) Analysis

Immunohistochemical analysis was performed to detect Caspase-1, NLRP3, and ASC expression in rat lung tissues. Tissue sections (5 µm) were baked at 60°C for 60 min, followed by deparaffinization and rehydration. Antigens were achieved using heating the sections in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 15 minutes, then allowing the sections to cool naturally. Subsequently, endogenous peroxidase was quenched by applying 3% H₂O₂ for 20 minutes. Non-specific binding sites were blocked using 5% normal goat serum at 37°C for 20 minutes, after which the sections were treated with respective primary antibodies against Caspase-1 p20 (1:100), NLRP3 (1:100), and ASC (1:100) at 4°C overnight. Following PBS rinses, sections were treated with horseradish peroxidase (HRP)-conjugated secondary antibody, applied at a 1:200 dilution, for 30 min at 37°C. Following PBS washing, sections were developed with DAB, counterstained with hematoxylin, cleared, and mounted. Positive staining appeared as brown granules under microscopy. Quantitative analysis was performed using Image J software.

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-1β, IL-18, MDA, SOD, mitochondrial respiratory chain complexes I–V, ROS, and ATP in plasma or lung tissue homogenates were analyzed using commercial ELISA kits. The general procedure included: coating, blocking, addition of samples or standards, incubation, washing, addition of biotinylated detection antibody (100 µL, 0.25 µg/mL, incubation at 37°C for 1 h), washing, enzyme labeling, color development, and absorbance measurement. Sample concentrations were calculated based on standard curves. Specific operational steps varied slightly depending on the factor being detected and were performed strictly according to the kit instructions. For cell samples, supernatants were collected after high-speed centrifugation, and when necessary, an ultrasonic cell disruptor was used for further disruption. The remaining procedures were similar to those for animal experiments.

Western Blot Analysis

Protein expression of Caspase-1 p20, NLRP3, and ASC was analyzed by Western blot. Pulverized lung tissues were homogenized in RIPA buffer containing phosphatase and protease inhibitor cocktails (Biosharp, China), followed by centrifugation at 12,000 ×g for 10 minutes at 4°C to collect the supernatant. Protein concentrations were measured with a BCA method (Biosharp) using a BSA standard curve (range: 0–2000 µg/mL). Equal amounts of protein were resolved on 12.5% SDS-PAGE gels and transferred to methanol-activated PVDF membranes (Millipore) at 320 mA for 90 min. Membranes were then blocked for one hour with 5% non-fat dry milk before an overnight incubation at 4°C with specific primary antibodies (internal control and target proteins at 1:1000 dilution). After three 10-minute washes with TBST, the blots were probed with an HRP-conjugated secondary antibody (1:10,000 dilution) for 2 h, and signals were captured by enhanced chemiluminescence (ECL) detection for subsequent quantification using ImageJ. For cell experiments, an ultrasonic cell disruptor was used for cell disruption, and the remaining procedures were similar to those for animal experiments.

Immunofluorescence (IF)

For immunofluorescence analysis, macrophages were cultured on coverslips in 48-well plates until 60–80% confluent. Cells were first washed gently with PBS to remove debris, then fixed with 4% PFA (20 min, RT), permeabilized with 0.5% Saponin (10 min, RT), and blocked with 1% BSA (30 min, RT). Primary antibodies diluted in 1% BSA-PBS were applied overnight at 4°C. After PBS washes, fluorophore-conjugated secondary antibodies in 1% BSA-PBS were applied for 30–60 min at RT in the dark. Following further washes in the dark, nuclei were stained with DAPI (5 µg/mL,

5–10 min). Finally, after final PBS washes, coverslips were mounted and images were acquired using fluorescence microscopy.

Statistical Analysis

All datasets initially underwent normality assessment with the Shapiro–Wilk test and evaluation of variance homogeneity using Levene's test. For data meeting both assumptions of normal distribution and equal variances, results are presented as mean $\pm$ standard deviation, and group comparisons were conducted with the independent samples *t*-test or one-way ANOVA followed by Tukey's post-hoc test where applicable. Conversely, for datasets violating these assumptions, values are reported as median (25th percentile, 75th percentile), and the non-parametric Mann–Whitney *U*-test was employed for inter-group analysis. A significance threshold of $\alpha = 0.05$ was established, where P-values below 0.05 and 0.01 were deemed statistically significant and highly significant, respectively. All statistical computations were carried out using SPSS 26.0 in conjunction with Microsoft Excel.

Results

Compositional Profiling of BFF Chemical Constituents

A total of 719 components were successfully characterized and classified into 11 distinct chemical categories (Figure 2A and Table S1). The dominant constituents were Flavonoids (25.26%), followed by Alkaloids and derivatives (13.68%), Terpenoids (13.51%), and Indoles and derivatives (13.40%). Collectively, these four major classes constituted approximately 65.85% of the total identified components. Other notable categories included Others (10.18%), Carbohydrates and derivatives (4.96%), Phenolic acids and derivatives (4.65%), and Amino acids and derivatives (4.66%). The total ion chromatograms (TIC) in negative (Figure 2B) and positive (Figure 2C) ion modes further illustrated the chemical complexity of BFF. The analysis confirmed the presence of numerous bioactive compounds consistent with the source herbs. Key components identified in the negative mode included organic acids and active constituents such as Salvianolic

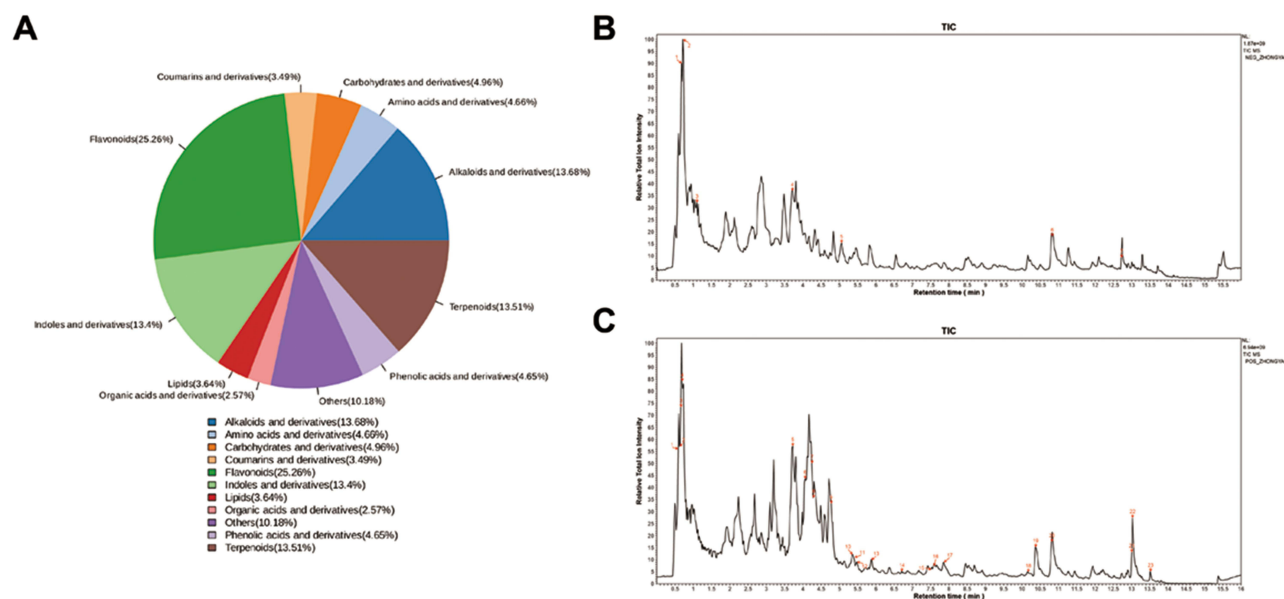


Figure 2 Comprehensive chemical profile of the Bu-Fei Formula (BFF) analyzed by UPLC-Q-Exactive-HRMS. **(A)** Chemical classification of identified compounds. The pie chart illustrates the percentage composition of the 11 major chemical categories identified in BFF. Detailed information on all 719 identified compounds is provided in Table S1. **(B)** Total ion chromatogram of BFF in negative ion mode. The chromatogram displays the overall chemical profile under negative ionization conditions. Key peaks are numbered and correspond to the following identified compounds: 1: D-(+)-Malic acid; 2: Citric acid; 3: 3-methylglutaric acid; 4: Isopsoralenoside; 5: Salvianolic acid b; 6: Neobavaisoflavone; 7: Isobavachalcone. **(C)** Total ion chromatogram of BFF in positive ion mode. The chromatogram displays the overall chemical profile under positive ionization conditions. Key peaks are numbered and correspond to the following identified compounds: 1: Choline; 2: L-proline; 3: Phloroglucinol; 4: DL-Pipecolic acid; 5: Isopsoralen; 6: Calycosin-7-O-beta-D-glucoside; 7: Oxotuberosmonine; 8: Tuberosmonine; 9: Karakoline; 10: Ononin; 11: Daidzein; 12: Sesamin; 13: Biochanin A; 14: Psoralen; 15: Licoflavanone; 16: Tuberosin; 17: Sauchinone; 18: Magnolignan A; 19: 14-Methylpentadecanoic acid; 20: 5'-Prenyllicodione; 21: Erysubin f; 22: Cryptotanshinone; 23: Tanshinone. The identification was based on accurate mass measurement and comparison with standard databases.

acid b, Isopsoralenoside, and Isobavachalcone. The positive mode revealed a rich profile of isoflavones and flavonoids (Calycosin-7-O-beta-D-glucoside, Daidzein, Biochanin A), alkaloids (Oxotuberosmonine, Tuberosmonine, Karakoline), and terpenoids, most notably the highly active components Cryptotanshinone and Tanshinone. These findings underscore the multi-component nature of BFF, providing a robust chemical basis for its observed therapeutic efficacy against COPD.

BFF Mitigates Alveolar Injury and Inflammatory Response in COPD-Like Rats

To evaluate the therapeutic effects of BFF on COPD, lung histopathology and systemic inflammation were assessed. H&E and Masson's staining demonstrated severe alveolar damage, emphysematous changes, marked inflammatory infiltration, and extensive collagen deposition in COPD-like rats compared with controls. BFF administration markedly mitigated these pathological changes in a dose-dependent fashion. The most pronounced therapeutic effect was observed in the high-dose cohort, which exhibited well-preserved alveolar structure, diminished inflammatory infiltration, and lower levels of collagen deposition (Figure 3A and B). Quantitative analysis confirmed lower lung injury scores and collagen content, indicating that BFF effectively protects against COPD-induced structural damage and fibrosis (Figure 3C and D). In parallel, plasma IL-1 β and IL-18 concentrations were markedly elevated in COPD-like rats but were significantly lowered after 8 weeks of BFF administration, with the high-dose group showing the strongest effect (Figure 3E and F). Collectively, these results support that BFF ameliorates COPD pathology by modulating inflammatory responses both systemically and within lung tissues.

BFF Regulates Mitochondrial Respiratory Chain Function and Oxidative Balance in COPD-Like Rats

The dysfunction of mitochondria is closely associated with the regulation of NLRP3 inflammasome activity. Moreover, some researchers have confirmed that fine-tuning the mitochondrial oxidative respiratory chain contributes to the suppression of NLRP3 inflammasome activation, highlighting mitochondrial homeostasis as a critical regulatory mechanism.²⁹ To assess the impact of BFF on oxidative stress and mitochondrial function, oxidative and bioenergetic parameters were measured. Compared with the control group, COPD-like rats showed markedly decreased SOD activity and increased MDA and ROS levels (Figure 3G–I), indicating severe oxidative stress. BFF treatment dose-dependently restored oxidative balance, with the high-dose group significantly enhancing SOD activity and reducing MDA and ROS levels. Mitochondrial functional assessment revealed that COPD-like rats exhibited hyperactivation of respiratory chain complexes I–V and elevated ATP levels (Figure 3J–O), reflecting abnormal energy metabolism and excessive ROS production. BFF administration effectively normalized these changes, significantly suppressing the overactivation of complexes I–V and reducing ATP overproduction, particularly in the BFF-H group. Collectively, these findings indicate that BFF alleviates oxidative stress and rebalances mitochondrial energy metabolism in COPD-like rats by inhibiting hyperactive respiratory chain function and excessive ROS generation.

BFF Ameliorates Ultrastructural Damage in Alveolar Cells

TEM analysis revealed that COPD-like rats exhibited severe ultrastructural damage in alveolar cells compared with controls, including disrupted cell membrane integrity, a marked decrease in lamellar bodies (LBs) and mitochondria, and evident mitochondrial degeneration such as swelling and fragmentation (red arrows in Model inset) (Figure 4). Following BFF intervention, these pathological alterations were markedly alleviated in a dose-dependent manner. BFF treatment restored membrane continuity, increased the number of LBs and mitochondria, and improved their structural integrity. Notably, the high-dose group showed the most pronounced recovery, characterized by well-organized LBs (green arrows in BFF-H inset) and intact mitochondrial morphology. These findings demonstrate that BFF effectively preserves alveolar cell ultrastructure and maintains mitochondrial integrity in COPD-like rats.

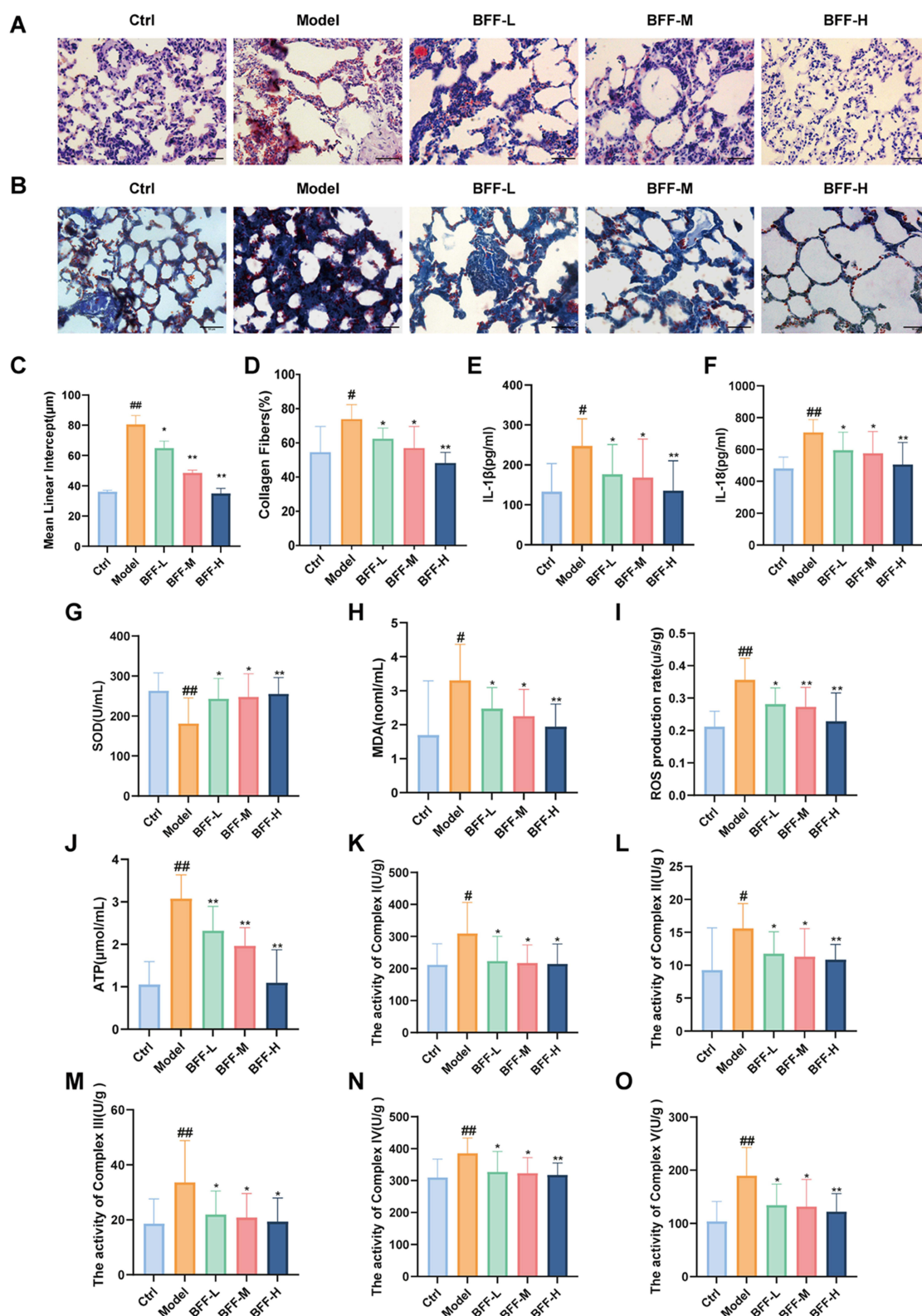


Figure 3 BFF ameliorates pulmonary histopathological damage, systemic inflammation, and mitochondrial dysfunction in COPD-like rats. **(A)** Representative images of lung tissue stained with Hematoxylin and Eosin (H&E). Scale bar: 50 μ m. **(B)** Representative images of lung tissue stained with Masson's trichrome. Blue staining indicates collagen deposition/fibrosis. Scale bar: 50 μ m. **(C)** Quantitative analysis of Mean Linear Intercept (MLI, μ m), a measure of alveolar enlargement and emphysema. **(D)** Quantitative analysis of pulmonary fibrosis represents the degree of pulmonary fibrosis. **(E)** Plasma level of IL-1 β . **(F)** Plasma level of IL-18. **(G)** Plasma SOD activity. **(H)** Plasma MDA level. **(I)** Plasma ROS production rate. **(J)** Lung tissue ATP level. **(K–O)** Activities of mitochondrial respiratory chain complexes I, II, III, IV, and V, respectively, in lung tissue. All data are presented as mean $\pm$ SD ($n = 10$). Statistical significance: [#] $P < 0.05$, ^{##} $P < 0.01$ vs. Ctrl group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. Model group.

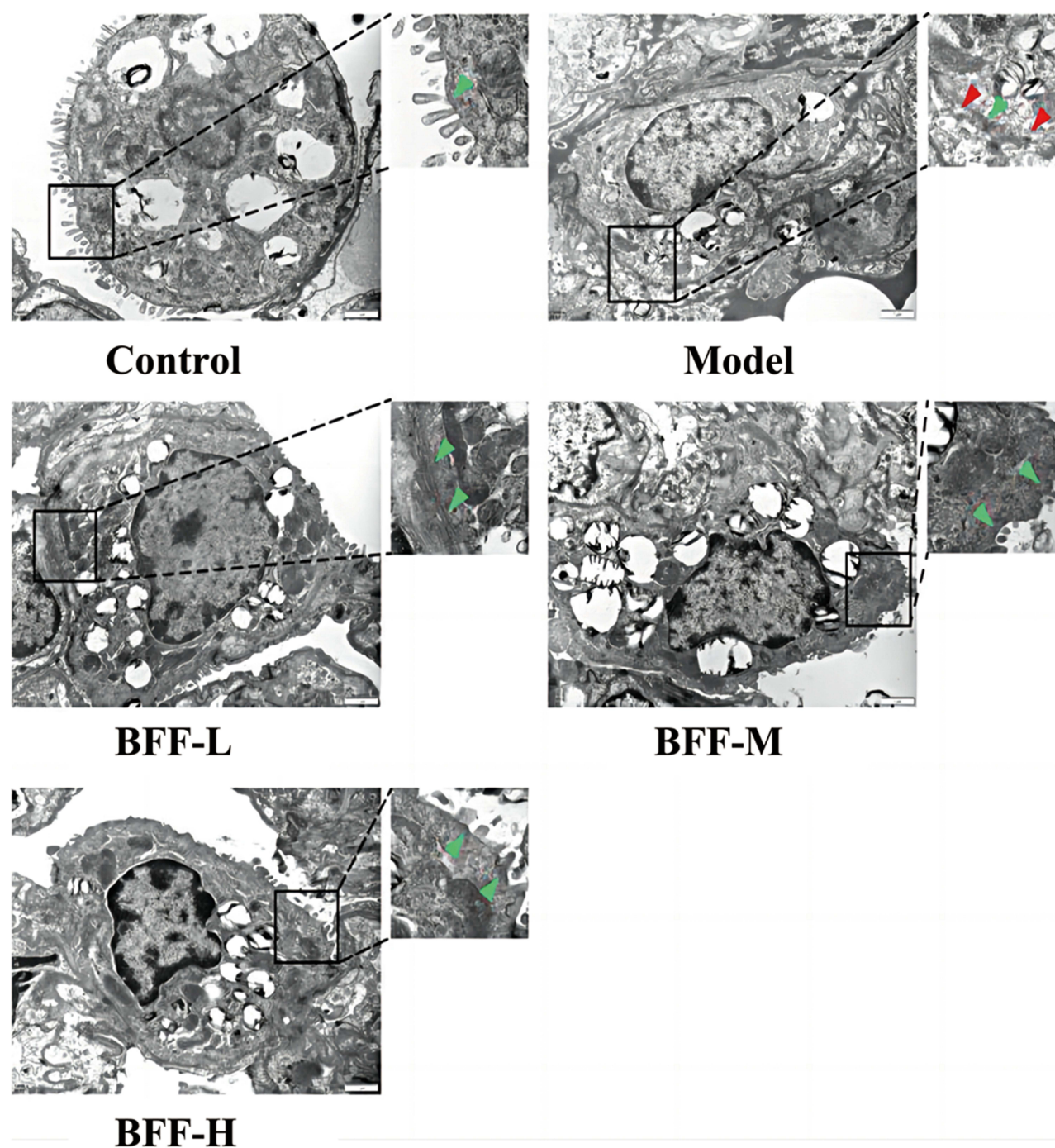


Figure 4 Representative TEM images of type II alveolar epithelial cells in the lung tissues of rats from different groups. The ultrastructural changes were observed in the Control, Model, BFF-L, BFF-M, and BFF-H groups. Green arrows indicate lamellar bodies. Red arrows point to damaged mitochondria, characterized by swelling and fragmentation. Scale bar = 2 μ m.

BFF Suppresses NLRP3 Inflammasome in COPD-Like Rats

Given the elevated IL-1 β and IL-18 levels and enhanced oxidative stress, the status of the NLRP3 inflammasome was examined via immunohistochemical staining and Western blot. Both analyses demonstrated that the expression of NLRP3, ASC, and the active fragment Caspase-1 p20 was markedly increased in the COPD pulmonary tissues relative to the control group, confirming inflammasome activation in COPD lungs. BFF treatment significantly and dose-dependently suppressed this activation. Immunohistochemistry revealed a marked reduction in the positive staining of

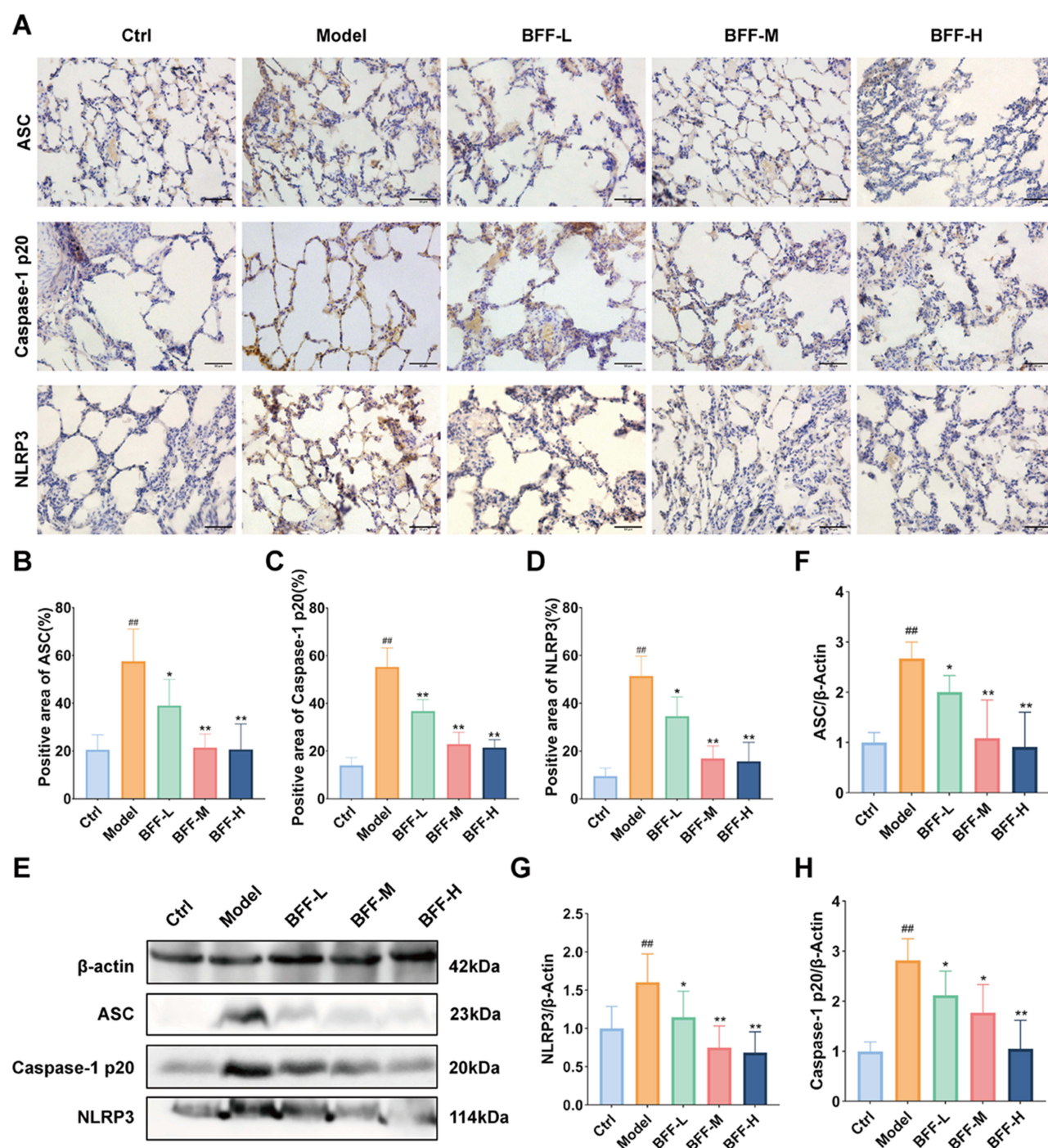


Figure 5 BFF inhibits the activation of the NLRP3 inflammasome pathway in COPD-like rats lung tissue. **(A)** IHC images show the protein expression and localization of ASC, Caspase-1 p20, and NLRP3 in the lung tissue of different groups (400× magnification). **(B–D)** Semi-quantitative analysis of IHC results. Bar graphs represent the positive staining area (%) for ASC **(B)**, Caspase-1 p20 **(C)**, and NLRP3 **(D)**. **(E)** Western blot analysis of NLRP3 inflammasome components. Representative immunoblots show the protein levels of ASC, Caspase-1 p20, and NLRP3 in lung tissue lysates. β-Actin served as the loading control. **(F–H)** Quantitative analysis of Western blot results. Bar graphs represent the relative protein expression levels of ASC **(F)**, NLRP3 **(G)**, and Caspase-1 p20 **(H)** normalized to β-Actin. All data are presented as mean ± SD (n = 5). Statistical significance: ^{##}P<0.01 vs. Ctrl group; ^{*}P<0.05, ^{**}P<0.01 vs. Model group.

NLRP3, ASC, and Caspase-1 p20 (Figure 5A–D), while Western blot analysis confirmed corresponding decreases in protein expression levels (Figure 5E–H). The most substantial inhibitory effect was observed in the high-dose group. Taken together, these results elucidate that BFF alleviates downstream inflammatory cascades in COPD-like rats through effective blockade of the NLRP3 inflammasome pathway.

Determination of Optimal CSE Stimulation and BFF-Containing Serum Concentrations in NR8383 Macrophages

To establish a reliable in vitro model of COPD-related inflammation and determine the effective therapeutic concentration of BFF medicated serum, we first optimized the CSE stimulation conditions on macrophages. We observed that CSE induced a time- and concentration-dependent increase in IL-1 β and IL-18 (Figure 6A and B), with the rate of increase slowing significantly after 24 hours. Concurrently, cell viability assays (Figure 6C and D) revealed that CSE concentrations up to 5% did not significantly impair macrophage viability, whereas higher concentrations (8% and 10%) were cytotoxic. Based on the robust inflammatory response and preserved cell viability, the optimal condition for the subsequent in vitro experiments was determined to be 5% CSE stimulation for 24 hours. Next, we assessed the cytotoxicity of BFF medicated serum and its anti-inflammatory effect on the established model. Concentrations of

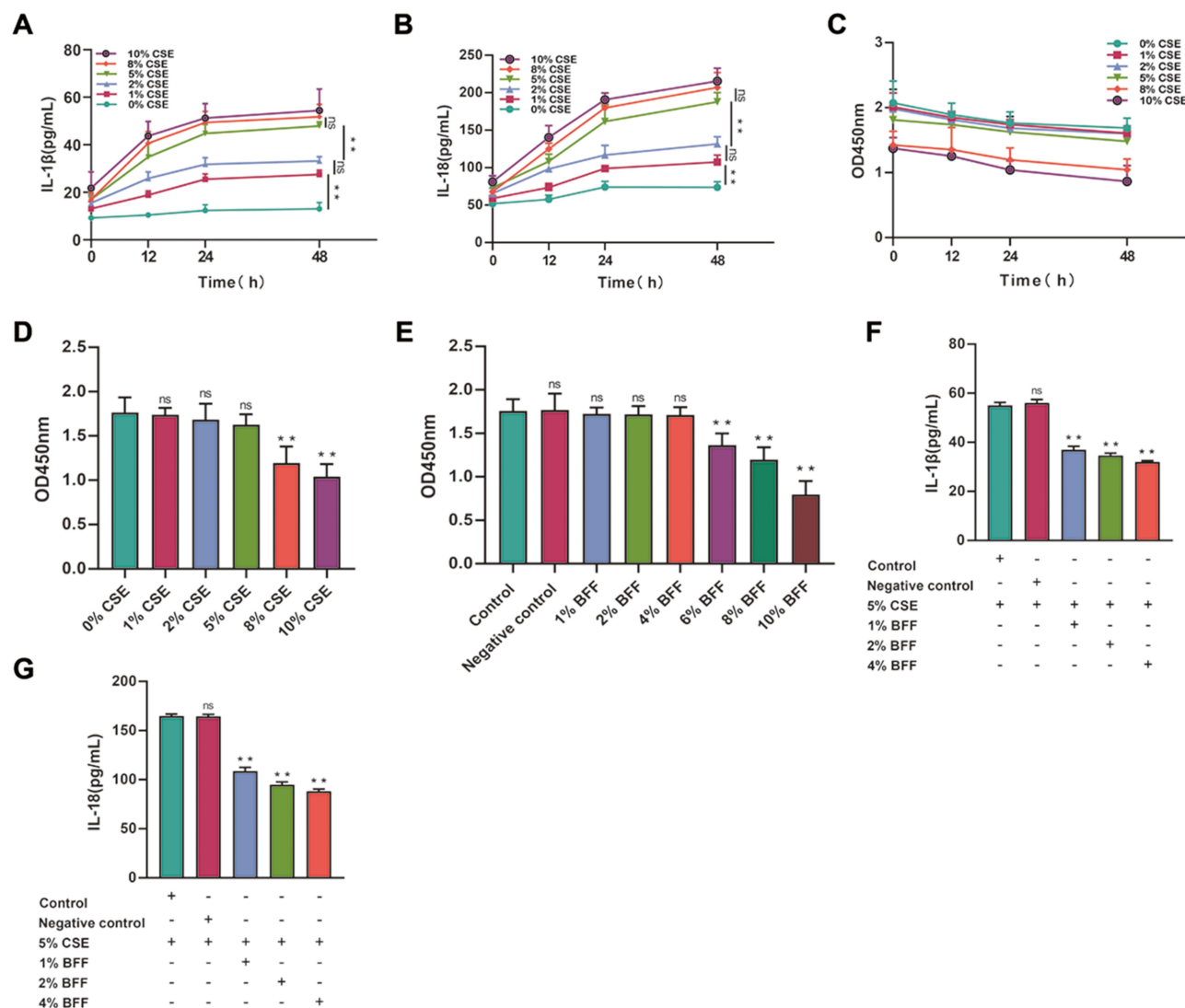


Figure 6 Determination of optimal CSE stimulation conditions and BFF medicated serum concentration for in vitro macrophage experiments. (A and B) Time- and concentration-dependent effects of CSE on IL-1 β (A) and IL-18 (B) concentration in macrophage culture supernatant, measured by ELISA. (C) Time- and concentration-dependent effects of CSE on macrophage viability, measured by CCK-8 assay. (D) Macrophage viability after 24 hours of stimulation with different CSE concentrations, measured by CCK-8 assay. (E) Macrophage viability after treatment with different concentrations of BFF medicated serum (1% to 10%), measured by CCK-8 assay. "Control" refers to serum-free medium; "Negative control" refers to 10% blank serum. (F and G) IL-1 β (F) and IL-18 (G) concentrations in macrophage culture supernatant after stimulation with 5% CSE for 24 hours and treatment with selected concentrations of BFF medicated serum (1%, 2%, 4%), measured by ELISA. In the tables below (F and G), the "+" symbol indicates the addition or presence of the specified treatment, while the "-" symbol indicates the absence of the specified treatment. All data are presented as mean $\pm$ SD (n = 5). Statistical significance: ns (not significant); *P<0.05; **P<0.01.

BFF medicated serum $\geq 6\%$ were found to be cytotoxic (Figure 6E). Conversely, treatment with 1%, 2%, or 4% BFF-containing serum was non-cytotoxic and effectively attenuated the 5% CSE-induced elevation of IL-1 β and IL-18 (Figure 6F and G). Consequently, these concentrations were designated as the low-dose (BFF-L, 1%), medium-dose (BFF-M, 2%), and high-dose (BFF-H, 4%) groups for all subsequent cell-based experiments.

BFF Regulates Mitochondrial Respiratory Chain Function and ROS Levels in COPD Macrophages

To confirm the mechanism observed in vivo and to eliminate systemic confounding factors, we examined the impact of BFF on mitochondrial function in a COPD cell model using CSE-stimulated NR8383 macrophages (Figure 7A–G). The

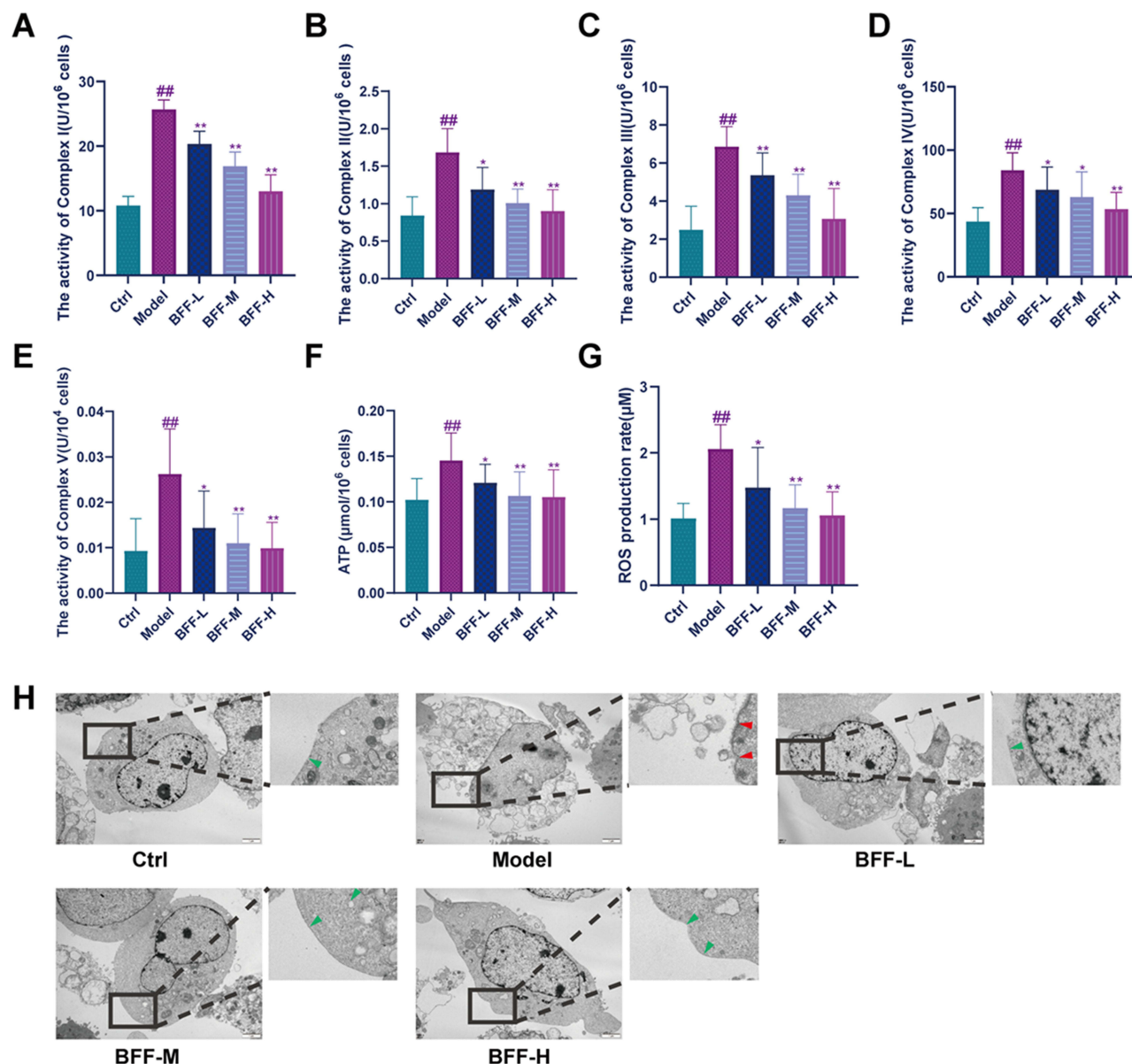


Figure 7 BFF regulates mitochondrial function and inhibits pyroptosis in CSE-stimulated COPD macrophages. (A–G) BFF regulates mitochondrial respiratory chain function and ROS levels. Activities of mitochondrial respiratory chain complexes I (A), II (B), III (C), IV (D), and V (E). Intracellular ATP level (F). Intracellular ROS production rate (G). All data are presented as mean $\pm$ SD ($n = 10$). Statistical significance: ^{##} $P < 0.01$ vs. Ctrl group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. Model group. (H) TEM images of macrophages. The images illustrate the ultrastructural changes of macrophages (Ctrl, Model, BFF-L, BFF-M, and BFF-H groups). The insets provide magnified views of the cell membrane. The Model group displays typical pyroptotic features, including significant cell swelling and severe cell membrane rupture (Red arrows). BFF treatment restores cell membrane integrity (Green arrows). Scale bar = 2 μ m.

Model group exhibited pronounced mitochondrial hyperactivity, with significantly increased activities of all respiratory chain complexes (I–V), elevated ATP levels, and enhanced ROS production compared to the Control group, indicating an aberrant energy metabolism state. BFF treatment markedly and dose-dependently suppressed this hyperactivation. High-dose BFF most effectively improved mitochondrial function by enhancing complexes I–V activities and ATP production, concomitantly suppressing ROS generation. These *in vitro* results strongly reinforce the *in vivo* data, demonstrating that BFF directly acts on macrophages to suppress the hyperactivation of the mitochondrial respiratory chain, thereby effectively reducing the production of excessive ATP and ROS.

BFF Ameliorates Ultrastructural Damage Indicative of Pyroptosis in COPD Macrophages

To visually validate the anti-pyrototic effect of BFF indicated by molecular findings, ultrastructural morphology was examined by TEM (Figure 7H). Control macrophages exhibited intact membranes, clear nuclei, and uniform cytoplasm, whereas the COPD Model group showed classic pyroptotic features—marked cell swelling, disrupted membranes with multiple pores and fissures, cytoplasmic vesiculation, and extensive leakage of intracellular contents. BFF treatment significantly alleviated these structural abnormalities in a dose-dependent manner. The BFF-L and BFF-M groups displayed reduced swelling and fewer membrane disruptions, while the high-dose group preserved nearly normal morphology, with intact membranes and minimal cytoplasmic leakage. These observations provide direct ultrastructural evidence that BFF effectively protects macrophages from NLRP3 inflammasome-mediated pyroptotic damage.

BFF Inhibits NLRP3 Inflammasome Activation in Macrophages

To validate the inhibitory impact of BFF on NLRP3 inflammasome activation observed *in vivo*, immunofluorescence and Western blot analyses were performed in CSE-stimulated macrophages. The Model group exhibited marked upregulation of NLRP3, ASC, and Caspase-1 p20, confirming inflammasome activation (Figure 8A–C). BFF treatment significantly and dose-dependently reduced the fluorescence intensity and integrated optical density of these proteins, indicating suppression of inflammasome assembly. Consistently, Western blot results showed that BFF, particularly at high doses, markedly downregulated NLRP3, ASC, and Caspase-1 p20 levels compared with the Model group (Figure 8D). This collective suppression of core inflammasome components confirms that BFF impedes NLRP inflammasome assembly in macrophages, mechanistically accounting for the curtailed IL-1 β /IL-18 release and diminished pyroptosis observed in COPD.

Discussion

Chronic inflammatory processes and structural damage to lung tissue drive the development of COPD, which manifests clinically as persistent respiratory symptoms and irreversible airflow obstruction.³⁰ Current pharmacological treatments often focus on bronchodilation and anti-inflammation but fail to halt disease progression.^{31,32} In this context, BFF holds promise as an adjunct therapy to standard bronchodilators or corticosteroids, rather than a standalone replacement. TCM, particularly complex herbal formulas like BFF, offers a multi-component, multi-target approach to managing chronic diseases. Our study aimed to elucidate the underlying mechanism of BFF's therapeutic effect on COPD, specifically focusing on the interplay between mitochondrial function and the NLRP3 inflammasome.

Our comprehensive chemical analysis of BFF revealed 719 compounds, with Flavonoids (25.26%), Alkaloids (13.68%), Terpenoids (13.51%), and Indoles (13.40%) as the four dominant chemical classes. These components, including compounds like astragaloside, ginsenosides, and tanshinones, are known to harbor multiple bioactivities; these include mitigation of inflammation, scavenging of oxidative species, and regulation of immune responses,^{33–35} providing a robust chemical basis for BFF's observed therapeutic efficacy. The pathological findings confirmed that the COPD model exhibited severe lung damage, including alveolar destruction (emphysematous changes) and significant pulmonary fibrosis, accompanied by elevated systemic pro-inflammatory cytokines IL-1 β and IL-18. BFF treatment produced dose-dependent attenuation of these impairments, accompanied by molecular and cellular evidence that uniformly highlighted the NLRP3 inflammasome as a key intervention target.

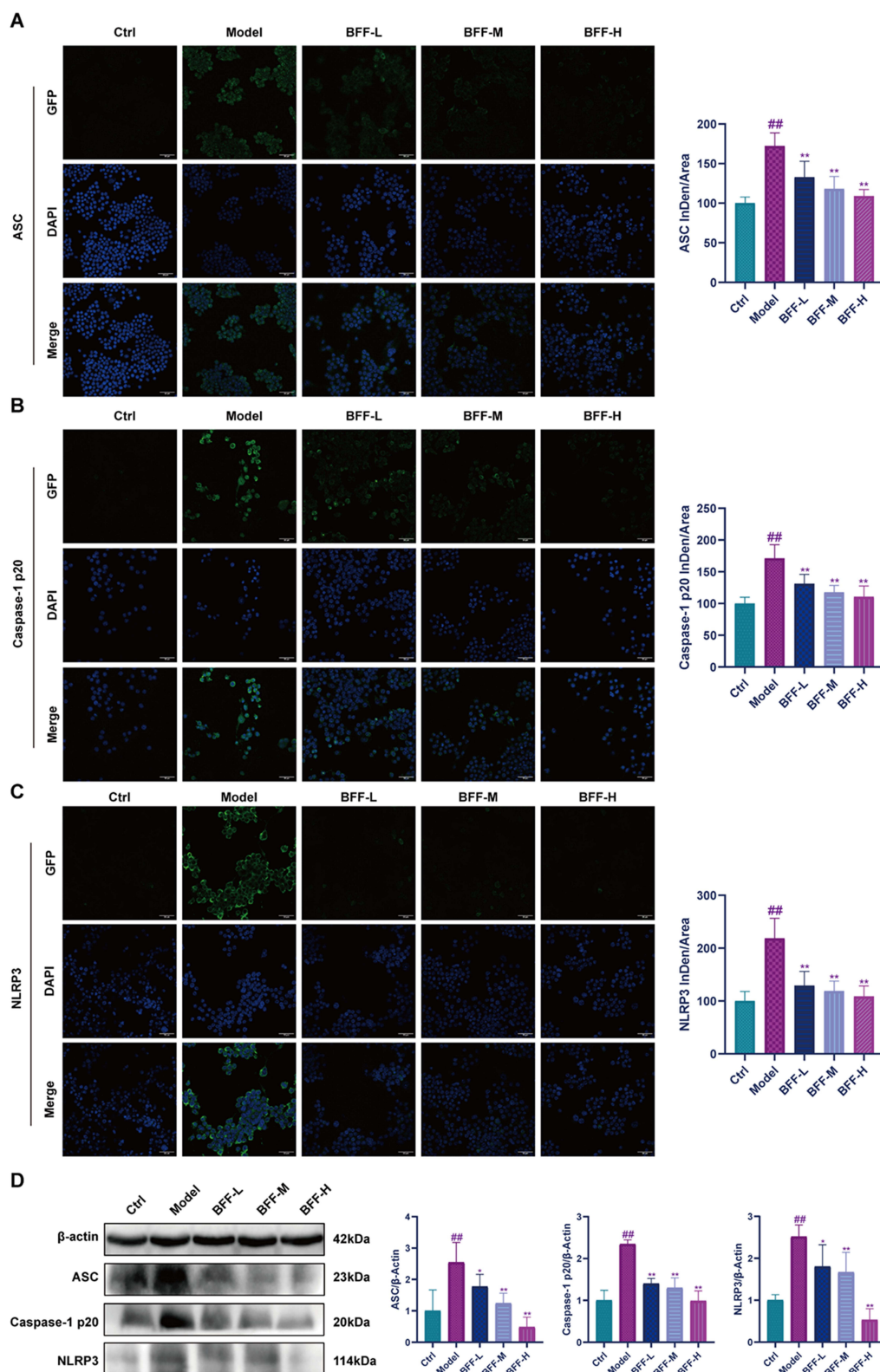


Figure 8 BFF inhibits the expression and activation of the NLRP3 inflammasome pathway in CSE-stimulated macrophages. **(A)** Immunofluorescence staining and quantitative analysis of ASC. The images show the expression (green) of ASC. DAPI (blue) stains the nuclei. The bar graph shows the integrated optical density (IOD) of ASC/Area. **(B)** Immunofluorescence staining and quantitative analysis of Caspase-1 p20. The images show the expression (green) of the active fragment Caspase-1 p20. The bar graph shows the IOD of Caspase-1 p20/Area. **(C)** Immunofluorescence staining and quantitative analysis of NLRP3. The images show the expression (green) of NLRP3. The bar graph shows the IOD of NLRP3/Area. Scale bar = 50 μ m. **(D)** Western blot analysis of NLRP3 inflammasome components. Representative immunoblots and quantitative bar graphs show the protein expression levels of ASC, Caspase-1 p20, and NLRP3 normalized to β -Actin. All data are presented as mean $\pm$ SD ($n = 5$ or $n = 6$). Statistical significance: ^{##} $P < 0.01$ vs. Ctrl group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. Model group.

The NLRP3 inflammasome serves as a crucial multi-protein platform that regulates inflammatory responses and programmed cell pyroptosis. Upon activation, its core biochemical function involves catalyzing the maturation of pro-IL-1 β and pro-IL-18, ultimately yielding their biologically active forms. The subsequent secretion of these cytokines potentially amplifies the inflammatory cascade, contributing to a cytokine storm.^{36,37} The data from this study clearly indicate a substantial increase in central NLRP3 inflammasome constituents (NLRP3, ASC, and active Caspase-1 p20) in the COPD model group, evident in both pulmonary tissue samples and in macrophages exposed to CSE stimulation. Crucially, BFF treatment effectively suppressed the expression and activation of these components, confirming that BFF acts as a potent inhibitor of NLRP3 inflammasome activation in COPD. These results thereby corroborate the recognized function of NLRP3 in promoting COPD progression.^{38–40}

As essential organelles, mitochondria generate the majority of cellular ATP through the activity of the ETC complexes I to V.^{41,42} While ETC activity naturally produces basal levels of ROS, ETC dysfunction can lead to the accumulation of excessive ROS, a potent DAMP that activates NLRP3.^{43,44} In this context, it is important to distinguish mitochondrial hyperactivity from classical mitochondrial dysfunction. While classical dysfunction typically involves structural impairment, energy failure, and reduced ATP production, mitochondrial hyperactivity represents a state of enhanced respiratory activity that paradoxically drives oxidative stress.⁴⁵ Recent studies have highlighted that in pro-inflammatory macrophages, metabolic reprogramming can induce reverse electron transport (RET) at complex I, leading to massive superoxide production that acts as a critical signal for NLRP3 activation and IL-1 β release.⁴⁶ Consistent with this paradigm of metabolic overactivation, we intriguingly observed that in both the COPD-like rat model and the CSE-stimulated macrophage model, the activities of all five ETC complexes (I–V) were significantly hyperactive compared to the control group. Rather than experiencing energy failure, these cells exhibited a simultaneous increase in ROS, MDA, and abnormally high ATP levels. This suggests that the observed mitochondrial hyperactivity, while potentially compensatory in origin, ultimately transitions into a pathologically damaging metabolic state. Such metabolic reprogramming and excessive ETC activity directly promote the inflammatory cascade, as both mitochondrial ROS overproduction and elevated ATP are well-established triggers for NLRP3 inflammasome assembly.^{47,48} In summary, our study establishes the mitochondrial hyperactive ETC/ROS/NLRP3 axis as a critical pathway in COPD pathogenesis and confirms that BFF exerts its therapeutic effect by intervening at this upstream level.

Despite these promising findings, our study has several limitations that should be acknowledged. First, a major limitation is the lack of functional respiratory measurements (such as lung function tests) to confirm a clinical COPD diagnosis, which is why we refer to our *in vivo* system as a COPD-like model. Second, while we observed morphological evidence of pyroptosis and significant changes in core inflammasome components (NLRP3, ASC, Caspase-1), functional pyroptosis markers such as GSDMD cleavage or LDH release were not assessed. Future studies incorporating comprehensive lung function evaluations and specific pyroptosis execution markers are warranted to further validate these preclinical findings.

Conclusion

The NLRP3 inflammasome is a pivotal event in the pathogenesis of pulmonary diseases. Our findings suggest that BFF shows potential in alleviating inflammation in a COPD-like model. This therapeutic effect is associated with the modulation of the mitochondrial energy metabolism/ROS/NLRP3 axis. Specifically, BFF treatment appears to attenuate the aberrant hyperactive state of mitochondrial ETC complexes and reduce the subsequent accumulation of ROS and ATP. These metabolic changes correlate with a suppression of NLRP3 inflammasome activation and a decrease in downstream inflammatory cytokines. This mechanism suggests that targeting the mitochondrial energy metabolism/ROS/NLRP3 axis represents a potential therapeutic approach. Future clinical trials and studies incorporating functional respiratory assessments are warranted to validate these preclinical findings.

Abbreviations

COPD, Chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; BFF, Bufe formula; BFF-L, BFF at low dose group; BFF-M, BFF at middle dose group; BFF-H, BFF at high dose group; NLRP3, NOD-like receptor family pyrin domain-containing protein 3 inflammasome; ASC, Apoptosis-associated Speck-like protein

containing a CARD; Caspase-1 p20, Interleukin-1 beta-converting enzyme p20 subunit; ROS, Reactive Oxygen Species; ATP, Adenosine Triphosphate; mtROS, Mitochondrial reactive oxygen species; ETC, Electron transport chain; DAMPs, Damage-associated molecular patterns; PAMPs, Pathogen-Associated Molecular Patterns; TLRs, Toll-like receptors; GSDMD, Gasdermin D; LPS, Lipopolysaccharide; IL-1 β , Interleukin-1 beta; IL-18, Interleukin-18; CSE, Cigarette Smoke Extract; HE, Hematoxylin and eosin; PBS, Phosphate-Buffered Saline; DNA, Deoxyribonucleic Acid; SOD, Superoxide Dismutase; MDA, Malondialdehyde; RT, Room Temperature; PFA, Paraformaldehyde.

Data Sharing Statement

The data that support the findings of this study are not publicly available due to privacy reasons but are available from the corresponding author upon request.

Ethics Statement

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) and received approval from the Animal Ethics Committee of Guizhou University of Traditional Chinese Medicine (Approval No. 2025101004). Animals were provided ad libitum access to food and water throughout the experimental period.

Author Contributions

Tong Yang: Conceptualization, Project administration, Methodology, Investigation, Formal analysis, Writing – original draft. Yang Liu: Conceptualization, Investigation, Formal analysis, Validation, Writing – original draft. Xingli Sun: Investigation, Formal analysis, Visualization, Writing – review and editing. Genyan Liu: Investigation, Formal analysis, Visualization, Writing – review and editing. Lang Liu: Investigation, Resources, Data curation, Writing – review & editing. Jiayin Ning: Investigation, Validation, Data curation, Writing – review and editing. Jiangqin Ou: Conceptualization, Project administration, Supervision, Methodology, Writing – review and editing, final approval of the version to be published. All authors 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; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Tong Yang and Yang Liu are co-first authors for this study. The authors declare no competing interests in this work.

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