

In vivo and in vitro Models of PM2.5 Induced COPD: Focus on the Role of RTA-408

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Introduction: Inflammation and oxidative stress are important factors in the pathogenesis of Chronic obstructive pulmonary disease (COPD). Current treatments for COPD focus on improving symptoms caused by inflammation rather than curing the disease, therefore, emerging research focusing on upstream pathways may help develop effective treatments. Epidemiological investigations have shown that exposure to fine particulate matter (PM2.5) can cause lung inflammation and oxidative stress through nuclear factor NF-E2-associated factor (Nrf2) pathway, leading to COPD. Nrf2 is an important transcription factor regulating anti-inflammatory and antioxidant stress, and its abnormal expression level or changes in transcriptional activity are related to the occurrence and development of COPD. Omaviloxone - RTA-408, a synthetic oleanane triterpene that acts as an Nrf2 activator, RTA-408 may play an important role in COPD.

Purpose: In this study, PM2.5 was used to establish HBE cell model in vitro and rat model in vivo to simulate COPD, and the effect of Nrf2 activator RTA-408 on PM2.5-induced COPD model and its mechanism were investigated.

Patients and Methods: The HBE cell model in vitro and rat model in vivo were established to simulate COPD, and the effect of RTA-408 on COPD was detected by various experimental methods.

Results: The results showed that RTA-408 could activate Nrf2 both in vivo and in vitro. By activating Nrf2/HO-1 pathway, RTA-408 inhibits NF- κ B and IFN- γ pathways, alleviates inflammation and oxidative stress of HBE cells in COPD model rats and PM2.5 exposed cells, and plays a therapeutic role in reversing cell damage and delaying disease progression in COPD. In addition, in vitro experiments, silencing Nrf2 eliminated the protective effect of RTA-408 on COPD cell models, which also confirmed the role of RTA-408.

Conclusion: We conclude that RTA-408 is well worth considering as a new strategy for the treatment of COPD, and may also have a positive preventive effect.

Keywords: chronic obstructive pulmonary disease, inflammation, oxidative stress, Nrf2, RTA-408

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic progressive lung disease characterized by limited airflow. Fine particulate matter (PM2.5) is a pollutant in the air. Long-term exposure to high concentrations of PM2.5 can induce the transcription of various inflammatory mediators by activating NF-E2-related factor (Nrf2)/Heme Oxidase-1 (HO-1), Nuclear Factor kappa B (NF- κ B) and other signaling pathways, promote the release of pro-inflammatory cytokines, and cause a large number of inflammatory cells to flood into the airway, causing lung inflammation, oxidative stress and airway epithelial cell damage. Destroy lung tissue, and then develop COPD, or aggravate the condition of the original COPD. As an antioxidant nuclear transcription factor, Nrf2 regulates the expression of a variety of antioxidant, anti-inflammatory and pro-survival genes through the antioxidant response elements in the promoter, neutralizes free radicals, accelerates the removal of environmental toxins,¹ and plays an important role in improving airway inflammation and

oxidative damage induced by PM2.5 in COPD and repairing damaged tissues.^{2–4} In COPD, Nrf2 levels are significantly reduced.

Nrf2 has a basic leucine zipper structure and belongs to the Cap'n'collar (CNC) transcription factor family.⁵ Activated Nrf2 translocates to the nucleus and binds to antioxidant response elements in various detoxification and antioxidant gene promoters are essential for maintaining intracellular oxidative stress balance. Research has shown that Nrf2 also plays a role in immune cell function and a variety of inflammatory diseases, including autoimmune diseases and allergies. Nrf2 activation has been shown to have anti-inflammatory effects, while Nrf2 deletion has been shown to have pro-inflammatory effects.^{6–8} The Nrf2 activator RTA-408 (Figure 1) can treat a variety of diseases by activating the Nrf2 signaling pathway, showing pharmacological effects such as anti-inflammatory, antioxidant, anti-tumor and improving mitochondrial function. RTA-408 increases Nrf2 levels by binding to Keap1 and blocking its ability to promote Nrf2 degradation. Therefore, the newly synthesized Nrf2 accumulates in the nucleus, increases the expression of antioxidant genes and decreases the expression of pro-inflammatory genes, thereby enhancing the ability of cells to resist oxidative stress, reduce inflammatory response, and promote the repair and regeneration process.^{9–11} More and more studies have shown that RTA-408 plays an antioxidant and anti-inflammatory role by activating Nrf2 signaling pathway and inhibiting NF- κ B signaling pathway. When cells are stimulated by oxidative stress, the NRF2-dependent cellular defense mechanism is activated, allowing Nrf2 to separate from Keap1 and be transported to the nucleus to activate downstream target genes. This evidence strongly supports the idea that RTA-408 regulates the balance of the oxidation-antioxidant system in vivo.^{12,13}

In PM2.5-induced COPD, Nrf2/HO-1, NF- κ B and other signaling pathways are activated, and the activation of Nrf2/HO-1 enhances the ability of cell defense and antioxidant damage, and the activation of NF- κ B aggravates the inflammatory response. Activated Nrf2 may reduce PM2.5-induced lung inflammation by regulating the NF- κ B pathway. Existing literature indicates that Nrf2/NF- κ B has a crosstalk mechanism aimed at cancelling each other's activities.¹⁴ In summary, RTA-408 may act through the Nrf2/NF- κ B pathway and serve as a potential treatment for improving lung function and reducing disease symptoms in patients with COPD. Therefore, in this study, we studied the changes of Nrf2/HO-1, NF- κ B, interferon-gamma (IFN- γ) signaling pathway, and endogenous active molecule Nitric Oxide (NO), to further explore the relationship between RTA-408 and COPD.

Materials and Methods

PM2.5 Collection and Treatment

The quartz fiber filter membrane of Whatman Company of the United States and the intelligent midstream atmospheric PM2.5 collector of Beijing ASEM Company were used for random sampling in Shijiazhuang Recycling Chemical Park (the parameter was set to flow rate of 100 L/min. The collection time was 22 h/d, and the interval was 2 h). Remove the screen from the sampler, cut it into small pieces as much as possible, soak it in distilled water, and place it in the ultrasonic vibrator. The eluent was filtered and centrifuged by gauze, the bottom sediment was collected, vacuum freeze-

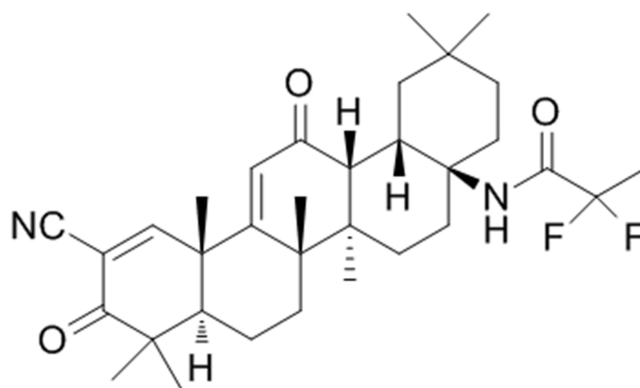


Figure 1 RTA-408 chemical formula.

dried, weighed and recorded, and kept at low temperature. Before use, saline suspensions of different concentrations were prepared and disinfected.

Medicines and Reagents

Antibodies used: anti-NRF2, anti-HO-1, anti-NF- κ B, anti-IFN- γ , anti-transforming growth factor β 1 (TGF β 1), anti-matrix metalloproteinase9 (MMP9), anti-fibronectin (FN) purchased from Affinity Corporation (Jiangsu, China). NovoStart SYBR qPCR SuperMix Plus, NovoScript Plus All-In-One-1st Strand cDNA Synthesis SuperMix (gDNA Purge) was purchased from Nearcoast Protein Company (Shanghai, China). TriQuick reagents are purchased from Solarbio (Beijing, China). RTA-408 was purchased from GLP BIO Corporation (Montclair, USA). Rat Nrf2, rat HO-1, rat NF- κ B, rat IFN- γ enzyme-linked immunoadsorption assay (ELISA) kits were purchased from Bioswamp Life Sciences Laboratory (Wuhan, China). NO assay kit (microassay) was purchased from Abbkine (Wuhan, China). Human specific Nrf2 siRNA and scrambled control double-stranded were provided by GenePharma (Suzhou, China). Enhanced chemiluminescence (ECL) reagents from Abbkine (Wuhan, China). Lipo3.0 highly effective transfection reagents were purchased from Abbkine (Wuhan, China) and dimethyl sulfoxide (DMSO) from Invitrogen (Carlsbad, CA, USA).

Construction of COPD Model in Rats

Fifty-four SPF grade male SD rats aged 6 to 8 weeks, weighing 180 to 200 g, were purchased from Wuhan Yuncun Animal Co., LTD. Quarantine for 3–5 days, after passing the test, weight was weighed, and randomly divided into three groups: normal saline control group, low-dose PM2.5 experimental group (5 mg/kg) and high-dose PM2.5 experimental group (50 mg/kg), 18 animals in each group. The PM2.5 experimental group was injected with PM2.5 suspension by inhalation tracheal drip to make rats infected, and the control group was injected with normal saline in the same way, with the same volume of each rat, once every four days for 3 months. The experiments were conducted in accordance with the Regulation of Animal Research Ethics in China (GB/T35892-2018). The experimental protocol was approved by the Experimental Animal Care and Ethics Committees at Hebei Medical University Fourth Affiliated Hospital (Approval NO. IACUC-4th hos Hebmu-20231101).

Growth Index and Pulmonary Respiratory Function Index Were Measured in Rats

Within 24 hours of the last exposure at 1 week, 1 month and 3 months, 6 rats were taken from each group and weighed to calculate the weight change during the exposure period. At the same time, the lung respiratory function, including inspiratory time (Ti), expiratory time (Te), peak expiratory time (PEF), lung compliance (C_{dyn}) and airway resistance (RI), was measured by the RC airway resistance and compliance analysis system of Tawangtech. The rats were anesthetized immediately after the lung respiratory function was measured.

Percentage of Inflammatory Cells in Rat Alveolar Lavage Fluid

After blood extraction, the thoracic cavity of the rat was exposed, the left lung lobe of the rat was clamped with hemostatic forceps, the bronchus was removed, a small incision was cut, the pulmonary irrigation needle was inserted and then ligated with surgical line, 3 mL of pre-cooled sterile saline was slowly injected into the lung, massaged into the lung and aspirated, the alveolar irrigation fluid was collected after two repetitions, centrifuged, and the bottom precipitation was suspended with sterile saline. After the number of cells was adjusted, the plates were coated and dried at room temperature. 100 cells were counted and the percentage of neutrophils was calculated by Reichsmard-Giemsa staining.

Lung Histopathology in Rats

The right lung lobe of the rat was removed and fixed with 10 times the volume and 10% formalin for 48 h. A slice of about 4 mm thick was cut from the middle part of the lung lobe tissue and placed in an embedding box, which was dehydrated and transparent and immersed in wax. The lung tissue was cut into 0.5 mm slices and stained by HE. Lung tissue injury and inflammatory cell infiltration were observed under microscope.

Intervention Experiment of RTA-408 on COPD Model in Rats

COPD rat models were established after exposure to high dose PM_{2.5} (50mg/kg) for 3 months, and 30 SPF grade SD male rats underwent the same modeling process as above. They were divided into normal control group, COPD group, COPD+ low-dose RTA-408 group (0.5mg/kg), COPD+ medium-dose RTA-408 group (1.5mg/kg), COPD+RTA-408 high-dose group (3mg/kg) (6 rats in each group). After 1 week of administration of RTA-408, 10% chloral hydrate (4mL/kg) was injected intraperitoneal anesthesia, and the right lung and serum were taken respectively. Appropriate amount of lung tissue was taken for RT-qPCR in RNA later, appropriate amount of fresh tissue was taken for WB detection, and the remaining lung tissue was stored in the refrigerator at -80°C.

Lung Inflammation and Oxidative Stress Cytokines Were Detected by RT-qPCR

The expression of Nrf2, HO-1, NF-κB and IFN-γ in lung tissue was detected by RT-qPCR. Appropriate amount of lung tissue was ground and 100 mg of total RNA was purified from rat lung tissue by RNA extraction kit. Reverse transcription and RT-qPCR were performed. All samples were repeated three times. Quantitative PCR analysis was performed using SYBR qPCR kit. All amplification results were normalized to GAPDH, and then the mRNA expression levels of Nrf2, HO-1, NF-κB and IFN-γ were determined using $2^{-\Delta\Delta Ct}$ as the target gene quantity. The following primers were used for PCR in this experiment:

① Nrf2 primer sequence

Forward primer sequence 5'-TTCCTCTGCTGCCATTAGTCAGTC-3'

Reverse primer sequence 5'-GCTCTTCCATTCCGAGTTCACTG-3'

② HO-1 primer sequence

Forward primer sequence 5'-CTTCTTCACCTTCCCCAACA-3'

Reverse primer sequence 5'-CAGGCATCTCCTTCCATT-3'

③ NF-κB p65 primer sequence

Forward primer sequence 5'-GGTGACATCTGCTTCTCCCT-3'

Reverse primer sequence 5'-TGGATTTGCTCGAACGGAAC-3'

④ IFN-γ primer sequence

Forward primer sequence 5'-ACAACCCACAGATCCAGCACAAAG-3'

Reverse primer sequence 5'-CACCGACTCCTTTTCCGCTTCC-3'

⑤ GAPDH primer sequence

Forward primer sequence 5'-GAAGGTGAAGGTCGGAGTC-3'

Reverse primer sequence 5'-GAAGATGGTGATGGGATTTC-3'

The Expression Levels of Nrf2, HO-1, NF-κB and IFN-γ in Rat Lung Were Detected by Western Blot

The test tube is shaken with a sterile steel ball and the RIPA buffer (Thermo, Rockford, IL) is used to extract protein from broken lung tissue according to the manufacturer's protocol. The protein concentration was determined by BCA method. SDS polyacrylamide gel electrophoresis was performed at 30 mA constant flow. Take 100V PVDF membrane, wet electrospinning for 1 ~ 2 h. The membrane was sealed in Tris-buffered Saline/0.1% Tween 20/5% nonfat milk powder (TBST/B) at room temperature for 1 h, gently shaken, and incubated with primary antibody solution at 4°C overnight. Then it was washed 3 times with TBST/B, immediately transferred to the secondary antibody solution and incubated at room temperature for 1 hour. The membrane was washed again for 3 times and developed with Luminata crescendo western HRP substrate. The images were obtained using the Molecular Imager ChemiDoc XRS System Gel Imaging Analyzer (Bio-Rad, PA). Image J analysis software was used to analyze gray values, and the levels of Nrf2, HO-1, NF-κB and IFN-γ were determined. The results were expressed by relative optical density.

The Levels of Nrf2, HO-1, NF- κ B and IFN- γ in Serum Were Detected by ELISA

The levels of Nrf2, HO-1, NF- κ B and IFN- γ in rat serum were determined according to the instructions provided by the ELISA kit manufacturer.

Determination of Serum NO Level in Rats

The NO content detection kit of Abbkine company (micromethod) was used to accurately determine the production of NO after the reduction of nitrate to nitrite by improved Gris method. The serum NO level of rats was determined.

Human Bronchial Epithelial (HBE) Cell Culture

HBE cells were purchased from Zhongqiao Xinzhou (Shanghai, China) in a special base medium (500mL base medium: 5mL keratinocyte growth factor; 5mL penicillin/streptomycin solution). The cell cultures were kept at 37°C in a humid atmosphere incubator containing 5% CO₂ and 95% air, the medium was changed every 2–3 days, and the passage was 1:2.

Preparation of PM2.5 Solution

5mg PM2.5 was dissolved in a complete medium and diluted into a PM2.5 mother liquor with a concentration of 1mg/mL, and a series of working liquids with final concentrations of 0, 50, 100, 150, 200, 250, 300, 350 and 400ug/mL were prepared (The solution is prepared on the spot, and the suspension is mixed with sufficient eddy current oscillation).

Cell Viability Analysis

Cell viability was measured using SuperKine TM hypersensitive cell proliferation assay (CKK-8) (Abbkin, Wuhan, China) according to the manufacturer's protocol. HBE (5×10^3 cells/ well) was inoculated in 96-well plate medium and cultured overnight in base medium. The cell density was observed to be about 85–90%, then the medium was changed, RTA-408 and PM2.5 were added at the same time, treated for 24 h, and 10 μ L CKK-8 solution was added to each well for 1 h. The absorbance at 450 nm was determined by Molecular Devices.

The Cytokine Expression in HBE Was Detected by Real-Time PCR

Total RNA was extracted by treating HBE with RNA extraction reagent. Real-time PCR on Stratagene Mx3005p multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA) Template RNA 2ul, 10 μ L 2x NovoStart SYBR qPCR SuperMix plus, forward and reverse primers 1 μ M each, the final reaction volume of 20 μ L. The sample was held at 95°C for 1 minute, then denatured at 95°C for 20 seconds, annealed at 60°C for 20 seconds, and extended at 72°C for 30 seconds, a total of 40 cycles. All amplification results were normalized to GAPDH, and then the mRNA expression levels of Nrf2, HO-1, NF- κ B, IFN- γ , TGF β 1, MMP9 and FN were determined using $2^{-\Delta\Delta C_t}$ as the target gene quantity. The real-time PCR primers are as follows:

①Nrf2 primer sequence

Forward primer sequence 5'-CTTGGCCTCAGTGATTCTGAAGTG-3'

Reverse primer sequence 5'-CCTGAGATGGTGACAAGGGTTGTA-3'

②HO-1 primer sequence

Forward primer sequence 5'-CTTCTTCACCTTCCCCAACA-3'

Reverse primer sequence 5'-ATTGCCTGGATGTGCTTTTC-3'

③NF- κ B p65 primer sequence

Forward primer sequence 5'-GGGATGGCTTCTATGAGGCTGAAC-3'

Reverse primer sequence 5'-CTTGCTCCAGGTCTCGCTTCTTC-3'

④IFN- γ primer sequence

Forward primer sequence 5'-AGAAATATTTTAATGCAGGTCA-3'

Reverse primer sequence 5'-CATTCAAGTCAGTTACCGAA-3'

⑤TGFβ1 primer sequence

Forward primer sequence 5'-GGACACCAACTATTGCTTCAG-3'

Reverse primer sequence 5'-TCCAGGCTCCAAATGTAGG-3'

⑥MMP9 primer sequence

Forward primer sequence 5'-TTTGAGTCCGGTGGACGATG-3'

Reverse primer sequence 5'-GCTCCTCAAAGACCGAGTCC-3'

⑦FN primer sequence

Forward primer sequence 5'-CCATCGCAAACCGCTGCCAT-3'

Reverse primer sequence 5'-AACACTTCTCAGCTATGGGCTT-3'

⑧GAPDH primer sequence

Forward primer sequence 5'-GAAGGTGAAGGTCGGAGTC-3'

Reverse primer sequence 5'-GAAGATGGTGATGGGATTTC-3'

The Cytokine Expression Levels in HBE Were Detected by Western Blotting

Cells inoculated in 6-well plates were washed, cleaved and harvested with PBS. After centrifugation at 14000 rpm at 4°C for 15 min, the supernatant was collected and stored at -80°C for later use. BCA protein assay kit (Abbkine, Wuhan, China) was used to determine the protein concentration. After adding the sample buffer, the protein was dissolved by 12% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to PVDF (Servicebio, Wuhan, China) membranes and then separated in 5% dry milk for 2h in PBS containing 0.1% tween-20e. The primary antibody diluted with PBST was incubated overnight at 4°C, washed with PBST 3 times, and the secondary antibody combined with PBST was incubated for 1h. The resulting immunoreactive protein complex was detected with ECL assay reagent (Abbkine, Wuhan, China), and the level changes of Nrf2, HO-1, NF-κB, IFN-γ, TGFβ1, MMP9, FN were detected according to the manufacturer's instructions.

Instantaneous Transfection of Nrf2-siRNA

According to the manufacturer's instructions, Lipo 3.0 high-efficiency transfection reagent (Abbkine, Wuhan, China) was used to transfect specifically targeted human Nrf2 sequence (S: 5'-GGUUGAGACUACCAUGGUUTT-3'; AS: 5'-AACCAUGGUAGUCUCAACCTT-3') siRNA. 48h after transfection, the cells were used for experiments (allowing the turnover of the remaining Nrf2 protein and preventing the re-synthesis of Nrf2). Western blotting confirmed that the target protein was successfully knocked out.

Determination of NO Level in Cell Culture Supernatant

NO content detection kit (Abbkine, Wuhan, China) was used to accurately determine the production of NO after the reduction of nitrate to nitrite by the modified Gris method. The NO content in the cell culture supernatant was determined according to the instructions provided by the manufacturer, and the OD value was obtained according to the steps.

Data Statistics and Analysis

The experimental data were analyzed statistically by GraphPad Prism 10 software. The measured data conform to normal distribution and are expressed as $\bar{x} \pm s$. The *t* test of two independent samples was used for comparison between groups, the count data was expressed as "rate (%)", and the comparison was performed by χ^2 test. Single-factor analysis of variance (ANOVA) was used for multi-group comparison, and Tukey multiple comparison test was used for posterior test. All data are expressed as mean $\pm$ SEM (standard error of the mean). $p < 0.05$ was considered statistically significant, * meant $p < 0.05$, ** meant $p < 0.01$.

Results

Analysis of Body Weight Gain in Rats Exposed to PM_{2.5} at Different Time Points of Modeling

During the exposure period, the body weight of the three groups of rats increased, but the weight growth rate of the normal saline control group was significantly faster than that of the PM_{2.5} experimental group, and the weight growth rate of the low-dose PM_{2.5} experimental group was faster than that of the high-dose PM_{2.5} experimental group, indicating that PM_{2.5} has an impact on the growth of rats, and the greater the exposure dose and the longer the exposure time, the more serious the impact. The weight gain of rats in each group at three time points was shown in Figure 2.

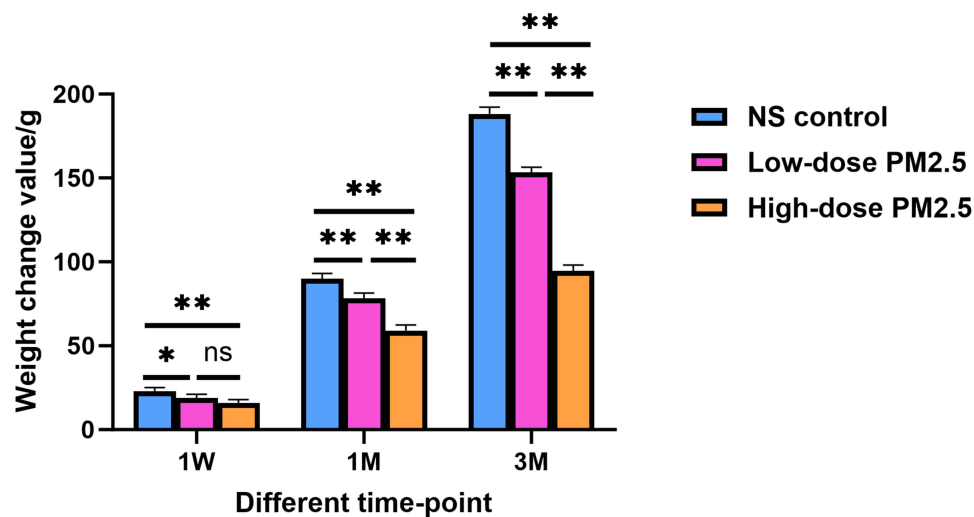


Figure 2 Body weight gain of SD rats in different treatment groups at different time points.

Notes: Control group: 0 mg/kg; Low dose group: 5 mg/kg; High dose group: 50 mg/kg. Compared with normal saline control group at the same time, * $P < 0.05$, ** $P < 0.01$.

Abbreviations: 1W, 1 week; 1M, 1 month; 3M, 3 months.

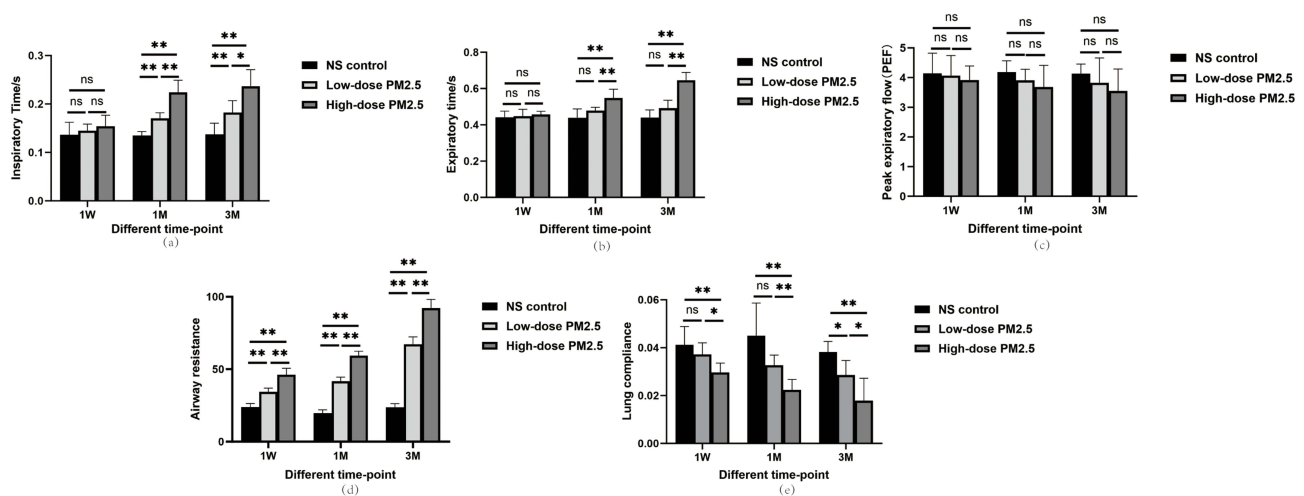


Figure 3 Changes of lung function in different treatment groups at different time points.

Notes: Control group: 0 mg/kg; Low dose group: 5 mg/kg; High dose group: 50 mg/kg. (a–e) illustrates the changes in respiratory function among rats in each group at three different time points. Compared with normal saline control group at the same time, * $P < 0.05$, ** $P < 0.01$.

Abbreviations: 1W, 1 week; 1M, 1 month; 3M, 3 months.

PM2.5 Exposure Resulted in Decreased Lung Respiratory Function in Rats

During PM2.5 exposure, compared with the control group, the inhalation time and expiratory time of the PM2.5 experimental group were significantly prolonged, the peak expiratory value was significantly reduced, the airway resistance was significantly increased, and the lung compliance was significantly decreased. The same trend existed between the high-dose PM2.5 group and the low-dose PM2.5 group, suggesting that PM2.5 can significantly reduce the pulmonary respiratory function of rats, and the degree of reduction is related to the exposure dose and exposure time. The changes of pulmonary respiratory function of rats in each group at three different time points were shown in Figure 3.

The Percentage of Neutrophils in Alveolar Lavage Fluid of PM2.5 Exposed Rats Was Up-Regulated

Compared with the normal saline control group, the percentage of neutrophils in BALF at 1 week, 1 month and 3 months in the high dose PM2.5 group was significantly higher than that in the normal saline control group. At the same time, the percentage of neutrophils in BALF of the low-dose PM2.5 experimental group was significantly higher than that of the saline control group at 3 months, indicating that the lungs of the PM2.5 experimental group had a significant inflammatory response at these time points, as shown in Figure 4.

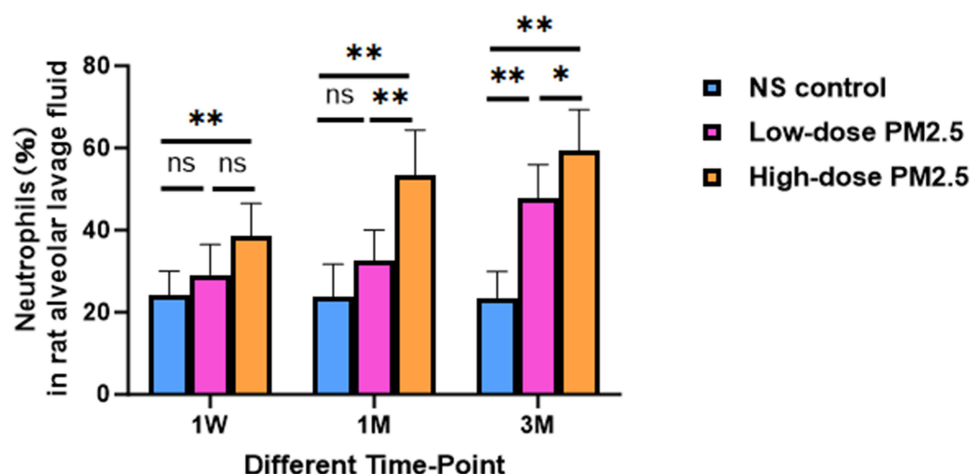


Figure 4 Percentage of BALF inflammatory cells in different treatment groups at different time points.

Notes: Control group: 0 mg/kg; Low dose group: 5 mg/kg; High dose group: 50 mg/kg. Compared with normal saline control group at the same time, * $P < 0.05$, ** $P < 0.01$.

Abbreviations: 1W, 1 week; 1M, 1 month; 3M, 3 months.

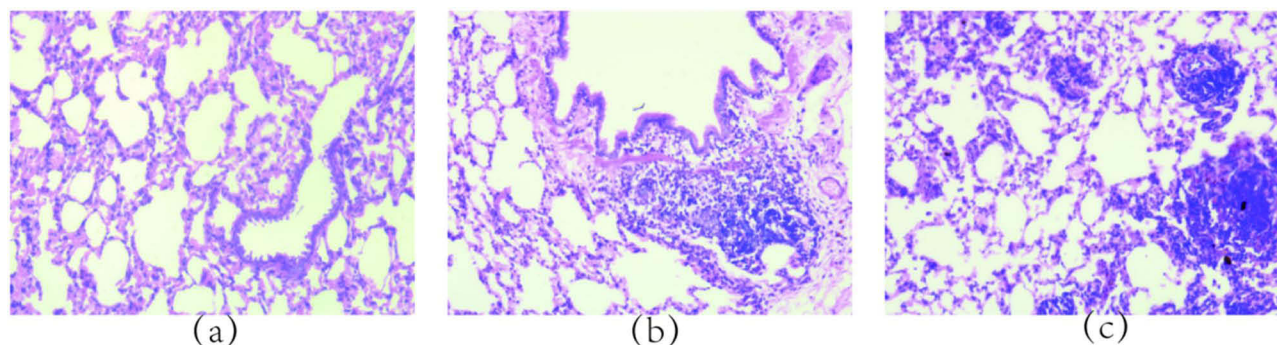


Figure 5 Pathological changes of lung tissue of rats in different treatment groups (HE×50).

Notes: (a) Normal saline control group; (b) Low-dose PM2.5 experimental group; (c) High-dose PM2.5 experimental group.

Changes of Lung Histopathology in Rats Exposed to PM2.5

After PM2.5 exposure, HE staining showed that the size of alveoli in the normal saline control group was basically normal at different time points, and no obvious exudate was found in the alveolar cavity. In the low-dose PM2.5 group, the lung interstitial widened, the alveolar septum became thin and broken, the bronchial wall smooth muscle thickened, and the focal inflammatory cells infiltrated. In the high-dose PM2.5 group, foreign particles were found in some alveolar cavities, lung interstitial widened and thickened, fibrous tissue hyperplasia, alveolar cavity increased, and focal inflammatory cell infiltration increased. The above phenomena indicate that the lungs of the PM2.5 experimental group showed obvious inflammatory response and injury, which is typical of COPD (Figure 5).

RTA-408 Can Increase the Activities of Nrf2 and HO-1, and Down-Regulate the Levels of NF- κ B and IFN- γ in COPD Rats

To investigate whether RTA-408 can reverse the decreased expression of Nrf2 and HO-1 and the increased expression of NF- κ B and IFN- γ induced by PM2.5, We studied the mRNA and protein levels of Nrf2, HO-1, NF- κ B and IFN- γ in lung tissues of rats in control group, COPD group and COPD+ RTA-408 group, and detected the changes of the above indexes in serum by ELISA. It can be seen that the expression levels of oxidative stress indicators Nrf2 and HO-1 in COPD group were significantly lower than those in control group, and the expression levels of oxidative stress indicators were increased after RTA-408 intervention, and the effect was dose-dependent. The expressions of inflammatory indexes NF- κ B and IFN- γ in COPD group were significantly higher than those in control group, and the inflammatory indexes decreased after drug intervention, with statistical significance (Figures 6–8).

RTA-408 Increased Serum NO Level in PM2.5 Exposed COPD Model Rats

The serum NO detection results of rats showed that PM2.5 exposure significantly reduced the serum NO expression level of COPD rats, and the level of NO expression increased after RTA-408 intervention (Figure 9).

RTA-408 Reversed the Decrease of HBE Activity Induced by PM2.5

CCK-8 test results showed that HBE cells were treated with a concentration of 0–400 μ g/mL PM2.5 solution, the cell viability showed a downward trend, and the IC50 value was 249.9 μ g/mL (Figure 10a and b). According to the cell state and experimental requirements, the cell experiment was conducted according to the intervention model with PM2.5 concentration of 200 μ g/mL. In order to select the optimal concentration of RTA-408, we investigated the effect of RTA-408 on the viability of HBE cells. After treating HBE cells with 5–1000 nM RTA-408 solution, we observed no significant difference in the cell viability of HBE cells in the 5–100 nM range compared with the blank control group. When RTA-408 concentration was greater than 100 nM, HBE cell viability decreased in a dose-dependent manner. The 100 nM RTA-408 virtually eliminated the decrease in cell viability caused by PM2.5 (Figure 10c). According to the above results, the effect concentration of the cell model was preliminarily determined as PM2.5 200 μ g/mL and RTA-408 100 nM. HBE cells were treated with PM2.5 and RTA-408 for 24 hours to detect cell viability (Figure 10d).

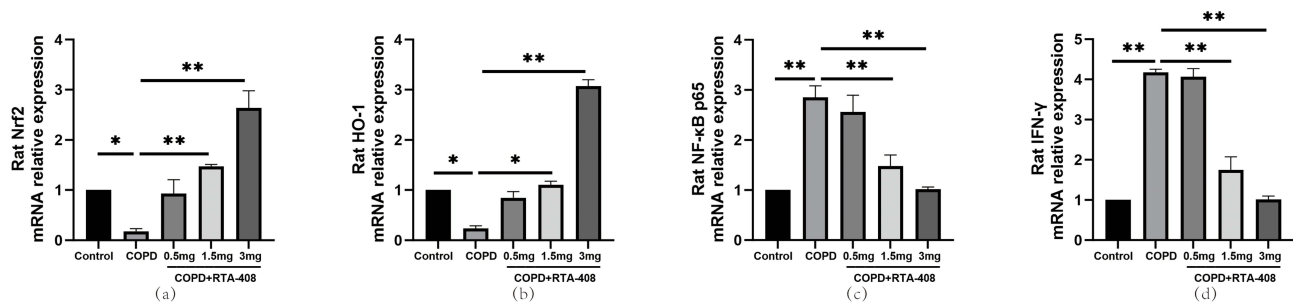


Figure 6 Effects of RTA-408 on gene levels of inflammatory factors and oxidative stress indexes in lung tissue of COPD model.

Notes: (a–d) Shows RT-qPCR in blank control group (normal saline), COPD model (PM2.5 exposure) group, COPD+ low-dose RTA-408 (0.5mg/kg) group, COPD+ medium-dose RTA-408 (1.5mg/kg) group, and COPD+ high-dose RTA-408 (3mg/kg) group, respectively. The mRNA expression levels of Nrf2, HO-1, NF- κ B and IFN- γ were measured by CR (6 rats per group). Data are expressed as mean $\pm$ SD, * p < 0.05, ** p < 0.01.

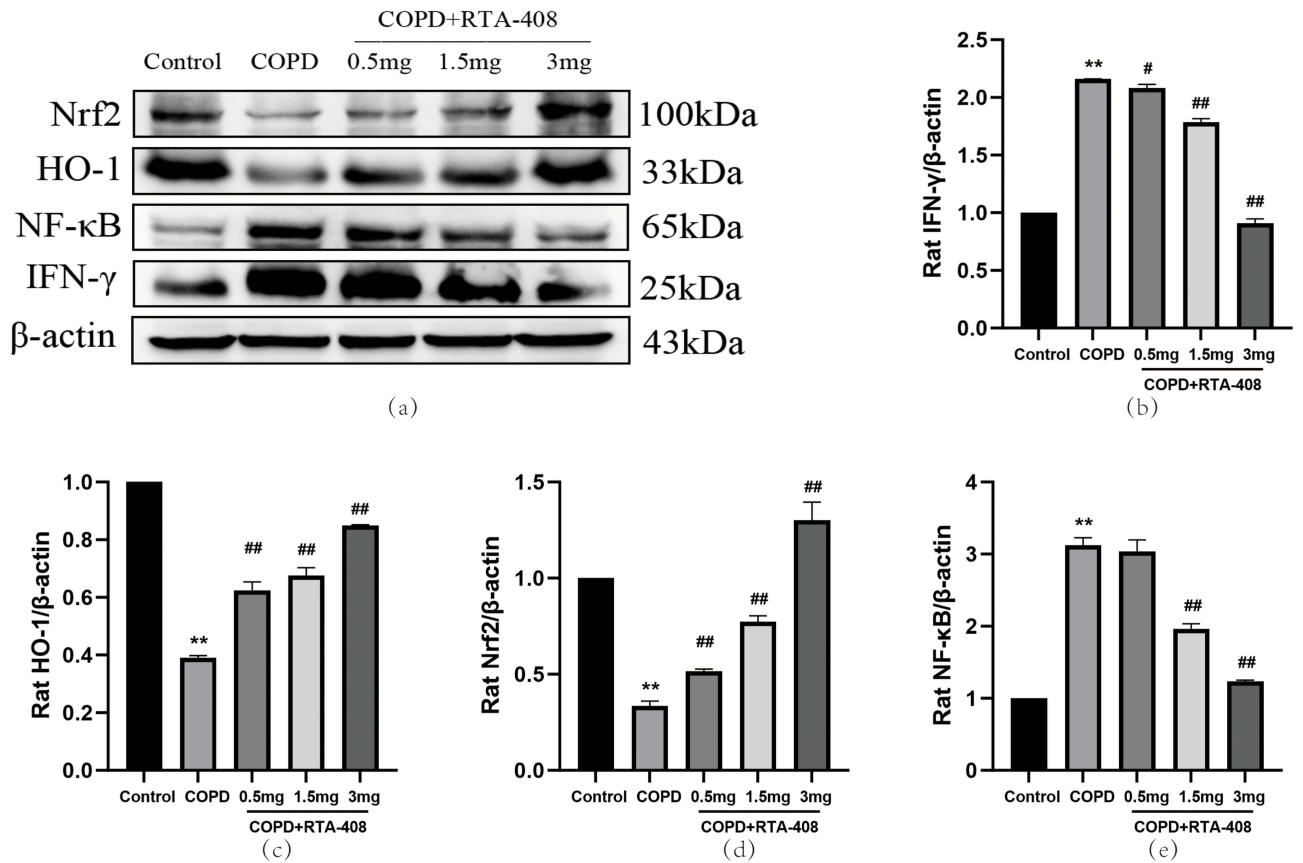


Figure 7 Effects of RTA-408 on the levels of inflammatory factors and oxidative stress index proteins in lung tissue of COPD model. **Notes:** (a) It shows blank control group (normal saline), COPD model (PM2.5 exposure) group, COPD+ low-dose RTA-408 (0.5mg/kg) group, COPD+ medium-dose RTA-408 (1.5mg/kg) group, and COPD+ high-dose RTA-408 (3mg/kg) group of target proteins Nrf2, HO-1, NF-κB, IFN-γ/WB bands (6 per group). (b–e) respectively depict the changes in protein content of Nrf2, HO-1, NF-κB, and IFN-γ among the groups. Data were expressed as mean±SD, **P<0.01 as compared to the control group, #P<0.05, ###P<0.01 as compared to the COPD group.

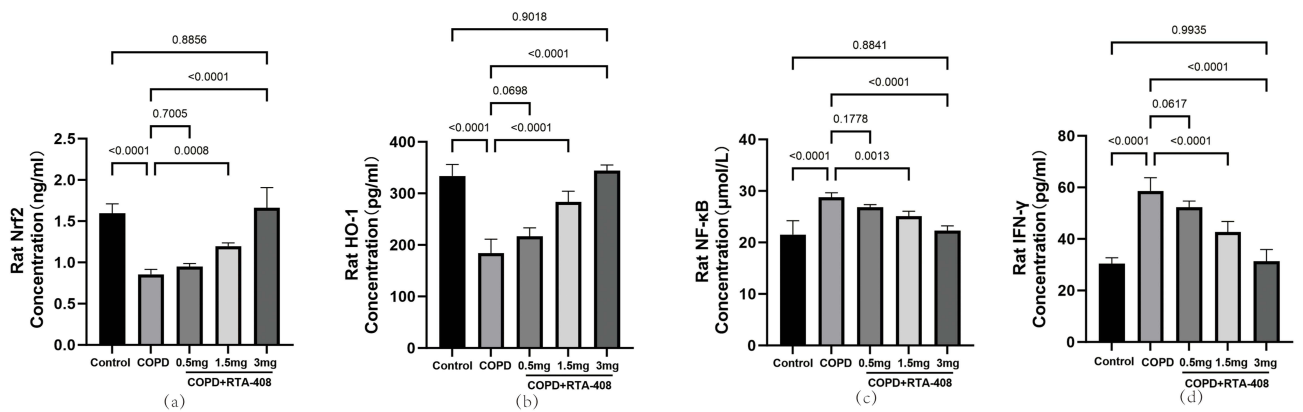


Figure 8 Effects of RTA-408 on serum inflammatory factors and oxidative stress indexes in COPD model rats. **Notes:** The expression levels of Nrf2, HO-1, NF-κB and IFN-γ proteins were detected by ELISA (a–d). Data are expressed as mean ±SD.

Effects of PM2.5 on HBE Airway Remodeling Factors TGF-β1, MMP9 and FN

HBE cells treated with PM2.5 showed morphological and structural destruction and fibrosis, inflammatory response, oxidative stress injury, and activation of inflammatory signaling pathways. The morphological changes of HBE and related airway remodeling cytokines were observed after PM2.5 treatment, and the in vitro cell model of COPD was

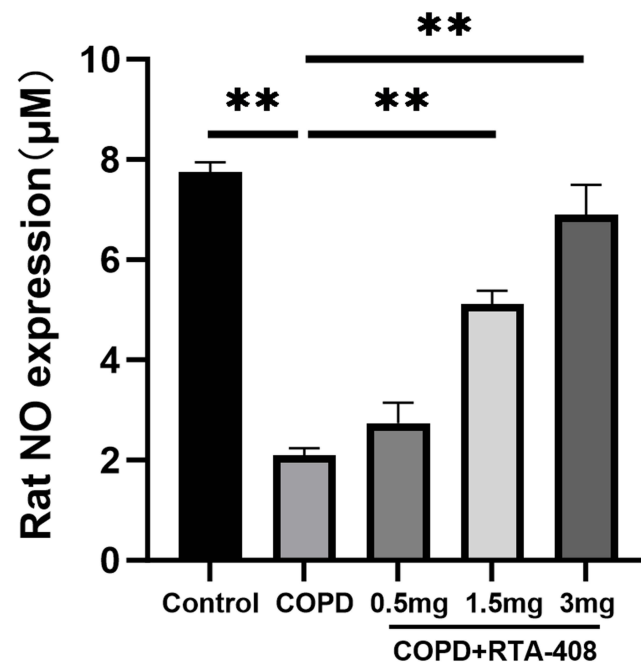


Figure 9 RTA-408 increased the decrease of NO in the serum of COPD model rats exposed to PM2.5.

Notes: The modified Griess method was used to detect the content of NO in serum of rats. The data were expressed as the mean $\pm$ SEM of three independent experiments, $n = 6$, ** $p < 0.01$.

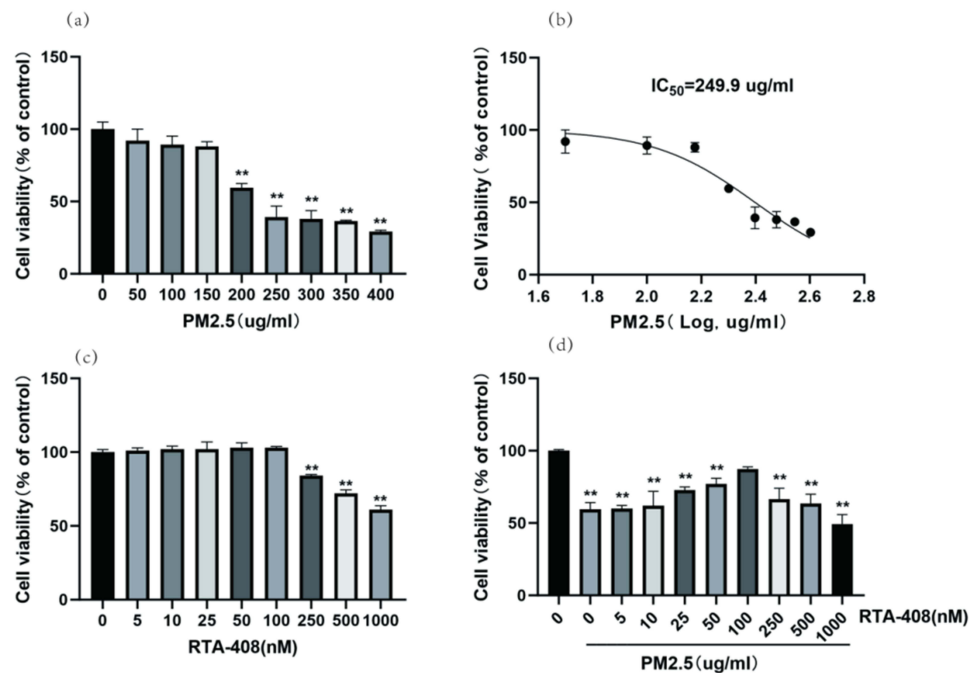


Figure 10 RTA-408 prevents PM2.5-induced decrease in HBE activity.

Notes: The cell viability of untreated HBE cells was 100% measured by CCK-8 method. (a) Incubation of HBE for 24h with 50–400 μ g/mL PM2.5. (b) The IC₅₀ of HBE treated with a series of concentration gradient PM2.5 solutions was 249.9 μ g/mL. (c) After incubation with 5–1000 nM RTA-408 for 24h, there was no significant difference in cell activity between 5–100 nM and blank group. (d) 200 μ g/mL PM2.5+100 nM RTA-408 for 24 h. The data were represented by mean $\pm$ SEM, $n = 4$, ** $p < 0.01$.

established successfully. In this study, it was observed that in the normal group, PM2.5 group, PM2.5+RTA-408 group and RTA-408 group, compared with the blank control group, HBE in the PM2.5 group showed inflammatory cell infiltration, fibrosis, cell gap widening, cell morphology destruction, cell number reduction, and decreased vitality, which

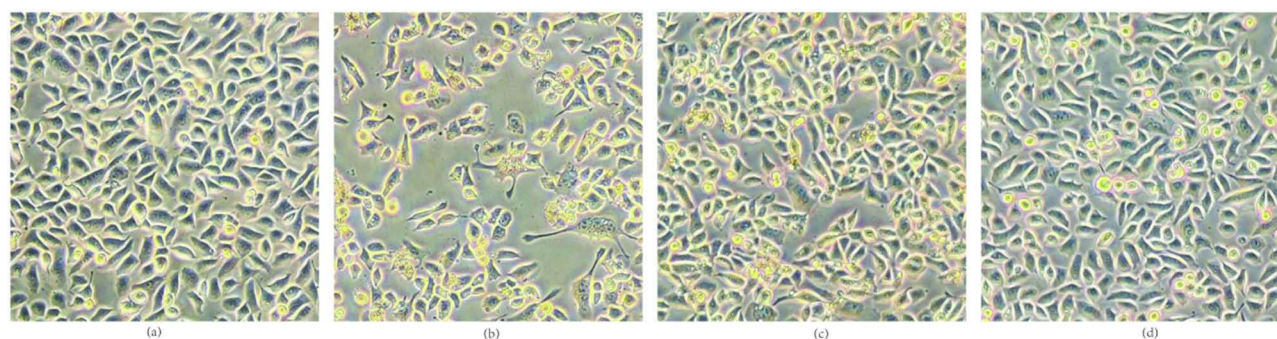


Figure 11 HBE cell morphology.

Notes: (a) Blank control group, (b) PM2.5 group: PM2.5 concentration was 200 μ g/mL, (c) PM2.5+RTA-408 group: PM2.5 concentration was 200 μ g/mL and RTA-408 concentration was 100nM, (d) RTA-408 group: RTA-408 concentration was 100nM.

were consistent with COPD cells. After appropriate RTA-408 intervention, the cell morphology was restored, the number of cells increased, and the vitality was enhanced. There was no significant difference between the blank control group and the RTA-408 group (Figure 11). According to the changes of TGF- β 1, MMP9 and FN mRNA and protein levels, the above airway remodeling indexes were significantly increased in the PM2.5 group, suggesting inflammation in HBE. After drug intervention, airway remodeling and inflammation indexes were lower than those in PM group. RTA-408 mitigated airway remodeling and inflammatory responses induced by PM2.5-treated HBE cells with significant differences in results. There was no significant difference between the blank control group and the RTA-408 group (Figure 12).

IFN- γ is a classical cytokine secreted by Th1 lymphocytes and its main inflammatory mediator, causing inflammatory infiltration and histopathological changes in the body, ultimately leading to the occurrence and development of diseases.¹⁵ In patients with COPD, increased IFN- γ further induces the production and release of matrix metalloproteinase (MMP) 9, which degrades extracellular matrix fibrin (FN), glycoprotein, mucin, and basement membrane protein components of the lung parenchyma, inhibits alpha trypsin, resulting in loss of lung tissue elasticity and enlargement of ineffective alveolar space. This forms emphysema. At the same time, MMP-9 can cause the chemotactic response of inflammatory cells, make a large number of inflammatory cells gather on the blood vessel wall, release inflammatory factors, aggravate local airway inflammation, cause histocyte infiltration and tissue destruction, and thus reduce the lung function of patients. In PM2.5-induced COPD, the expression of transforming growth factor (TGF- β 1) is elevated, leading to increased epithelial interstitial transformation (EMT), which is involved in airway remodeling in COPD, aggravating inflammation and damaging alveolar structure.¹⁶ The above indicators are closely related to COPD airway

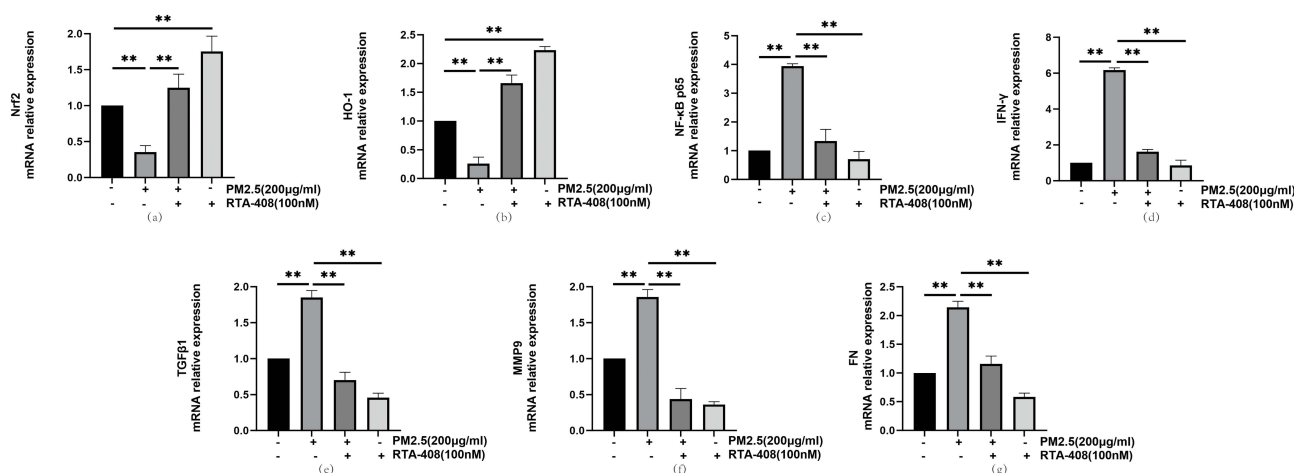


Figure 12 Changes of mRNA levels of inflammation and oxidative stress markers and airway remodeling markers in HBE cell models of COPD in vitro.

Notes: (a–g) Shows the summary data of Nrf2, HO-1, NF- κ B, IFN- γ , TGF β 1, MMP9 and FN mRNA expression in HBE cell model control group, PM2.5 group, PM2.5+RTA-408 group and RTA-408 group, respectively. The data were expressed as the mean $\pm$ SEM of three independent experiments. ** p < 0.01.

remodeling, which is of great value to understand the status of COPD airway remodeling. Therefore, we further investigated the level changes of these indicators in an in vitro cell model of COPD. The results of this study further verified the relationship between the cell model of COPD in vitro and the above three indicators.

RTA 408 Enhanced the Activity of Nrf2 and HO-1 in HBE Treated with PM2.5, and Down-Regulated the Levels of NF- κ B and IFN- γ

To investigate whether RTA-408 can reverse the PM2.5-induced decline in Nrf2 and HO-1 expression and increase in NF- κ B expression, We studied the changes of Nrf2, HO-1, NF- κ B and IFN- γ mRNA levels and protein levels in normal group, PM2.5 group, PM2.5+RTA-408 group and RTA-408 group. It can be seen that, compared with the control group, the oxidative stress indexes Nrf2 and HO-1 in PM2.5 group were significantly decreased, and the expression of oxidative stress indexes was increased after RTA-408 intervention. In the PM2.5 group, inflammatory indexes NF- κ B and IFN- γ increased significantly, and decreased after drug intervention, with statistical significance (Figure 13). In summary, we

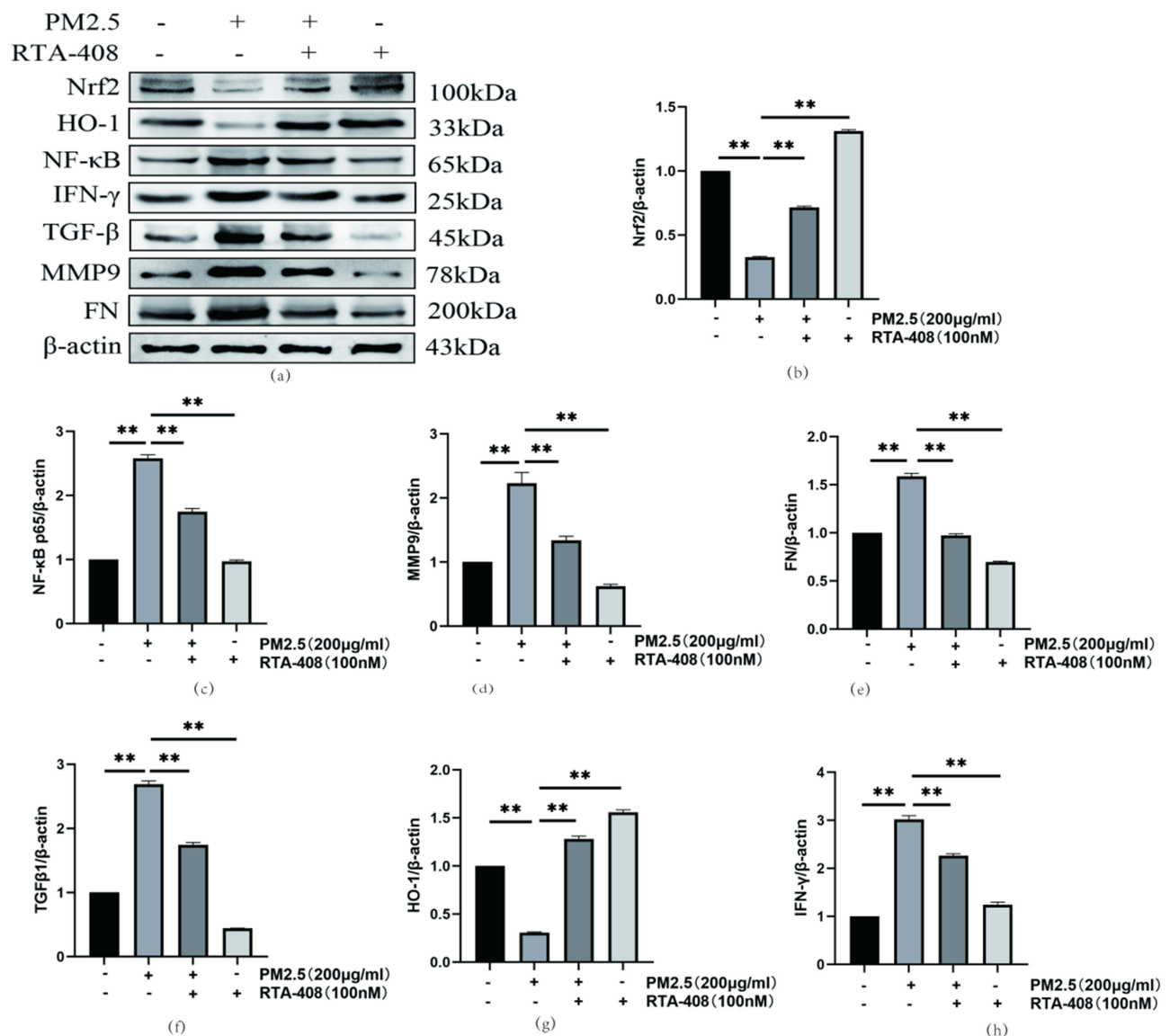


Figure 13 Changes of inflammation and oxidative stress indexes and airway remodeling index proteins in HBE cell models of COPD in vitro.

Notes: (a) Representative images of Nrf2, HO-1, NF- κ B, IFN- γ , TGF β 1, MMP9 and FN protein expression in HBE cell model control group, PM2.5 group, PM2.5+RTA-408 group and RTA-408 group. (b–h) Summary data on the level of gray values of target proteins in HBE. The data were expressed as the mean $\pm$ SEM of three independent experiments. ** $p < 0.01$.

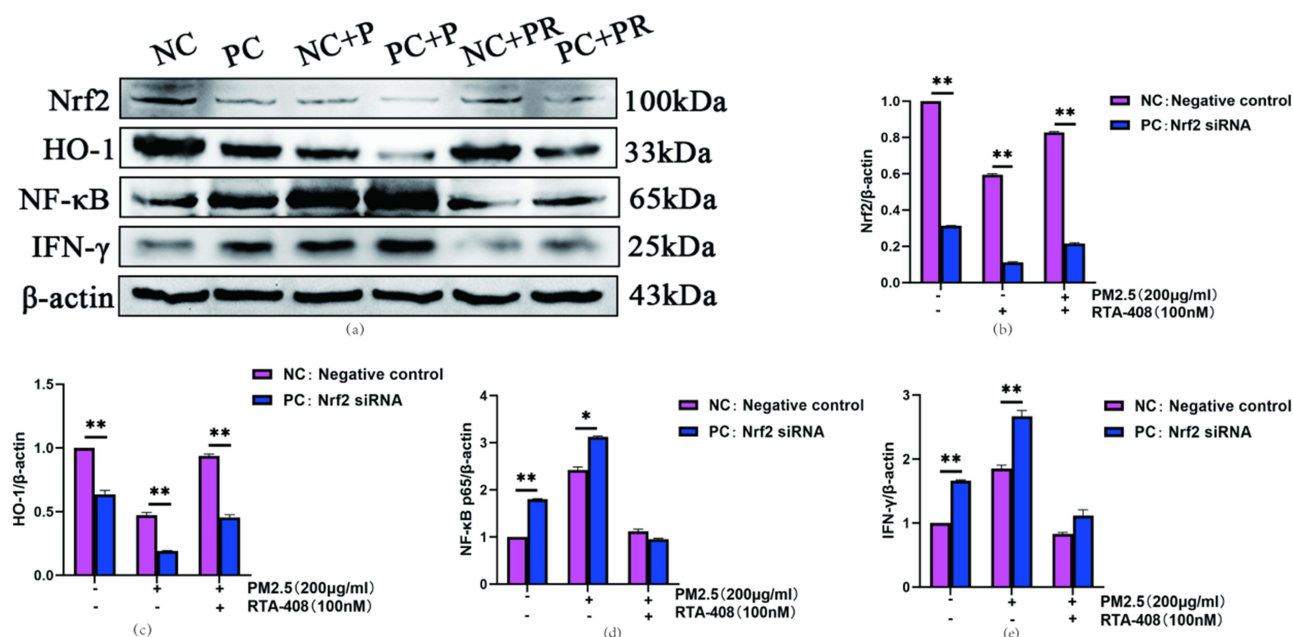


Figure 14 In PM2.5-treated HBE, Nrf2 silences can eliminate PM2.5-induced Nrf2 and HO-1 downregulation and NF-κB and IFN-γ upregulation.

Notes: PC: HBE was transfected at 60%-70% concentration of Nrf2-siRNA (100 nM, Nrf2-siRNA), and Nrf2 was silenced for 24 hours. NC: NS-siRNA, transfected HBE with non-specific siRNA (100 nM, Nrf2-SIRNA-non-silenced control). (a) Western blotting showed the effectiveness of Nrf2 silencing with β-actin as the load control. The representative images of Nrf2, HO-1, NF-κB, and IFN-γ protein expression in HBE treated according to the figure (b-e) It is the gray value of WB expression, and the summary data is expressed as the average of three independent experiments ±SEM. **p* < 0.05, ***p* < 0.01.

successfully established a cell model of COPD in vitro, and demonstrated that RTA-408 reversed the HBE-induced inflammation and oxidation response treated with PM2.5 through the Nrf2/HO-1 and NF-κB, IFN-γ pathways.

Nrf2 siRNA Can Eliminate the Cellular Protective Effect of RTA 408

In this experiment, the level of Nrf2 in the Nrf2 siRNA treated group was significantly lower than that in the negative control siRNA transfection group (Figure 14a). When PM2.5 was exposed to HBE, the expression of Nrf2 and HO-1 proteins in cells transfected with Nrf2 siRNA was significantly lower than that in cells transfected with negative control siRNA, and the inflammatory proteins NF-κB and IFN-γ were significantly increased (Figure 14b-e). In addition, as shown in the figure, the cell protective effect of RTA 408 was significantly blocked by Nrf2 siRNA, indicating that RTA 408 promotes cell survival by activating Nrf2.

RTA-408 Reversed the PM2.5-Induced Decline in HBE NO

NO detection results showed that 200μg/mL PM2.5 significantly decreased the expression level of NO. When RTA-408 concentration was 100nM, RTA-408 alone had NO significant effect on ROS production, but completely offset the decrease in NO production caused by PM2.5 (Figure 15).

Discussion

Chronic obstructive pulmonary disease (COPD) is a very common chronic respiratory disease in clinic. In recent years, the prevalence rate of COPD has been on the rise, seriously endangering human life and health, and causing heavy economic burden to China and even the world.¹⁷⁻¹⁹ The main causes of COPD include smoking, air pollution and occupational exposure.²⁰ Air particulate matter is the most harmful component of air pollution, among which PM2.5 and PM1.0 have strong permeability and can be inhaled into the distal airway through the nasal cavity through all levels of respiratory tract and deposited in the alveolar region to cause inflammation, which is believed to play a key role in the occurrence and development of COPD.^{21,22} PM2.5 exposure was reported to have contributed to the premature deaths of 860,000 COPD patients worldwide in 2015, of which 18.7% were in China.^{23,24} Although studies have shown that

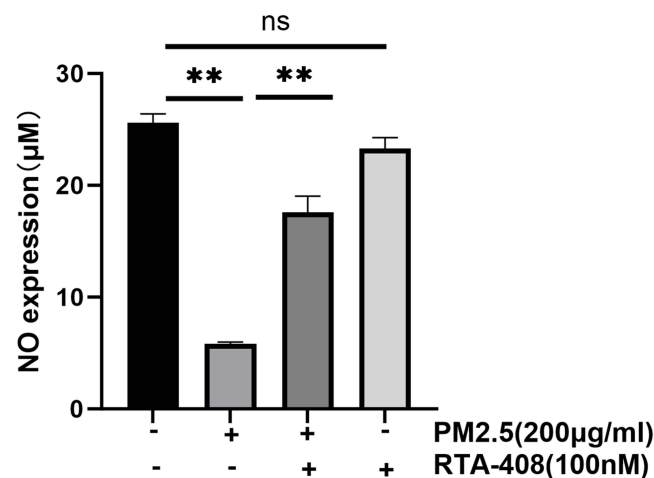


Figure 15 RTA-408 increased the PM2.5-induced reduction of NO in HBE.

Notes: The level of NO in the supernatant of cell culture was detected by the improved Griess method. Blank control group was incubated with 200 μg/mL PM2.5 for 24h, 200 μg/mL PM2.5+RTA-408 100 nM for 24h, and RTA-408 100 nM alone for 24h. Data were expressed as the mean $\pm$ SEM of three independent experiments, $n = 3$, ** $p < 0.01$.

airborne particulate exposure is significantly correlated with the occurrence and development of COPD,^{25,26} there is still insufficient evidence to prove the pathogenicity of airborne particulate matter to COPD.²⁷ Cough, sputum and dyspnea are common clinical symptoms of COPD, often accompanied by acute exacerbation.²⁸ Studies have found that chronic inflammation of airway, pulmonary parenchyma and pulmonary blood vessels induces infiltration and aggregation of inflammatory cells, resulting in high cytokine expression and strong immune defense capability of the body, which is the pathological basis for the occurrence and development of COPD.²⁹ The activation of neutrophils participates in the inflammatory response of COPD and induces chronic inflammation of the lungs, among which neutrophils are also considered to be the main inflammatory cells in the pathogenesis of COPD, and their elevation is the main feature of COPD.³⁰ The role of oxidative stress in the pathogenesis of COPD is well known. Exposure to PM2.5 can lead to oxidative stress imbalances, resulting in organelle damage and disruption of homeostasis.³¹ Oxidative stress can promote and enhance chronic inflammation and emphysema through activation of inflammatory protein transcription, destruction of anti-protease defense function, cell aging, mitochondrial damage, DNA damage, cell death and other pathways. It plays a central role in the pathogenesis of COPD. Therefore, inhibition of oxidation and antioxidant imbalance is of great significance in inhibiting the progression of COPD.^{32–34} Nuclear factor erythrocyte 2-associated factor 2 (Nrf2) is a cytoprotective transcription factor that regulates the expression of Phase II antioxidant enzymes. The Nrf2-Keap1 pathway is a major regulator of cellular protective responses to oxidative and electrophilic stress.³⁵ In animal models, the improvement of Nrf2 activators on oxidative stress and inflammation showed a protective effect of Nrf2. Therefore, Nrf2 appears to be an attractive drug target for the treatment or prevention of several oxidative stress-related diseases. Some studies have shown that some natural small molecules can activate the Nrf2 system and play a role in several disease models associated with increased inflammation or oxidative stress, such as autoimmune diseases, atherosclerosis, and cancer.⁴

As a model animal commonly used in biological studies, rats are ideal experimental subjects for COPD models. In the past, the common COPD animal models were airway inhalation models, such as cigarette smoke exposure model, air pollutant exposure model, tracheal infusion model-tracheal infusion protease model, tracheal infusion lipopolysaccharide model, etc., and other models. In this study, airway infusion of PM2.5 suspension was used to successfully establish a PM2.5-induced COPD model.^{36–38} The pathogenicity of PM2.5 in COPD was confirmed. At present, there is no mature experimental scheme for the airway drip PM2.5 model. The PM2.5 dose in this experiment was summarized on the basis of previous experimental data and was representative, providing valuable experience for the establishment of COPD models in rats exposed to PM2.5.^{39–42} Different modeling cycles and different doses of PM2.5 were designed to verify the pulmonary function, the proportion of inflammatory cells in alveolar lavage fluid and the pulmonary pathology of rats. Based on the analysis of the above indicators, the COPD modeling dosimetry was performed on rats exposed to

different doses of PM2.5. Finally, the COPD rat model was successfully constructed with a high dose of PM2.5 (50mg/kg). This study showed that the changes of oxidative stress and inflammatory factors *in vivo* were alleviated after RTA-408 treatment of COPD model rats, providing a theoretical basis for studying the protective effect of RTA-408 on COPD. The results showed that after drug treatment, the level of antioxidant factor Nrf2 increased, the level of inflammatory factors NF- κ B and IFN- γ decreased, and the level of NO increased. It was further confirmed that Nrf2 activator RTA-408 had anti-inflammatory and antioxidant effects in COPD.

In this study, we also demonstrated that PM2.5 can induce inflammation and oxidative stress in airway epithelial cells. In HBE cells, PM2.5 reduced the expression of antioxidant genes, enhanced the expression of inflammatory factors, and reduced the level of NO in the cells, and RTA-408 was able to counteract the damage caused by PM2.5. PM2.5 exposure increases the expression of airway remodeling proteins TGF- β 1, MMP9 and FN.^{43–45} Considering the combination of cell morphology, mRNA and protein expression levels, it can be concluded that PM2.5 can cause HBE changes similar to COPD, providing a more reliable cell model for studying the pathogenesis of PM2.5-induced COPD *in vitro*. In this study, we speculate that the anti-inflammatory effects of RTA-408 may be attributed to two mechanisms. First, the antioxidant function of RTA-408 promotes the expression of Nrf2 and downstream proteins associated with enhanced antioxidant capacity. Second, this may be due to the fact that RTA-408 inhibits p65 phosphorylation, thereby reducing inflammation levels. RTA-408 plays a beneficial role in a variety of diseases, but studies on the role of RTA-408 in COPD are lacking. In this study, we first investigated the effect of RTA-408 on HBE activity after exposure to PM2.5. At concentrations of RTA-408 below or equal to 100 nM, HBE activity was increased when exposed to PM2.5, but there was no significant cytotoxicity. Markers associated with inflammation and oxidative stress were then examined. The treatment of PM2.5-exposed HBE cells with RTA-408 (100nM) significantly upregulated the expression levels of Nrf2 and HO-1, while downregulating the expression levels of NF- κ B and IFN- γ , suggesting that RTA-408 can treat PM2.5-mediated oxidative stress in HBE cells. In addition, 100nM RTA-408 can effectively inhibit the expression of TGF- β 1, MMP9 and FN, indicating that RTA-408 can inhibit airway inflammation and airway remodeling in HBE. Inflammation and oxidation imbalance are important pathogenic factors of COPD. In this study, transfection of Nrf2 siRNA, that is, silencing Nrf2 gene, can significantly reduce the expression of intracellular antioxidant markers Nrf2/HO-1, increase the levels of NF- κ B and IFN- γ , and return to normal after RTA-408 intervention. These results suggest that Nrf2/HO-1, NF- κ B and IFN- γ pathways play an important role in PM2.5-induced airway epithelial cell injury. Previous studies have shown that RTA-408 may inhibit the NF- κ B signaling pathway, and RTA-408 can play a protective role in cartilage by activating the Nrf2/HO-1 signaling pathway.⁹ In this study, the expression levels of Nrf2/HO-1 and NF- κ B and IFN- γ signaling pathways were evaluated, and the trend was consistent with previous studies. These results suggest that RTA-408 plays an important role in PM2.5-induced COPD cell model *in vitro* through Nrf2/HO-1, NF- κ B and IFN- γ signaling pathways. NO is a well-known vasodilator that fundamentally controls various lung functions such as macrophage activity, pulmonary artery vasodilation, and bronchoconstriction. In addition, NO plays an important role in pulmonary neurotransmission, mucosal ciliary clearance, airway mucus secretion, and host defense. Studies have shown that serum NO levels decreased significantly in patients with simple COPD and in rats with COPD, which may be related to pulmonary vascular hypoxia, endothelial cell injury and increased pulmonary vascular resistance caused by long-term ventilation disorders caused by COPD. Lung tissue is exposed to the internal environment of oxidation/antioxidant imbalance for a long time, which further aggravates lung injury. The above factors lead to the injury of vascular endothelial cells, the decrease of NOS activity, and the decrease of NO synthesis and release. Similarly, the experimental results of NO in this study were the same as those in previous COPD studies.^{45–47}

In summary, in this study, we conducted an *in vivo* study with rats as subjects, supplemented by *in vitro* cell experiments, to explore the role of RTA-408 in COPD. First of all, *in vitro* experiments, experimental data show that by activating Nrf2/HO-1 and inhibiting NF- κ B and IFN- γ pathways, RTA-408 not only reduces the inflammatory response and oxidative stress of human bronchial epithelial cells, but also reverses cell fibrosis, cell gap widening, and cell morphological destruction. We observed that the cell morphology was restored, the number of cells increased, and the vitality was enhanced. Similarly, in the rat model, we observed that the changes of inflammation and oxidative stress indicators after drug intervention were consistent with the changes of cell experiments, and we observed that the intervention effect of RTA-408 on COPD increased in a drug dose dependent manner. Secondly, COPD is a serious

global public health problem. At present, there are very few drugs that can reverse the progression of the disease. The study on RTA-408 in COPD is of great significance, and this study provides valuable theoretical experience for subsequent relevant studies. Thirdly, at present, the health hazards of PM_{2.5} to various systems of the human body are complex and diverse, and even some harmful effects have not been fully understood by scientists, and the protection and self-protection of PM_{2.5} only remain external means. Therefore, we propose: Can RTA-408 play a preventive role in respiratory diseases caused by PM_{2.5}? This will be the next research direction of our research group, which is of great significance for the study of PM_{2.5} induced COPD. Therefore, we conclude that RTA-408 is well worth considering as a new strategy for the treatment of COPD, and may also have a positive preventive effect. However, as more research and clinical trial data become available, the safety and toxicological profile of RTA-408 may change. This study also has limitations. In addition to oxidative stress and inflammation, the pathogenesis of COPD in humans is influenced by many factors and the clinical phenotype is complex. Whether RTA-408-activated Nrf2 can also produce different effects by influencing these factors is unclear, and this study did not analyze this in detail.

Conclusion

In this study, we successfully constructed PM_{2.5} exposed rat COPD model and HBE in vitro cellular COPD model, proving the pathogenicity of PM_{2.5} on COPD, and explored the anti-inflammatory and antioxidant effects of Nrf2 activator RTA-408 in COPD and the related mechanisms through experiments. Therefore, the study of RTA-408 in COPD is of great significance, especially for COPD caused by PM_{2.5}. This study provides very valuable theoretical experience for subsequent relevant research.

Abbreviations

COPD, Chronic obstructive pulmonary disease; Nrf2, NF-E2-related factor; HO-1, Heme Oxidase-1; NF-κB, Nuclear Factor kappa B; IFN-γ, interferon-gamma; HBE, Human Bronchial Epithelioid; CNC, Cap'n'collar; NO, Nitric Oxide; CCK-8, cell proliferation assay; TGFβ1, transforming growth factor β1; MMP9, matrix metalloproteinase9; FN, fibronectin; PM_{2.5}, fine particulate matter; PM_{1.0}, ultra-fine particulate matter; EMT, epithelial interstitial transformation; Ti, inspiratory time; Te, expiratory time; PEF, peak expiratory time; Cydn, lung compliance; RI, airway resistance.

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Disclosure

The authors report no conflicts of interest in this work.

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