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ORIGINAL RESEARCH

Efficacy of Nebulized Pentoxifylline in a Mouse Model of Emphysema Induced by Cigarette Smoke and Aerosolized Lipopolysaccharide

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Objective: To investigate the impact of pentoxifylline dosage on lung pathological changes and inflammation triggered by the combined exposure to cigarette smoke and aerosolized lipopolysaccharides.

Methods: Female C57BL/6 mice were subjected to either cigarette smoke (CS) plus lipopolysaccharide (LPS) exposure or sham smoke (SCS) exposure for a duration of 10 weeks. Starting from the 9th week, the mice were randomly assigned to distinct intervention groups, where they received nebulized treatments of pentoxifylline (at varying doses), theophylline, or budesonide suspension for 2 weeks. A co-solvent control group was also included. At the end of the 10th week, the mice were euthanized. Enzyme-linked immunosorbent assay (ELISA) was employed to detect the expression levels of tumor necrosis factor-α (TNF-α), keratinocyte chemoattractant/C-X-C motif chemokine ligand 1 (KC/CXCL1, the murine functional homolog), and interleukin-1β (IL-1β) in bronchoalveolar lavage fluid (BALF). After tissue homogenization, ELISA was also used to measure the expression of matrix metalloproteinase-12 (MMP-12) and the level of histone deacetylase 2 (HDAC2). Hematoxylin and eosin (H&E) staining was performed on lung tissue sections to determine the alveolar mean linear intercept (Lm) and alveolar destruction index (ADI). Additionally, Wright-Giemsa staining was utilized for the classification and quantification of cells in BALF.

Results: Chronic exposure to CS+LPS resulted in a significant exacerbation of pulmonary inflammation, which was characterized by elevated levels of TNF-α, KC/CXCL1, IL-1β, and MMP-12 (CS+LPS group vs SCS group, all $p<0.05$). Moreover, a notable reduction in HDAC2 level was observed, accompanied by significant increases in Lm and ADI (all $p<0.05$). The total cell count in BALF was also significantly higher in the CS+LPS group ($p<0.05$). Budesonide treatment led to a significant reduction in the levels of inflammatory cytokines and MMP-12; however, it had no significant effect on HDAC2 level. Both pentoxifylline (PTX) at different doses and theophylline (THEO) effectively decreased the expression of inflammatory markers and increased HDAC2 level ($p<0.05$). Notably, none of the treatments resulted in a significant improvement in Lm, whereas ADI was significantly reduced following treatment. Although THEO induced a reduction in TNF-α levels, this change did not reach statistical significance.

Conclusion: Nebulized pentoxifylline and theophylline can alleviate lung inflammation induced by CS and LPS exposure, as evidenced by the decreased expression of TNF-α, KC/CXCL1, IL-1β, and MMP-12, and the restoration of HDAC2 level (which was reduced by CS+LPS exposure). In contrast, budesonide alone was capable of reducing inflammation but failed to exert an impact on HDAC2 level. A numerical trend indicated that pentoxifylline at a concentration of 22.0 mg/mL might exhibit greater efficacy; however, no statistically significant differences were observed among the various PTX doses tested. Further research is required to identify the optimal concentration of pentoxifylline for this application.

Keywords: pulmonary inflammation, cigarette smoke, pentoxifylline, histone deacetyltransferase 2, cytokines

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Introduction

Chronic obstructive pulmonary disease (COPD) is a highly prevalent respiratory disorder demonstrated by persistent airflow limitation and chronic inflammation.[1] Cigarette smoke exposure represents the primary risk factor, driving key pathological alterations including emphysema, small airway fibrosis, and chronic bronchitis.[2–4] As a complex, progressive disease, COPD is characterized by a prolonged clinical course, recurrent exacerbations, and a high burden of comorbidities—such as cor pulmonale, pulmonary encephalopathy, and cardiovascular diseases—which further worsen patient outcomes.[5,6] Notably, recent research has underscored that COPD patients exhibit increased susceptibility to respiratory infections, including coronavirus disease 2019 (COVID-19), leading to heightened disease burden and mortality.[7,8]

A major clinical challenge in COPD management is the limited efficacy of corticosteroids for suppressing inflammation during stable disease.[9] This corticosteroid resistance is particularly problematic, as COPD exacerbations and comorbidities significantly impair patient quality of life and reduce survival.[10,11] Furthermore, the use of inhaled corticosteroids (ICS) in COPD has been linked to an increased risk of pneumonia, further complicating treatment decision-making.[12]

The anti-inflammatory effects of corticosteroids (eg, budesonide, BUD) are mediated via the glucocorticoid receptor, which recruits histone deacetylase 2 (HDAC2).[13] HDAC2 functions to deacetylate histones, thereby suppressing the transcription of pro-inflammatory genes. Critically, in COPD, oxidative stress and chronic inflammation induce a marked reduction in HDAC2 expression and activity. This impairment is a central mechanism underlying corticosteroid resistance, directly explaining the limited efficacy of these agents in stable COPD.[14] Thus, therapeutic strategies aimed at restoring HDAC2 levels or activity represent a promising approach to overcome this barrier.

Theophylline (THEO) has a long history of use in COPD treatment; however, its clinical application is limited by a narrow therapeutic window and dose-related side effects at conventional bronchodilator doses.[15,16] Intriguingly, at lower concentrations, theophylline can upregulate HDAC2 levels, potentially reversing corticosteroid resistance[17,18]—a property that makes it a valuable reference compound for evaluating HDAC2-enhancing therapies.

Pentoxifylline (PTX), another non-selective phosphodiesterase inhibitor, is well recognized for its broad anti-inflammatory and antioxidant activities. It exerts these effects by inhibiting the release of key pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α), matrix metalloproteinase (MMP)-9, and MMP-12.[19,20] Emerging clinical evidence suggests that PTX may confer therapeutic benefits in respiratory conditions such as acute respiratory distress syndrome (ARDS) and COPD, primarily through its modulatory effects on inflammation and oxidative stress.[21] Compared to other anti-inflammatory agents for COPD (eg, phosphodiesterase-4 [PDE4] inhibitors like roflumilast), PTX offers a more diverse mechanism of action but lacks extensive clinical validation in respiratory settings.[22] Notably, recent preclinical studies have demonstrated that PTX can reverse smoke-induced reductions in lung HDAC2 levels;[23] however, its therapeutic potential when administered via inhalation (a route highly relevant to respiratory diseases) remains unexplored.

Based on this body of evidence, we hypothesize that nebulized PTX may restore HDAC2 levels and exert synergistic anti-inflammatory effects with corticosteroids. Using a mouse model of emphysema induced by chronic cigarette smoke and lipopolysaccharide (LPS) exposure, we aimed to test the primary hypothesis: nebulized PTX attenuates smoke- and LPS-induced lung inflammation and emphysematous changes by enhancing HDAC2 levels and reducing pro-inflammatory cytokine production. Key study endpoints included the levels of cytokines (keratinocyte chemoattractant/C-X-C motif chemokine ligand 1 [KC/CXCL1], macrophage inflammatory protein-2/C-X-C motif chemokine ligand 2 [MIP-2/CXCL2], TNF-α, and interleukin-1β [IL-1β]) in bronchoalveolar lavage fluid (BALF), lung MMP-12 expression, HDAC2 levels, and lung morphometric parameters. The effects of nebulized PTX (tested across five concentrations) were compared against those of theophylline, budesonide, and relevant control groups.

Materials and Methods

Ethics Approval

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and strictly adhered to the 3R principles (Replacement, Reduction,

and Refinement). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (Approval Number: 3269).

Inhalation Exposure System

The inhalation system comprised a glass reservoir and four sealed white plastic exposure chambers, with each chamber connected to the reservoir via a transfer tube of equal diameter. For cigarette smoke (CS) generation: one commercially available Hongjinlong cigarette (Wuhan Tobacco Group, China; tar content: 11 mg) was burned in the glass reservoir, with compressed air supplied at a flow rate of 4.4 L/min for ventilation. This whole-smoke exposure setup delivered a mixture of mainstream and sidestream smoke to the exposure chambers, simulating real-world environmental tobacco smoke. The mainstream-to-sidestream smoke ratio was not quantified, as it is an inherent characteristic of whole-smoke exposure designs. Under these conditions, each cigarette burned completely over approximately 6 minutes.

For lipopolysaccharide (LPS) delivery: LPS derived from *Pseudomonas aeruginosa* was prepared as a 2.5 mg/mL stock solution and stored at −20°C. Prior to use, the stock was thawed at 4°C, and 0.7 mL of stock was diluted with phosphate-buffered saline (PBS) to a total volume of 20 mL. This diluted solution was divided into 4 aliquots (5 mL each), each placed into a nebulizer cup positioned inside an exposure chamber. An AXD-302 nebulizer (Etienne, China; output rate: ~0.20 mL/min; mass median aerodynamic diameter [MMAD]: 1–5 μm) was used to aerosolize LPS for inhalation by mice. Nebulization of 5 mL of the LPS solution required approximately 15 minutes.

Mice in the exposure chambers were simultaneously exposed to both cigarette smoke (from the glass reservoir) and aerosolized LPS (from the nebulizer). To minimize positional bias (eg, uneven smoke/LPS distribution), the chambers were rotated daily.

Study Design

Ninety female C57BL/6 mice (6–8 weeks old; Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were acclimated to the housing environment for 1 week prior to randomization. Mice were then assigned to two primary exposure groups for 10 weeks:

- CS+LPS group: Exposed to cigarette smoke combined with aerosolized LPS (n = 80);
- Sham smoke (SCS) group: Exposed to filtered air only (n = 10, blank control).

At the end of Week 8, the 80 CS+LPS-exposed mice were further randomized into 8 intervention subgroups (n = 10 per subgroup), resulting in a total of 9 experimental groups:

- SCS group (blank control);
- CS+LPS group (untreated control);
- CS+LPS+PTX1–PTX5 groups (pentoxifylline [PTX] intervention, with concentrations of 9.8, 14.7, 22.0, 33.0, and 49.5 mg/mL, respectively);
- CS+LPS+THEO group (theophylline [THEO] intervention);
- CS+LPS+BUD group (budesonide [BUD] intervention).

Exposure Protocol

All mice had ad libitum access to a standard chow diet and sterile water throughout the study.

CS+LPS exposure: Mice in the CS+LPS group were exposed to 10 cigarettes (total smoke) and 20 mL of aerosolized LPS solution daily, with each exposure session lasting approximately 1 hour.

SCS exposure: Mice in the SCS group were housed in identical chambers but exposed to filtered air for 1 hour daily.

Both CS+LPS and SCS exposures were conducted 5 days per week for 10 consecutive weeks.

Interventional Medication and Route of Administration

All drugs were delivered via nebulization (consistent with the inhalation exposure route, ensuring targeted lung delivery). Drug dosages were calculated using human-to-animal dose conversion formulas referenced in the literature[24,25] as the followings:

PTX was prepared as sterile PBS-based suspensions at 5 concentrations (9.8, 14.7, 22.0, 33.0, and 49.5 mg/mL), THEO was prepared as a sterile PBS-based suspension at 25 mg/mL and BUD was used as an inhaled suspension at 0.5 mg/mL (commercially formulated, reconstituted per manufacturer guidelines if required).

Drug nebulization was initiated at the start of Week 9 and continued through Week 10 (total 2 weeks), coinciding with the final 2 weeks of CS+LPS exposure. Treatments were administered 5 days per week, with nebulization performed 1 hour prior to daily CS+LPS exposure to maximize drug deposition and efficacy. The SCS group and CS+LPS (untreated control) group received nebulization with 5 mL of sterile PBS (vehicle control) on the same schedule.

Morphological Analysis of Lung Tissue

At the end of Week 10 (24 hours after the final CS+LPS exposure), mice were euthanized in accordance with the updated American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. Euthanasia was performed via intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight), followed by exsanguination via abdominal aorta transection.

Lung tissue processing followed previously established protocols:[26]

The left lung was fixed by intratracheal instillation of buffered formalin (pH 7.4) at a transpulmonary pressure of 20 cmH_2O for 48 hours; Sagittal sections of the fixed left lung were paraffin-embedded, and 5-μm-thick sections were stained with Hematoxylin and eosin (H&E) for general histopathological assessment and Periodic acid-Schiff (PAS) combined with Alcian blue (pH 2.5) was for mucin detection.

Lung morphological parameters were quantified using a Stepanizer (image analysis tool) following the method described by Zheng et al.[23]

Mean Linear Intercept (Lm): A measure of alveolar airspace size (increased Lm indicates alveolar enlargement, a hallmark of emphysema);

Alveolar Destruction Index (ADI): A semi-quantitative score reflecting the degree of alveolar wall destruction.

The right lung was rinsed via intratracheal instillation of 0.5 mL ice-cold PBS (3 consecutive instillations), with the contralateral main bronchus ligated to prevent fluid leakage. The average bronchoalveolar lavage fluid (BALF) recovery volume was ~1.2 mL.

Supernatant for Cytokine Analysis: BALF was filtered through a 70-μm cell strainer and centrifuged at 750 × g for 10 minutes at 4°C. The supernatant was aliquoted and stored at −80°C for subsequent cytokine measurements.

Cell Pellet for Cellular Analysis: The cell pellet was resuspended in 1.0 mL sterile PBS. A 10-μL aliquot was used for total cell counting with a hemocytometer under light microscopy. The remaining suspension was centrifuged again at 1500 rpm (4°C) for 10 minutes. The supernatant was partially retained (volume adjusted based on total cell count and number of slides needed) to resuspend the pellet, which was then smeared onto glass slides. Slides were air-dried, fixed in 10% neutral buffered formalin for 10 minutes, and stained with Wright-Giemsa for differential cell counting. Stained slides were dried at 60°C, cleared in xylene (5 minutes), mounted with neutral balsam, and examined under light microscopy (low magnification for overview, oil immersion lens for differential counting).

BALF Cytokines: Levels of KC/CXCL1, TNF-α, and IL-1β in BALF supernatants were measured using commercial ELISA kits (Neobioscience, China) according to the manufacturer's instructions.

Lung Tissue Homogenization and Biomarker Analysis

The right lung (after BALF collection) was dissected, weighed, and stored at −80°C until analysis. Lung tissue was thawed on ice, and 0.9% sterile sodium chloride solution was added at a ratio of 1:9 (tissue weight [mg]: solution volume [μL]). Tissue was homogenized in an ice-water bath (0–4°C) using a mechanical homogenizer to prepare a 10% (w/v)

homogenate, and the homogenate was centrifuged at 2500–3000 rpm for 10 minutes at 4°C. The supernatant was collected (pellet discarded) and stored at −80°C.

Levels of MMP-12 and HDAC2 in lung homogenates were measured using commercial ELISA kits (Neobioscience, China) following the manufacturer's protocols.

Statistical Analysis

Data were statistically analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

First, the normality of data distribution was evaluated via the D'Agostino–Pearson omnibus K2 test, while the homogeneity of variances was assessed using Levene's test. For data that satisfied both normality and variance homogeneity assumptions, parametric statistical tests were applied. One-way analysis of variance (ANOVA) was used for comparisons across multiple groups (followed by Tukey's post-hoc test for pairwise comparisons), and independent samples t-tests were used for two-group comparisons. For data that failed to meet these assumptions, non-parametric tests were employed: the Kruskal–Wallis *H*-test was used for multi-group comparisons (followed by Dunn's post-hoc test for pairwise comparisons), and the Mann–Whitney *U*-test was used for two-group comparisons.

Bar graphs illustrating comparisons between multiple groups were generated using GraphPad Prism 8.0, with statistical significance determined via one-way ANOVA. A two-tailed p-value < 0.05 was considered statistically significant.

Results

Pulmonary Morphology and Morphometry

Consistent with our hypothesis, mice exposed to cigarette smoke combined with lipopolysaccharide (CS+LPS) exhibited a significant increase in the Lm compared to the SCS group ($p < 0.05$) (Figure 1A and B; Table 1). Additionally, the ADI was significantly elevated in the CS+LPS group ($p < 0.05$) (Figure 1C and D; Table 1), indicating the successful establishment of the emphysema model.

Following 2 weeks of drug intervention, none of the treatment groups showed a statistically significant change in Lm relative to the CS+LPS control group ($p > 0.05$). In contrast, all drug intervention groups exhibited varying degrees of reduction in ADI compared to the CS+LPS group ($p < 0.05$) (Figure 1A and B). However, no significant differences in ADI were observed either within the pentoxifylline (PTX) dose groups or between the PTX, theophylline (THEO), and budesonide (BUD) groups ($p > 0.05$) (Figure 1C and D).

Pro-Inflammatory Cytokines in Bronchoalveolar Lavage Fluid (BALF)

Ten-week CS+LPS exposure significantly upregulated the levels of tumor necrosis factor-α (TNF-α), keratinocyte chemoattractant/C-X-C motif chemokine ligand 1 (KC/CXCL1, the murine homolog of human IL-8), and interleukin-1β (IL-1β) in BALF compared to the SCS group ($p < 0.05$) (Figure 2 and Table 2).

Monotherapy with PTX (all tested doses), THEO, or BUD effectively downregulated the expression of TNF-α, KC/CXCL1 and IL-1β in BALF ($p < 0.05$) (Figure 2 and Table 2).

Among the five tested PTX concentrations (9.8, 14.7, 22.0, 33.0, and 49.5 mg/mL), the 22.0 mg/mL dose showed the most pronounced trend toward reducing the levels of these pro-inflammatory cytokines. Nevertheless, no significant differences in cytokine levels were detected between the different PTX dose groups ($p > 0.05$).

MMP-12 and HDAC2 Levels in Lung Tissue

Similar to the pro-inflammatory cytokines in BALF, the level of MMP-12 in lung tissue was significantly higher in the CS+LPS group than in the SCS group ($p < 0.05$) (Figure 3A and Table 2). Treatment with PTX (all doses), THEO, or BUD significantly downregulated lung MMP-12 expression ($p < 0.05$). Notably, PTX (across all tested concentrations) exhibited a significantly stronger inhibitory effect on MMP-12 than THEO ($p < 0.05$) (Figure 3A). The 22.0 mg/mL PTX

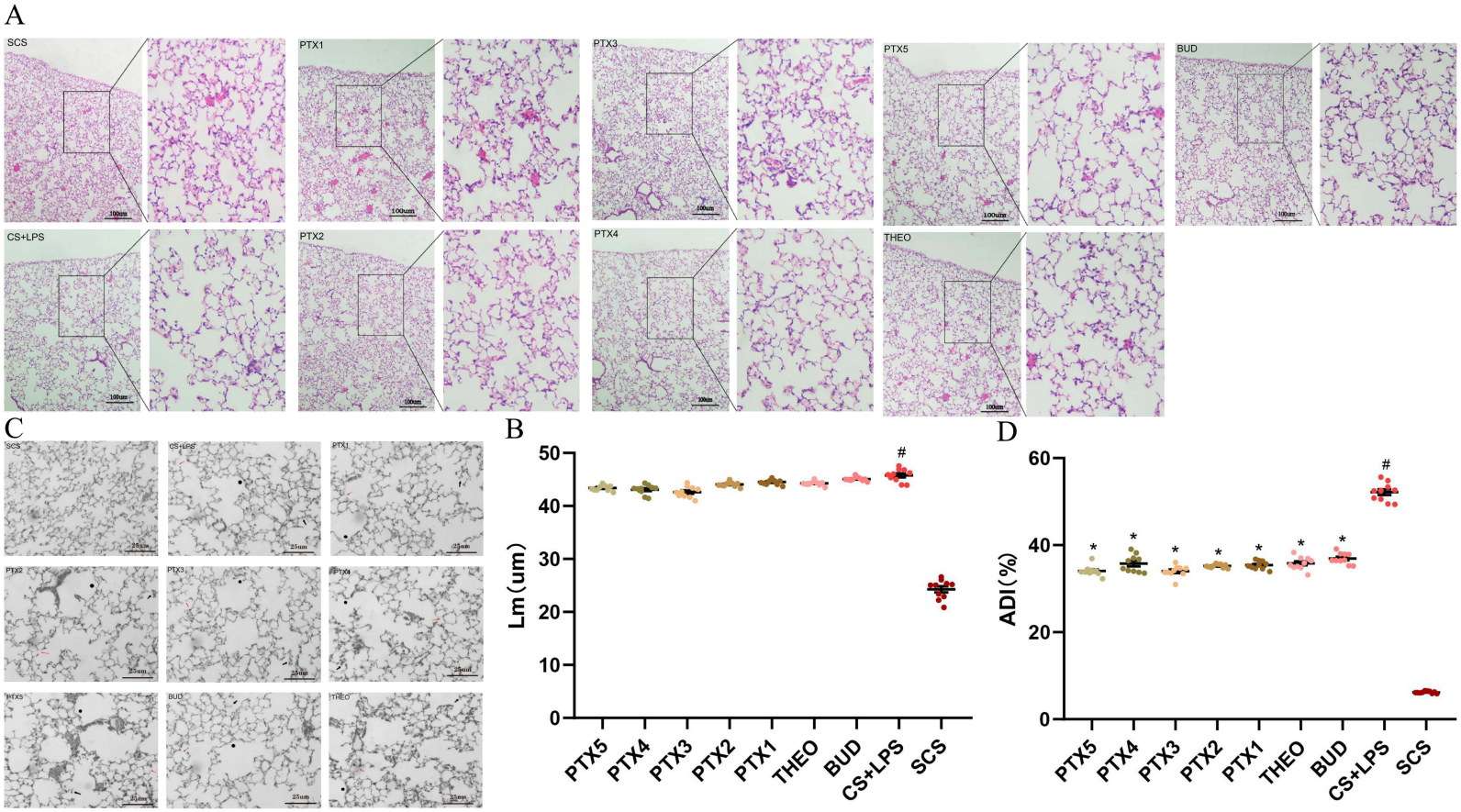

Figure 1 Lung Tissue Pathology. (**A**) Comparison of lung tissue pathological morphology. Chronic exposure to lipopolysaccharide (LPS) combined with cigarette smoke resulted in increased abnormal pathological structures in mouse lung tissues, including thinning of alveolar walls, and a significant number of alveoli becoming enlarged, ruptured, or fused to form bullae, presenting emphysema-like changes under microscopy. After drug intervention, the microscopic morphological changes in lung tissues of mice in each experimental group showed no significant difference from those in the control group (CS + LPS) (Hematoxylin-eosin staining, ×100 magnification). (**B**) Chronic exposure to cigarette smoke combined with lipopolysaccharide (LPS) significantly increases the mean linear intercept (Lm) in experimental mice. Compared with mice in the CS + LPS group, pentoxifylline (PTX), theophylline (THEO), and budesonide (BUD) show no significant effect on the mean linear intercept. $^{\#}p<0.05$ vs SCS. (**C**) Comparison of mouse lung tissue morphology under high-power microscopy. (●: Alveolar fusion, disruption/disappearance of alveolar septa, abnormal dilation of alveolar spaces, and formation of pulmonary bullae. ↑: Residual fragments of lung parenchyma in alveolar ducts. ↑: Thinning, discontinuity, or disruption of alveolar walls. Staining: Hematoxylin-eosin staining; grayscale image; magnification: ×400). (**D**). Chronic exposure to cigarette smoke combined with lipopolysaccharide (LPS) significantly increases the alveolar destruction index (ADI) in experimental mice. Compared with mice in the CS + LPS group, pentoxifylline (PTX), theophylline (THEO), and budesonide (BUD) all reduce the alveolar destruction index. $^{\#}p<0.05$ vs SCS; $^{*}p<0.05$ vs CS+LPS.

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Table 1 Morphological and Cellular Indices in BALF (mean ± SEM)

Group (n=10)	Lm (um)	ADI (%)	Total Cells (10^5/mL)	Neutrophils (10^5/mL)	Macrophages (10^5/mL)	Lymphocytes (10^5/mL)	Eosinophils (10^5/mL)
SCS	24.14±2.93	6.29±0.20	28.20±3.50	2.25±0.28	23.68±2.94	1.18±0.15	0.26±0.03
CS+LPS	45.58±0.50$^{\#}$	51.90±1.90$^{\#}$	156.20±18.88$^{@}$	12.45±1.51$^{@}$	131.1±15.86$^{@}$	6.53±0.15$^{@}$	1.42±0.17$^{@}$
CS+LPS+PTX5	43.40±1.31	34.81±1.05*	77.25±4.47$^{\$}$	6.61±0.36$^{\$}$	64.87±3.76$^{\$}$	3.23±0.19$^{\$}$	0.70±0.04$^{\$}$
CS+LPS+PTX4	43.16±1.51	34.78±1.00*	79.79±3.17$^{\$}$	6.36±0.25$^{\$}$	67.01±2.66$^{\$}$	3.34±0.13$^{\$}$	0.73±0.03$^{\$}$
CS+LPS+PTX3	43.04±1.42	34.43±0.81*	68.08±9.89$^{\$}$	5.43±0.79$^{\$}$	57.18±8.31$^{\$}$	2.85±0.41$^{\$}$	0.62±0.09$^{\$}$
CS+LPS+PTX2	44.05±1.27	35.08±0.30*	73.08±3.66$^{\$}$	5.83±0.29$^{\$}$	61.37±3.07$^{\$}$	3.06±0.15$^{\$}$	0.66±0.03$^{\$}$
CS+LPS+PTX1	44.29±1.22	35.61±0.63*	79.71±7.52$^{\$}$	6.36±0.60$^{\$}$	66.94±6.31$^{\$}$	3.33±0.31$^{\$}$	0.72±0.07$^{\$}$
CS+LPS+THEO	44.32±1.20	35.12±1.11*	76.16±4.64$^{\$}$	6.07±0.37$^{\$}$	63.95±3.90$^{\$}$	3.19±0.19$^{\$}$	0.69±0.04$^{\$}$
CS+LPS+BUD	45.23±1.17	36.55±0.79*	65.72±4.46$^{\$}$	5.24±0.28$^{\$}$	55.19±2.00$^{\$}$	2.75±0.14$^{\$}$	0.60±0.03$^{\$}$

Notes: $^{\#}$Compared to the counterpart variable in SCS exposure control group, $p < 0.05$. *Compared to the counterpart variable in CS+LPS exposure control group, $p < 0.05$. $^{@}$Compared to the counterpart variable in SCS exposure control group, $p < 0.05$. $^{\$}$Compared to the counterpart variable in CS+LPS exposure control group, $p < 0.05$. **Abbreviations**: SCS, shame cigarette smoke; CS, cigarette smoke; LPS, Lipopolysaccharide; PTX, pentoxifylline; THEO, theophylline; BUD, Budesonide; n = 10 for each group; Lm, mean linear intercept; ADI, Alveolar destruction index.

dose showed the most prominent trend in reducing MMP-12, though no significant differences were observed between the PTX dose groups ($p > 0.05$).

In contrast to MMP-12, the level of HDAC2 in lung tissue was significantly lower in the CS+LPS group than in the SCS group ($p < 0.05$) (Figure 3B and Table 2). All intervention groups except the BUD group significantly restored HDAC2 levels in lung tissue ($p < 0.05$) (Figure 3B). Among these, the 22.0 mg/mL PTX dose showed the greatest numerical increase in HDAC2, but no significant differences were found between the PTX dose groups or between PTX and THEO ($p > 0.05$).

Cell Classification and Counting in BALF

After 10 weeks of CS+LPS exposure, the total cell count in BALF was significantly higher in the CS+LPS group than in the SCS group ($p < 0.05$). This increase was accompanied by a significant elevation in the counts of differential inflammatory cells, including neutrophils, macrophages, lymphocytes, and eosinophils (Figure 4 and Table 1).

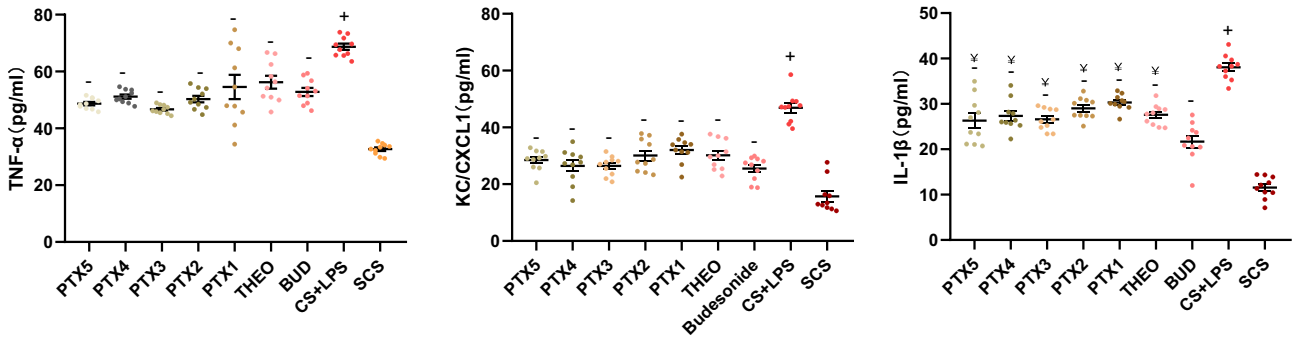

Figure 2 Chronic exposure to cigarette smoke combined with lipopolysaccharide (LPS) results in a significant increase in the levels of proinflammatory cytokines TNF-α, KC/CXCL1, and IL-1β in bronchoalveolar lavage fluid (BALF). Both pentoxifylline (PTX) and budesonide (BUD) at different concentrations can down-regulate the expression of TNF-α; among them, the intervention group with PTX at a concentration of 22.0 mg/mL shows the greatest numerical reduction in TNF-α expression, while there is no statistically significant difference between other intervention groups. Theophylline (THEO) can decrease the expression of TNF-α in BALF, but the difference is not statistically significant. PTX, THEO, and BUD all reduce the expressions of KC/CXCL1 and IL-1β in BALF, with BUD exerting the most significant effect in lowering IL-1β expression in BALF. $^{+}p<0.05$ vs SCS; $^{-}p<0.05$ vs CS+LPS; $^{¥}p<0.05$ vs BUD.

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Table 2 Inflammatory Mediators and Molecular Markers in Lung Tissue (mean ± SEM)

Group (n=10)	TNF-α (pg/mL)	KC/CXCL1 (pg/mL)	IL-1β (pg/mL)	MMP-12 (ng/mL)	HDAC2 (U/mL)
SCS	32.67±0.90	15.72±1.84	11.58±0.76	31.96±1.84	20.44±0.60
CS+LPS	68.70±1.75^{+}	45.66±1.72^{+}	36.81±1.02^{+}	103.57±1.87$^{!}$	8.86±0.29$^{!}$
CS+LPS+PTX5	48.71±1.12^{-}	28.74±1.42^{-}	27.63±1.72$^{-¥}$	66.47±0.96$^{-\%}$	14.50±0.28$^{-\wedge}$
CS+LPS+PTX4	51.19±1.33^{-}	27.54±2.36^{-}	27.98±1.30$^{-¥}$	68.61±2.66$^{-\%}$	15.68±1.00$^{-\wedge}$
CS+LPS+PTX3	46.68±0.87^{-}	26.15±1.53^{-}	26.61±0.99$^{-¥}$	62.75±2.76$^{-\%}$	16.60±1.16$^{-\wedge}$
CS+LPS+PTX2	50.31±2.10^{-}	29.89±2.54^{-}	28.60±0.95$^{-¥}$	64.71±1.36$^{-\%}$	16.06±0.34$^{-\wedge}$
CS+LPS+PTX1	54.58±7.76^{-}	32.17±2.17^{-}	30.48±0.94$^{-¥}$	72.15±1.31$^{-\%}$	14.07±0.27$^{-\wedge}$
CS+LPS+THEO	56.24±3.57^{-}	29.90±1.73^{-}	27.42±0.90$^{-¥}$	84.74±2.38^{-}	16.22±0.24$^{-\wedge}$
CS+LPS+BUD	53.54±2.21^{-}	24.78±1.54^{-}	21.67±1.37^{-}	70.22±1.06$^{-\&}$	10.54±0.18

Notes: $^{+}$Compared to the counterpart variable in SCS exposure control group, $p < 0.05$. $^{-}$Compared to the counterpart variable in CS+LPS exposure control group, $p < 0.05$. $^{¥}$Compared to the counterpart variable in BUD intervention group, $p < 0.05$. $^{!}$Compared to the counterpart variable in SCS exposure control group, $p < 0.05$. $^{-}$Compared to the counterpart variable in CS+LPS exposure control group, $p < 0.05$. $^{\%}$Compared to the counterpart variable in THEO and PTX intervention group, $p < 0.05$. $^{\&}$Compared to the counterpart variable in THEO and BUD intervention group, $p < 0.05$. $^{\wedge}$Compared to the counterpart variable in BUD intervention group, $p < 0.05$.

Abbreviations: SCS, shame cigarette smoke; CS, cigarette smoke; LPS, Lipopolysaccharide; PTX, pentoxifylline; THEO, theophylline; BUD, Budesonide; n = 10 for each group; TNF-α, Tumor necrosis factor alpha; KC/CXCL1, Interleukin-8; IL-1β, Interleukin-1β; MMP-12, Matrix metallopeptidase 12; HDAC2, Histone Deacetylase-2.

Treatment with PTX (all doses), THEO, or BUD significantly reduced both the total BALF cell count and the counts of individual inflammatory cell subsets ($p < 0.05$) (Figure 5). Numerically, BUD showed the strongest trend toward reducing total and differential cell counts, while the 22.0 mg/mL PTX dose exhibited the most prominent effect among the PTX groups. However, no significant differences in cell counts were observed between the treatment groups ($p > 0.05$) (Figure 5).

Discussion

The progression of COPD and the severity of its clinical manifestations are tightly linked to chronic pulmonary inflammation: AECOPD represents an amplification of this inflammatory process, characterized by heightened systemic

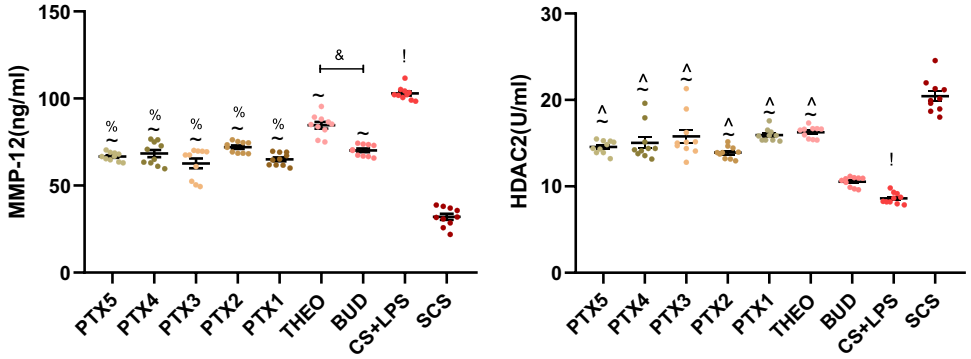

Figure 3 Chronic exposure to cigarette smoke combined with lipopolysaccharide (LPS) leads to a significant increase in the concentration of matrix metalloproteinase-12 (MMP-12) in lung tissues. Theophylline (THEO), budesonide (BUD), and pentoxifylline (PTX) at different concentrations can all down-regulate the expression of MMP-12. Among them, the CS+LPS+PTX3 group (22.0 mg/mL) shows the greatest numerical reduction in MMP-12 expression; however, there is no statistically significant difference between this group and other intervention groups, nor between this group and the CS+LPS+BUD group. The level of histone deacetylase 2 (HDAC2) in mouse lung tissues decreases significantly after chronic exposure to cigarette smoke combined with LPS. Budesonide (BUD) fails to significantly restore HDAC2 level, while theophylline (THEO) and pentoxifylline (PTX) at different concentrations can all enhance HDAC2 level. Specifically, PTX at a concentration of 22.0 mg/mL results in a more pronounced trend in enhancement of HDAC2 level, but no statistically significant differences are observed between this group and other intervention groups, or between this group and the CS+LPS+THEO group. $^{!}p<0.05$ vs SCS; $^{-}p<0.05$ vs CS+LPS; $^{\%}p<0.05$ VS THEO; $^{\wedge}p<0.05$ vs BUD; $^{\&}p<0.05$.

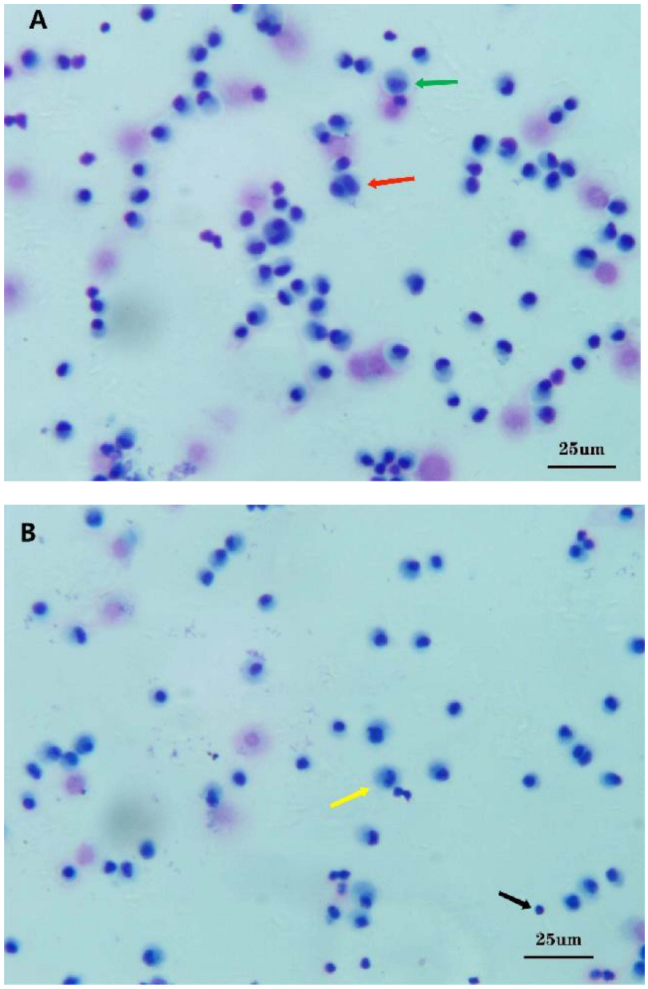

Figure 4 Cell classification staining of bronchoalveolar lavage fluid (BALF). (**A**) contain eosinophils and macrophages; (**B**) contain neutrophils and lymphocytes. (↑: Eosinophils; ↑: Macrophages; ↑: Neutrophils; ↑: Lymphocytes. Staining: Wright-Giemsa stain; Magnification: ×400.).

inflammation that accelerates disease progression, increases complication rates, and significantly elevates mortality risk.[27,28] Thus, effective anti-inflammatory and anti-infective strategies are critical for modifying the COPD disease course, reducing adverse health outcomes, and lowering mortality.

Inflammatory Mechanisms in COPD and Relevance to the Study Model

It is well established that pulmonary inflammation and oxidative stress are central to COPD development, progression, and symptom severity. A hallmark of COPD is the persistent inflammatory response driven by increased numbers of inflammatory cells—even during the stable phase of the disease, not just during acute exacerbations.[28,29] Approximately 82% of AECOPD episodes are triggered by lower respiratory tract infections,[30] which exacerbate pulmonary inflammatory cell recruitment and oxidative stress, leading to the production and release of pro-inflammatory mediators such as reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), keratinocyte chemoattractant/C-X-C motif chemokine ligand 1 (KC/CXCL1), interleukin-1 (IL-1), and matrix metalloproteinases (MMPs).[31–33]

Among these mediators, TNF-α acts as a key inflammatory regulator: it cooperates with chemokines to initiate inflammatory cascades, amplify the production of inflammatory mediators, and promote the chemotaxis of inflammatory cells into the lungs. MMP-12, in particular, sustains and amplifies inflammatory responses, further exacerbating lung tissue damage.[31,34] These mechanisms align with the pathological features of our CS+LPS-induced mouse model, where

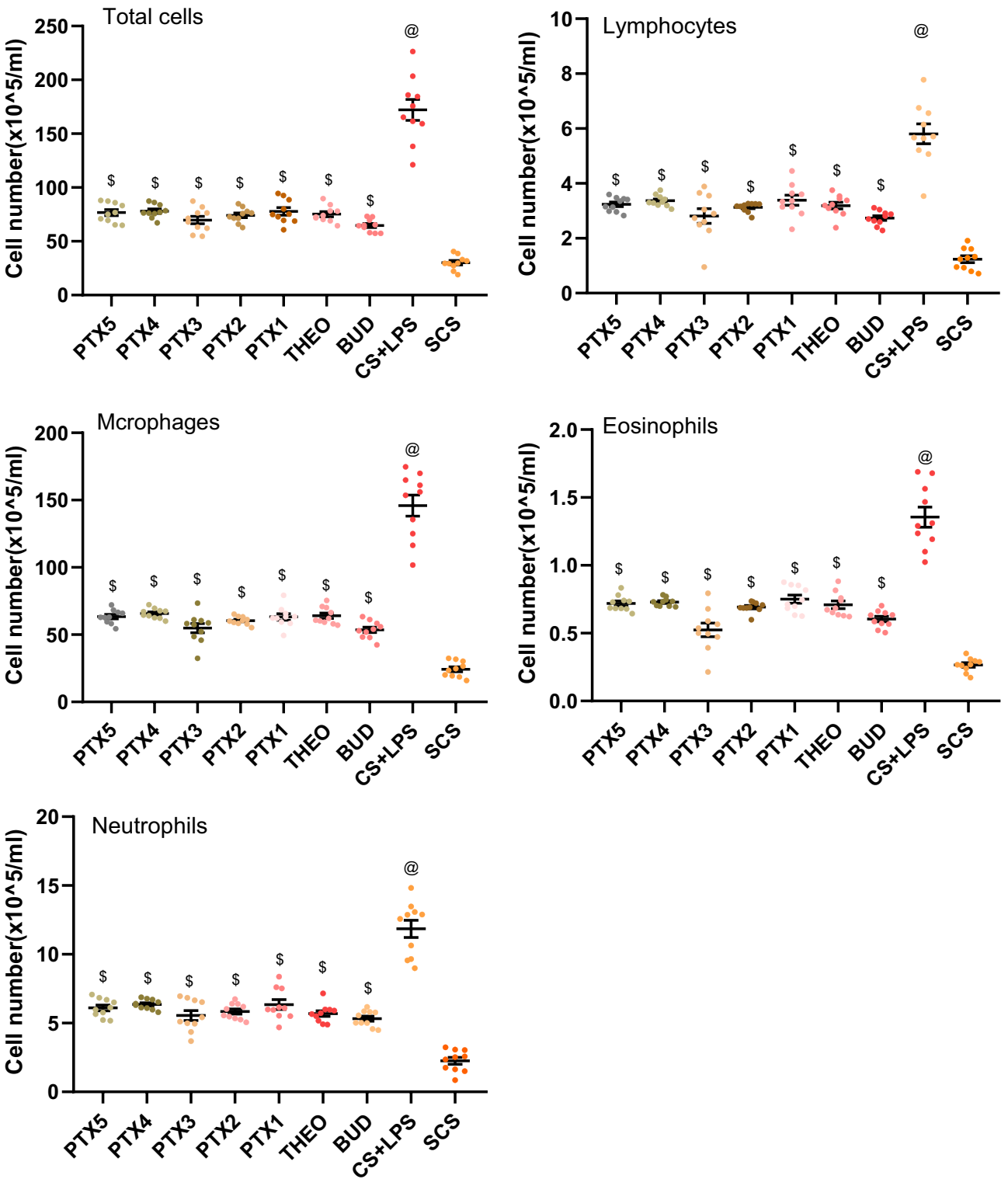

Figure 5 Chronic exposure to cigarette smoke combined with lipopolysaccharide (LPS) leads to a significant increase in the total number of cells and the counts of various inflammatory cells (neutrophils, macrophages, lymphocytes, and eosinophils) in the bronchoalveolar lavage fluid (BALF) of mice. Pentoxifylline (PTX), theophylline (THEO), and budesonide (BUD) all reduce the total cell number and the counts of each type of inflammatory cell in BALF. Among them, BUD shows a relatively more significant effect in reducing inflammatory cell counts, though the difference is not statistically significant. In the PTX intervention groups, the CS+LPS+PTX3 group (22.0 mg/mL) exhibits the greatest numerical reduction in inflammatory cell counts; however, there is no statistically significant difference within the groups. $^{@}p<0.05$ vs SCS; $^{\$}p<0.05$ vs CS+LPS.

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we observed elevated levels of TNF-α, KC/CXCL1, IL-1β, and MMP-12—validating the model's relevance to human COPD.

Rationale for "Late-Stage Intervention" Design

Many preclinical studies of emphysema initiate intervention at the onset of risk factor exposure (eg, cigarette smoke) —before the establishment of the emphysema model. This "preventive" approach differs fundamentally from the clinical scenario, where COPD treatment is initiated after diagnosis. Consequently, such studies have limited translational value for guiding therapies in patients with established COPD. In contrast, "late-stage intervention" —administering treatment after disease model establishment—more accurately recapitulates the natural history and therapeutic context of human diseases, providing clinically relevant insights.[35]

Consistent with this rationale, Zheng et al[23] administered therapeutic agents to mice after smoke-induced emphysema had developed, demonstrating that late-stage intervention significantly alleviated pulmonary inflammation and oxidative stress, improved overall mouse health, and yielded insights applicable to clinical COPD management. In the present study, we adopted the same late-stage intervention design: we first established the CS+LPS-induced emphysema model, then administered 2 weeks of drug treatment via inhalation (pentoxifylline [PTX], theophylline [THEO], or budesonide [BUD]) while maintaining CS+LPS exposure. This design allowed us to evaluate the therapeutic potential of the tested agents—rather than their preventive effects—enhancing the study's translational relevance.

Mechanisms and Efficacy of Tested Agents

Theophylline: Anti-Inflammatory Effects via HDAC2 Restoration

Theophylline, a classic non-selective phosphodiesterase inhibitor (PDEI), is widely used clinically for acute asthma exacerbations. Notably, at low plasma concentrations, theophylline exerts anti-inflammatory effects independent of its bronchodilatory actions (eg, PDE inhibition or adenosine receptor antagonism);[36] these effects are thought to be mediated by modulation of cyclic adenosine monophosphate (cAMP) levels, which regulate inflammatory signaling.

In our study, nebulized THEO (25 mg/mL) reduced BALF levels of KC/CXCL1, TNF-α, and IL-1β, and decreased total BALF cell counts—consistent with findings from Masato et al,[37] who showed that repeated nebulization of aminophylline (25 mg/mL) inhibited eosinophil infiltration and reduced BALF total cell counts in ovalbumin-sensitized guinea pigs. Importantly, we also observed that nebulized THEO significantly restored lung histone deacetylase 2 (HDAC2) levels. This aligns with Zheng et al,[23] who reported that intraperitoneal theophylline partially reversed smoke-induced HDAC2 downregulation, reduced ROS production, and linked HDAC2 restoration to alleviated oxidative stress. Collectively, these findings suggest that nebulized THEO exerts anti-inflammatory and antioxidant effects by restoring HDAC2— a key mechanism for overcoming steroid resistance in COPD.

Pentoxifylline: Broad Anti-Inflammatory and HDAC2-Modulating Effects

Like theophylline, PTX is a non-selective PDEI that potently inhibits PDE3, PDE4, and PDE7.[38] Preclinical studies have shown that PTX specifically antagonizes TNF-α (dose-dependently inhibiting its production in alveolar macrophages), suppresses cytokine-induced neutrophil chemoattractants, nuclear factor-κB (NF-κB), gelatinase B, and peroxidase, and downregulates pro-inflammatory cytokines (eg, IL-6, IL-1, IL-4).[39–41] While we did not directly measure oxidative stress markers (eg, malondialdehyde [MDA], 8-isoprostane) or cAMP levels, our observations—reduced pro-inflammatory cytokines and restored HDAC2—are consistent with PTX's established pharmacological profile as an anti-inflammatory and antioxidant agent.[22,42]

In our study, nebulized PTX (all tested doses) significantly reduced BALF levels of TNF-α, KC/CXCL1, and IL-1β, enhanced lung HDAC2 levels, and decreased lung MMP-12 expression—findings that align with Zheng et al,[23] despite the use of nebulization (a more clinically relevant route for respiratory diseases) in our study. To identify a potential optimal dose, we tested five PTX concentrations (9.8, 14.7, 22.0, 33.0, and 49.5 mg/mL). While no linear dose-response relationship was observed, the 22.0 mg/mL dose consistently showed the most pronounced therapeutic trend: it reduced BALF inflammatory cytokines, decreased lung MMP-12, and upregulated HDAC2 more effectively than other

concentrations. Although these differences were not statistically significant, they highlight 22.0 mg/mL as a candidate for further optimization in future preclinical studies.

Budesonide: Anti-Inflammatory Efficacy without HDAC2 Restoration

Budesonide (BUD), a corticosteroid widely used clinically for anti-inflammatory treatment of AECOPD, asthma, and acute respiratory distress syndrome (ARDS), significantly reduced BALF levels of TNF-α, KC/CXCL1, and IL-1β, and decreased lung MMP-12 expression in our model. However, critically, BUD did not restore lung HDAC2 levels—consistent with its known mechanism of action: corticosteroids mediate anti-inflammatory effects via the glucocorticoid receptor, which requires functional HDAC2 to suppress pro-inflammatory gene transcription.[13] In COPD, reduced HDAC2 levels contribute to steroid resistance,[14] explaining why BUD is less effective in patients with stable COPD (where HDAC2 is downregulated).

Notably, PTX and THEO both restored HDAC2 levels, while BUD did not. This raises the hypothesis that combining BUD with PTX or THEO could synergistically enhance anti-inflammatory efficacy: PTX/THEO-mediated HDAC2 restoration may reverse steroid resistance, allowing BUD to exert more potent effects. However, this hypothesis requires validation in future studies evaluating combination therapies.

Key Observations: Morphological Improvements vs Emphysema Reversal

It is critical to clarify that the "morphological improvements" observed in our study primarily reflect reduced inflammatory cell infiltration—not reversal of established emphysematous destruction. This is evidenced by the lack of significant change in the mean linear intercept (Lm)—a key metric of alveolar airspace enlargement—across all treatment groups. This finding aligns with the irreversible nature of emphysema in human COPD, where current therapies aim to alleviate inflammation and slow progression rather than reverse existing structural damage. Our results thus confirm that PTX, THEO, and BUD target the inflammatory component of COPD but do not reverse established emphysematous changes—consistent with clinical expectations.

Study Limitations

Despite the translational value of our findings, several limitations should be acknowledged:

1. Sample Size and Statistical Power: While a sample size of n=10 per group is standard in preclinical studies,[23,] we did not perform a formal a priori power analysis. This may have limited our ability to detect subtle intergroup differences—particularly among PTX dose groups.
2. Short Intervention Duration: The 2-week treatment period was insufficient to assess the sustainability of PTX's effects or its ability to halt long-term disease progression. A longer intervention (eg, 4–8 weeks) would be needed to evaluate whether PTX can slow Lm increase (a marker of emphysema progression).
3. Model Limitations: The CS+LPS model effectively induces emphysema-like pathology but does not fully recapitulate the slow, multifactorial progression of human COPD (eg, it lacks comorbidities, long-term smoke exposure, and genetic variability).
4. Sex-Specific Bias: This study was conducted exclusively in female mice to control for hormonal variability. We acknowledge that this choice limits the generalizability of our findings, as sex is a known biological variable that can modulate inflammatory responses to cigarette smoke.[43–45] For instance, distinct sex-specific transcriptional signatures in airway epithelial cells after smoke exposure have been reported.[43] Therefore, the efficacy of pentoxifylline observed here may be sex-dependent. Future studies including both male and female animals are necessary to confirm the broader applicability of our results.
5. Exposure and Dose Monitoring: Technical constraints prevented real-time monitoring of particulate matter/carbon monoxide in exposure chambers and precise measurement of lung-deposited drug doses. We report nominal nebulizer cup concentrations, which enable valid intergroup comparisons but limit precise dose-response analysis and clinical extrapolation.

6. HDAC2 Functional Assessment: We measured HDAC2 protein levels via ELISA but did not assess its enzymatic activity. While increased protein levels suggest functional restoration, future studies should confirm this using enzymatic assays and complementary techniques (eg, Western blotting).
7. Lack of Combination and Comparator Groups: We only evaluated monotherapies; the absence of combination groups (eg, PTX+BUD) precludes assessment of synergistic effects. Additionally, we did not include a comparator with a clinically relevant PDE4 inhibitor (eg, roflumilast), limiting direct benchmarking of PTX's efficacy.
8. Mechanistic Gaps: Our in vivo findings do not include complementary in vitro experiments to elucidate the precise molecular mechanisms underlying PTX's effects (eg, its impact on NF-κB or AP-1 signaling, or direct targets like macrophages/epithelial cells). Future cell-based studies (eg, using cigarette smoke extract [CSE]- or LPS-stimulated cells) are needed to confirm these mechanisms and clarify how PTX upregulates HDAC2.

Conclusion

In summary, this study demonstrates that nebulized pentoxifylline attenuates pulmonary inflammation and upregulates lung HDAC2 levels in a murine model of CS+LPS-induced emphysema—supporting our initial hypothesis and validating PTX's potential as a novel therapeutic agent for targeting chronic inflammation and steroid resistance in COPD. Theophylline also partially restored HDAC2 levels, while budesonide (a clinical standard) exerted anti-inflammatory effects but did not modulate HDAC2. Among the tested PTX concentrations, 22.0 mg/mL showed the most promising therapeutic trend, though intergroup differences were not statistically significant.

Despite limitations (eg, small sample size, short intervention duration), our findings provide a strong rationale for future investigations: larger preclinical studies with longer treatment periods, combination therapies (eg, PTX+BUD), direct comparisons with standard COPD medications (eg, roflumilast), and inclusion of both sexes are warranted to further validate PTX's efficacy and optimize its clinical application. Ultimately, these efforts could lead to improved therapeutic options for patients with COPD—particularly those with steroid resistance.

Abbreviations

ADI, Alveolar Destruction Index; Lm, Mean Linear Intercept; COPD, Chronic Obstructive Pulmonary Disease; CS, Cigarette Smoke; SCS, Sham Cigarette Smoke; LPS, Lipopolysaccharide; PTX, Pentoxifylline; BUD, Budesonide; THEO, Theophylline; PBS, Phosphate-Buffered Saline; BALF, Bronchoalveolar Lavage Fluid; ELISA, Enzyme-Linked Immunosorbent Assay; IL-1β, Interleukin-1 Beta; KC/CXCL1, Keratinocyte Chemoattractant/CX-C Motif Chemokine Ligand 1; TNF-α, Tumor Necrosis Factor-Alpha; HDAC2, Histone Deacetylase 2; MMP-12, Matrix Metalloproteinase-12.

Data Sharing Statement

The datasets used and analyzed during the current study are not publicly available due to contractual agreements with the study funder (CSPC Pharmaceutical Group Limited) concerning intellectual property. However, de-identified data are available from the corresponding author (Jinnong Zhang, zjnwhhb@163.com) on reasonable request, subject to approval by the Data Access Committee of Huazhong University of Science and Technology and CSPC Pharmaceutical Group Limited.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (Ethics Approval Number: [2021] IACUC No. 3269). The study strictly complied with the ARRIVE Guidelines 2.0 and relevant Chinese regulations for the ethical use of laboratory animals. All procedures were designed to minimize animal suffering, including the use of appropriate anesthesia, analgesia, and humane endpoints. The C57BL/6 mice were commercially obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). As animals were sourced from a professional laboratory animal provider and not privately owned, informed consent from owners was not required. Consent to participate is not applicable as this study utilized animal subjects.

Consent for Publication

This manuscript does not contain any individual person's data. All experimental data derived from animal studies are published with the approval of the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (Approval ID: [2021] IACUC No. 3269).

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Author Contributions

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

This study received financial support and in-kind supply of pentoxifylline from CSPC Pharmaceutical Group Limited (Shijiazhuang, China). The manufacturer did not participate in the design of the study, collection/analysis/interpretation of data, drafting of the manuscript, or the decision to submit the manuscript for publication. All authors declare that they have no other personal, financial, or professional interests that could inappropriately influence the results or interpretation of this work. A copy of the funding agreement and drug supply certificate is available upon request from the corresponding author.

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