

Efficacy of salmeterol and formoterol combination treatment in mice with chronic obstructive pulmonary disease

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Abstract. Chronic obstructive pulmonary disease (COPD) is a severe lung disease characterized by progressive airflow limitation. Salmeterol and formoterol are two commonly used drugs in COPD therapy, which act as β_2 -receptor agonists. In the current study, a mouse model of COPD induced by airway lipopolysaccharide inhalation was established. The therapeutic efficacy of salmeterol and formoterol co-treatment was investigated in this model over a 56-day-long observation period. It was also identified that functional residual capacity and inspiratory resistance were significantly improved after salmeterol and/or formoterol treatment compared with the control group (all $P < 0.01$). Furthermore, histological staining of lung tissue samples indicated that inflammation, thickening of the smooth muscle, goblet cell hyperplasia and pulmonary small vessel obstruction were reduced in the mice treated with salmeterol and/or formoterol, suggesting that salmeterol and formoterol were beneficial for ongoing airway and blood vessel remodeling in mice with COPD. The most common treatment-associated adverse events were hypertension and proteinuria. In conclusion, combined salmeterol and formoterol treatment was more effective compared with either single agent, suggesting that salmeterol and formoterol combined treatment has therapeutic value for the clinical treatment of patients with COPD.

Introduction

Chronic obstructive pulmonary disease (COPD), also known as obstructive emphysema, is caused by the narrowing of airways and lung damage (1,2). In recent years, the incidence of COPD has increased markedly due to air pollution, smoking and chronic bronchitis (3). The symptoms of COPD cause a large global burden of disease (1,4). COPD also has a notable impact on the quality of life of patients, frequently leading to the

impairment of activity, an increased risk of hospitalization and increased numerous symptoms including chronic bronchitis and emphysema (5,6). In addition, a previous study reported that ~80% of patients with COPD still exhibit deterioration of pulmonary function in a 3-year clinical investigation (7). Although the patients received different treatments, normal pulmonary function deteriorated heterogeneously (7).

Salmeterol and formoterol are two commonly used drugs in COPD therapy, which act as β_2 -receptor agonists (8). Salmeterol and formoterol were identified as β_2 -receptor agonists that inhibit early and late phase antigen-induced airway hyper-responsiveness (9,10). Previous reports indicate that the combination of inhaled salmeterol and formoterol is a commonly used treatment protocol for patients with COPD (11,12). In addition, Frampton (13) have reported that the combined use of inhaled formoterol and the corticosteroid mometasone significantly improved the lung function in patients with asthma. Furthermore, Yip *et al* (14) evaluated the effect of changing from fluticasone/salmeterol to mometasone/formoterol treatment in patients with COPD and identified that patients experienced COPD exacerbation after this conversion. However, the underlying mechanism of the effect of combined salmeterol and formoterol treatment in COPD is not well understood, and few reports have evaluated its efficacy in preclinical and clinical trials.

In the current study, the therapeutic efficacy of salmeterol and formoterol was evaluated in mice with COPD induced by airway lipopolysaccharide (LPS) inhalation. The therapeutic efficacy and side effects of this treatment were evaluated in a 56-day-long observation. Although salmeterol/formoterol is not currently approved for COPD therapy by the US Food and Drug Administration, evidences from clinical trials of mometasone/formoterol and salmeterol/formoterol have indicated that salmeterol/formoterol treatment is well tolerated and could improve lung function in patients with COPD (15,16). Based on previous clinical data (9), a combined salmeterol/formoterol therapeutic approach was designed to treat mice with COPD.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Yucheng City People's Hospital (Dezhou, China; Approval number: YCCPH: 20130567LPX1) and performed in accordance with the recommendations of the

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Guide for the Care and Use of Laboratory Animals of Yucheng City People's Hospital. All surgery and euthanasia procedures were performed under intraperitoneal injection of sodium pentobarbital anesthesia (50 mg/kg), and all efforts were made to minimize suffering.

Cell culture. MRC-5 normal lung fibroblast cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Lonza Group, Inc., Walkersville, MD, USA), 3 mM L-glutamine, 50 µg/ml gentamicin and 1% penicillin/streptomycin. All cells were cultured at 37°C and in 5% CO₂. MRC-5 cell models of COPD were established through 48 h incubation of LPS (2.5 mg/kg, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The MRC-5 cells (MOCK), and LPS-induced MRC-5 cells were treated with PBS (Control), salmeterol (0.16 mg/ml) and/or formoterol (0.32 mg/ml) for 24 h at 37°C for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from MRC-5 lung cells after treatment with salmeterol and/or formoterol using an RNeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed into cDNA using a QuantiTect Reverse Transcription kit (Cat no. 205310; Qiagen Sciences, Inc.) according to the manufacturer's protocol. The cDNA (10 ng) was subjected to qPCR using SYBR Green Master mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction conditions were performed as follows: 95°C for 10 min and 35 cycles of 95°C for 20 sec and 58°C for 1 min. Relative mRNA expression changes were calculated using the 2^{-ΔΔC_q} method (17). The results are expressed as a fold of the control. All primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. and are presented in Table I.

Evaluation of toxicity. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (CTC; version 3.0) (18). Mice received 0.08, 0.16, 0.32 or 0.40 mg salmeterol or formoterol once a day for a total of 10 days (n=5 in each group). Hypertension, diarrhea, proteinuria, vomiting, lethargy, rash, fatigue, constipation, weight, appetite, epistaxis, hypertriglyceridemia and peripheral edema were evaluated and recorded every 2 days during therapy. A dose-limiting toxicity (DLT) was defined as any of the drug-associated toxicities described previously (19).

Animal experiments. A total of 80 male C57BL/6 mice (6-8 weeks old, body weight: 32-35 g) were purchased from the West China Experimental Animal Center of Sichuan University (Sichuan, China). All mice were maintained at 22-25°C and relative humidity of 50±5% in a 12-h light/dark cycle with *ad libitum* access to food and water. Mice were used to establish a COPD model as described previously (20) and divided into five groups (Mock, Mock, salmeterol, formoterol and co-treatment of salmeterol and formoterol; n=16/group). Each mouse in the treatment groups received salmeterol (0.16 mg/kg) and/or formoterol (0.32 mg/kg) at the maximum tolerated dose (MTD) with PBS treatment used as the control.

Experimental mice were administered with drugs once daily for a total of 56 days. The therapeutic effects of salmeterol and/or formoterol on mice with COPD were measured as described previously (21).

Pulmonary function test. The pulmonary function of the mice after treatment with salmeterol and/or formoterol was assessed by recording changes in airway resistance and functional residual capacity using a Buxco Pulmonary Mechanics system (Buxco Electronics, Inc., Wilmington, NC, USA) according to the manufacturer's protocol. The efficacy of salmeterol and/or formoterol on mice with COPD was analyzed by COPD assessment test scores (22). The severity is determined based on score: low, <4, medium, ≥4 and <9 and high, ≥9.

Histopathology. Hypertrophy and hyperplasia of the goblet cells on airway surfaces, inflammatory cell infiltration, alveolar structural changes, and airway smooth muscle and intima cells were examined in the lungs of experimental mice by histopathology. On day 28, lung tissue samples were removed from the central airway, as previously described (23), then sectioned, fixed with 4% paraformaldehyde at 4°C for 12 h. Lung tissues were embedded in paraffin and cut into 4 µm thick sections for hematoxylin and eosin staining at 37°C for 2 h. Lung tissue sections were also stained with masson staining for 2 h at 37°C to analyze the efficacy of salmeterol and/or formoterol, according to previous study (24).

Statistical analysis. All data are presented as the mean ± standard error of the mean. Differences between groups were compared using an unpaired Student's t-test. Comparisons between multiple groups were performed using analysis of the one-way ANOVA variance. Analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). *P<0.05 and **P<0.01 was considered to indicate a statistically significant difference.

Results

Duration of treatment and MTD of salmeterol and formoterol. All mice salmeterol and/or formoterol (n=16/group). The median overall duration of treatment for the mice was 10 days for this analysis. Mice received 0.08, 0.16, 0.32 or 0.40 mg of salmeterol or formoterol. A dose of 0.16 mg salmeterol and 0.32 mg formoterol once daily were identified as the MTD (Table II) as previously described (25). The most common CTC grade ≥3 treatment-associated adverse events at these dosages were proteinuria and hypertension (≥15% each; Table III).

Efficacy of salmeterol and formoterol treatment in vitro. After confirming the MTD, biological markers of COPD were used to evaluate the efficacy of salmeterol and/or formoterol in the LPS-induced MRC-5 cells. Krebs von den Lungen-6 (KL-6), C-C motif chemokine ligand 18 (CCL-18), matrix metalloproteinase-7 (MMP-7) and surfactant protein A (SP-A) mRNA expression levels were analyzed in LPS-induced MRC-5 cells. The results indicated that KL-6, CCL-18 and MMP-7 mRNA expression levels were significantly higher in LPS-induced

Table I. Primer sequences of used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
KL-6	ATTTGTCTGGCTGGAAAGGCAACC	AAGCACTGAAAGACGCTGCTTTGG
CCL-18	AAGACCTTCTGCCACTGGGAACAT	AGTTGCCATCTCCTGTGTGATCCT
MMP-7	TGCCCACCGTCTCTTTCTTGTT	GCTGACTTGACTCATGGCT
SP-A	TCTGACGAATCTCCTCCAC	AAAGTTTTCAATGACCAAGC
TNF- α	TCCAGACTTCCTTGAGACA	GGCGATTACAGACACAACCT
IL-1	GGCTGCTTCCAAACCTTTGA	GAAGACACGGATTCCATGGT
IL-18	ACACCTGTCTCGCAGACCAC	TCAGCTGCTCCAGCACCAA
TGF- β	ATCCATGTGTGACCATGAGGAAATG	TCGGCTAGTTAGGGTACACTTC
IL-4	GTGAGGAACAAGCCAGAG	TGACCAGAAGAAGGAATGC
IFN- γ	CACCTCTGTCTACCTGCTCAA	ATGGCGCTGAGAAGACTTGGT
β -actin	GTGGGCGCCCAGGCACCA	CTCCTTAATGTACGCACGATTT

KL-6, Krebs von den Lungen-6; CCL-18, C-C motif chemokine ligand 18; MMP-7, matrix metalloproteinase-7; SP-A, surfactant protein A; TNF- α , tumor necrosis factor α ; IL, interleukin; TGF- β , transforming growth factor β ; IFN- γ , interferon γ .

Table II. Treatment-related adverse event with an overall incidence $\geq 10\%$.

Adverse event	Number of adverse events									
	Salmeterol, mg (n=16/group)					Formoterol, mg (n=16/group)				
	0.08	0.16	0.32	0.40	Total	0.08	0.16	0.32	0.40	Total
Hypertension	2	3	3	4	12	2	2	4	5	13
Diarrhea	0	1	1	1	3	0	1	1	2	4
Proteinuria	2	3	4	5	14	2	4	4	5	15
Vomiting	1	0	1	2	4	1	1	1	1	4
Lethargy	0	0	1	1	2	0	0	1	2	3
Rash	0	1	1	1	3	1	1	1	2	5
Fatigue	1	1	1	2	5	1	1	2	2	6
Constipation	1	1	2	2	6	1	1	1	2	5
Weight decrease	3	2	3	3	11	1	2	3	4	10
Decreased appetite	1	1	2	3	7	1	1	2	4	8
Epistaxis	2	1	2	3	8	2	1	2	4	9
Hypertriglyceridemia	1	1	1	2	5	0	1	1	2	4
Peripheral edema	1	1	2	3	7	1	1	2	2	6
Total	15	16	24	32	87	13	17	25	37	92

MRC-5 control cells compared with the non-LPS-induced mock group, while salmeterol and/or formoterol significantly inhibited KL-6, CCL-18 and MMP-7 expression compared with the control group (all $P < 0.01$; Fig. 1A-C). In addition, the data demonstrated that combination treatment with salmeterol and formoterol significantly reduced the expression of KL-6, CCL-18 MMP-7 compared with either single treatment ($P < 0.01$; Fig. 1A-C). Furthermore, the results indicated that SP-A expression was significantly lower in LPS-induced MRC-5 control cells compared with the non-induced mock group, while salmeterol and/or formoterol significantly increased SP-A expression (all $P < 0.01$; Fig. 1D). Salmeterol and formoterol combination treatment significantly increased

the expression of SP-A compared with either single treatment ($P < 0.01$; Fig. 1D). These data indicate that salmeterol, formoterol and, particularly, combined salmeterol/formoterol treatment efficiently reduces the symptoms of COPD.

Efficacy of salmeterol and formoterol in vivo. The therapeutic effects of salmeterol and/or formoterol treatment on mice with COPD were evaluated in the present study. The results indicated that the body weight of the control mice decreased over the study period, while treatment with salmeterol, formoterol or a combination of the two significantly inhibited this weight loss (all $P < 0.01$; Fig. 2A). In addition, COPD assessment test scores were markedly improved by

Table III. Treatment-related hypertension and proteinuria by Common Toxicity Criteria grade.

Adverse event	Number of adverse events		Total
	Salmeterol 0.16 mg (n=14)	Formoterol 0.32 mg (n=14)	
Hypertension	3	3	6
Grade 1	1	1	2
Grade 2	2	1	3
Grade 3	0	1	1
Proteinuria	5	6	11
Grade 1	1	1	2
Grade 2	2	3	5
Grade 3	2	2	4

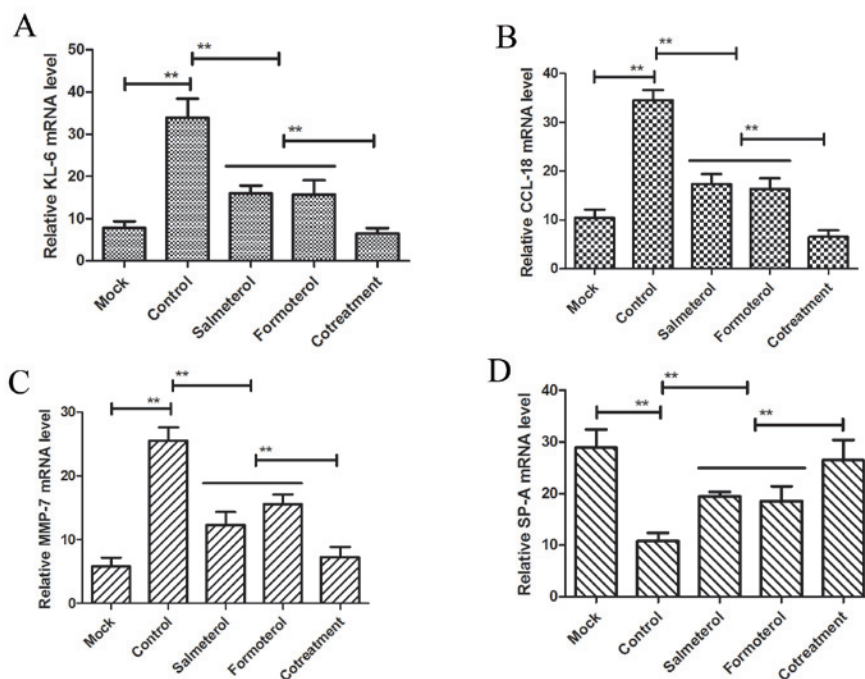


Figure 1. Expression of biological markers of chronic obstructive pulmonary disease in experimental mice. (A) KL-6, (B) CCL-18, (C) MMP-7 and (D) SP-A mRNA expression levels were analyzed in mice treated with salmeterol and/or formoterol. **P<0.01 vs. the control group. KL-6, Krebs von den Lungen-6; CCL-18, C-C motif chemokine ligand 18; MMP-7, matrix metalloproteinase-7; SP-A, surfactant protein A.

salmeterol and/or formoterol in mice with COPD (Fig. 2B). Furthermore, the functional residual capacity and inspiratory resistance data were evaluated. As presented in Fig. 2C, the functional residual capacity was 0.382 ± 0.052 , 0.410 ± 0.038 and 0.442 ± 0.058 ml in the salmeterol, formoterol and combined treatment groups, respectively, which was significantly higher compared with that of the control group (0.210 ± 0.022 ml; $P < 0.01$). As illustrated in Fig. 2D, the inspiratory resistance was 0.728 ± 0.032 , 0.744 ± 0.042 and 0.652 ± 0.058 cm H₂O/ml/sec in the salmeterol, formoterol and combination treatment groups, respectively, which was significantly lower compared with that of the control group (1.078 ± 0.062 cm H₂O/ml/sec; $P < 0.01$). These data suggest that salmeterol, formoterol and combined treatment have therapeutic effects in mice with COPD.

Levels of inflammatory factors in mice with COPD. COPD is associated with the increased production of inflammatory factors and is characterized by a progressive worsening of airflow (26). Therefore, in the current study, the mRNA levels of the inflammatory factors interleukin (IL)-1, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , transforming growth factor (TGF)- β , IL-18 and IL-4 were evaluated in the lung cells of mice with COPD after salmeterol and/or formoterol treatment using RT-qPCR. As illustrated in Fig. 3A-C, the results demonstrated that IFN- γ , IL-18 and IL-4 expression levels were significantly downregulated after salmeterol and/or formoterol treatment compared with the control group (all $P < 0.01$). In addition, co-treatment with salmeterol and formoterol significantly reduced IFN- γ , IL-18 and IL-4 expression compared with single salmeterol or formoterol

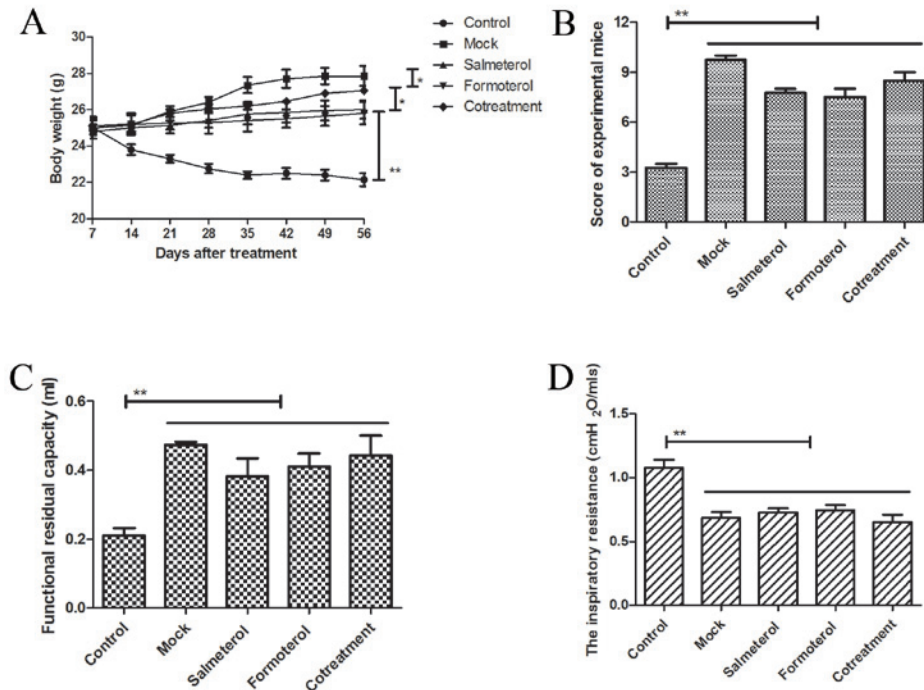


Figure 2. Therapeutic effects of salmeterol and/or formoterol on mice with COPD. (A) Body weight of mice with COPD during treatment. (B) COPD assessment test scores of experimental mice treated with salmeterol and/or formoterol. (C) Functional residual capacity in mice with COPD after salmeterol and/or formoterol treatment. (D) Inspiratory resistance in mice with COPD after salmeterol and/or formoterol treatment. **P<0.01 vs. the control group. COPD, chronic obstructive pulmonary disease.

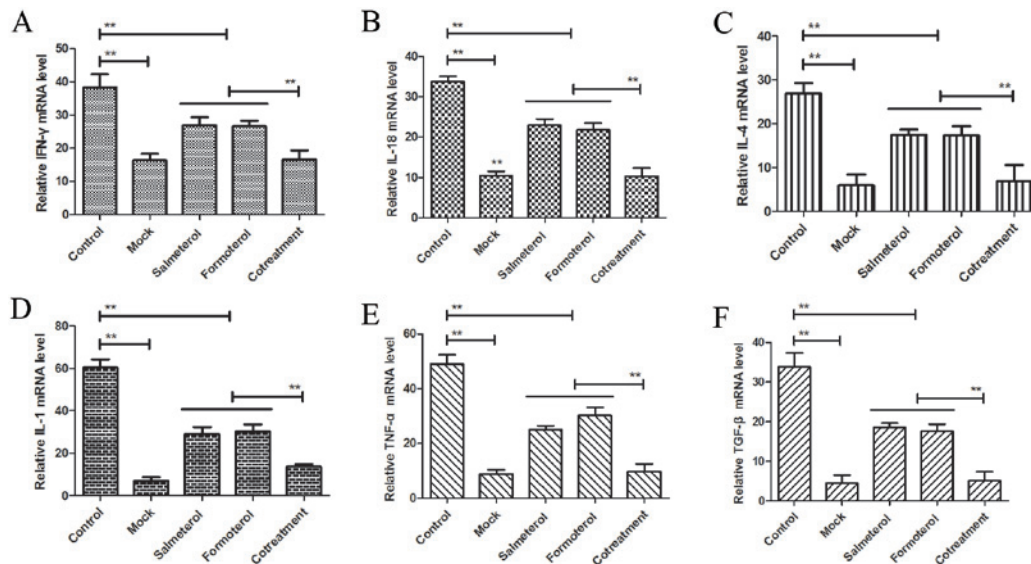


Figure 3. Expression of inflammatory factors in mice with COPD after salmeterol and/or formoterol treatment. (A) IFN- γ , (B) IL-18, (C) IL-4, (D) IL-1, (E) TNF- α and (F) TGF- β mRNA expression in the lung cells of mice with COPD treated with salmeterol and/or formoterol. **P<0.01 vs. the control group. COPD, chronic obstructive pulmonary disease; IFN- γ , interferon γ ; IL, interleukin; TNF- α , tumor necrosis factor α ; TGF- β , transforming growth factor β .

treatment (all P<0.01; Fig. 3A-C). As presented in Fig. 3D-F, IL-1, TNF- α and TGF- β expression levels were significantly downregulated after salmeterol and/or formoterol treatment compared with the control group (all P<0.01). However, co-treatment with salmeterol and formoterol significantly reduced IL-1, TNF- α and TGF- β expression compared with either single treatment (all P<0.01; Fig. 3D-F). These results

suggest that inflammatory factors were decreased after salmeterol and/or formoterol treatment.

Pathological analysis of the efficacy of salmeterol and formoterol treatment in mice with COPD. The pathological features and long-term effects of salmeterol and formoterol treatment were evaluated in mice with COPD.

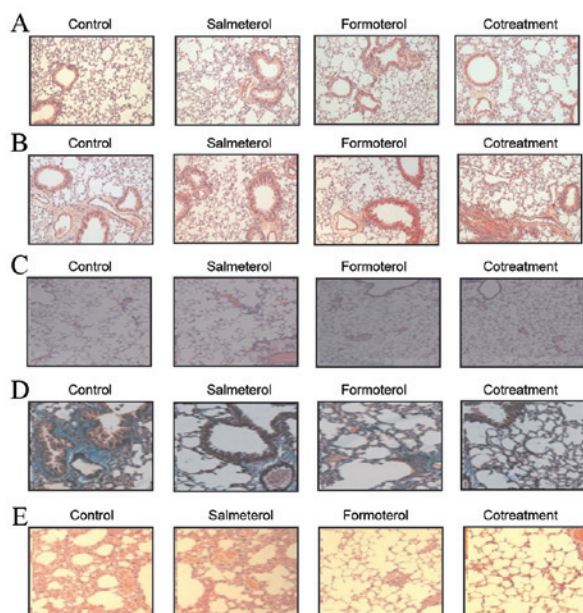


Figure 4. Histopathological assessment of lung tissue samples from mice with chronic obstructive pulmonary disease after salmeterol and/or formoterol treatment. Representative images of (A) hypertrophy and hyperplasia of goblet cells on airway surface, (B) alveolar structure, (C) airway smooth muscle, (D) smooth muscle cells in the small arteries and (E) inflammatory cell infiltration and smooth muscle cells. Magnification, x40.

As demonstrated in Fig. 4A, hypertrophy and hyperplasia of goblet cells on the airway surface was inhibited in salmeterol and/or formoterol-treated mice compared with the control group. Salmeterol and/or formoterol treatment also contributed to repair of alveolar structure (Fig. 4B). In addition, Masson staining indicated that airway smooth muscle was thinned and collagen deposition was decreased in the lungs of salmeterol and/or formoterol-treated mice compared with the control group, suggesting that these treatments aid in airway repair (Fig. 4C). It was also observed that intima cells presented typical morphology and smooth muscle cells were arranged more regularly in the small arteries of salmeterol and/or formoterol-treated mice compared with the control group (Fig. 4D). The present study demonstrated that inflammatory cell infiltration and smooth muscle cell thickening was reduced in salmeterol and/or formoterol-treated mice compared with the control group (Fig. 4E). It was observed that co-treatment markedly improved pathological features of COPD compared with salmeterol, formoterol and control groups. These results indicate that combined salmeterol and formoterol treatment is more efficient for the treatment of COPD.

Discussion

The results of the present study indicate that combined salmeterol and formoterol treatment exhibits therapeutic efficacy for mice with COPD induced by LPS. The present study revealed that beneficial inflammatory factors in the lung cells were increased in mice treated with salmeterol and formoterol. Among these inflammatory factors, IFN- γ , IL-18 and IL-4 expression levels were downregulated and IL-1, TNF- α and TGF- β expression levels were upregulated after salmeterol

and/or formoterol treatment. The results of the current study also demonstrated that no inflammatory cell infiltration or smooth muscle cell thickening was observed in salmeterol and formoterol-treated mice with COPD. Hypertrophy and hyperplasia of goblet cells on the airway surface was inhibited in salmeterol and/or formoterol-treated mice with COPD compared with the control group. In addition, airway smooth muscle was thinned, collagen deposition was decreased, intima cells exhibited typical morphology and smooth muscle cells were arranged more regularly in the small arteries of the lungs after salmeterol and formoterol treatment. Collectively, these data suggest that salmeterol and formoterol inhibit inflammation and reduce the histopathological characteristics of COPD.

Currently, chronic lung diseases, including asthma and COPD, are a global medical treatment burden and this burden is increasing (27-32). Statistics indicate that COPD is a major cause of morbidity and mortality worldwide (33). The World Health Organization has predicted that COPD will become the third most common non-cancer cause of mortality worldwide in 2020 (34). Drug therapies for COPD suppress inflammation in the small airways and lung parenchyma (35). Salmeterol, a β_2 -receptor agonist, is recommended as a long-acting bronchodilator for patients with COPD (36). Restrepo *et al* (8) identified that salmeterol xinafoate and fluticasone propionate treatment improved the survival rate of patients with severe COPD. In addition, Ryan *et al* (37) indicated that salmeterol also improved nocturnal sleeping arterial oxygen saturation and sleep quality. These reports suggest that salmeterol is an effective drug for treatment of COPD.

Formoterol is one of the most commonly used long-acting β_2 -receptor agonist drugs that is licensed for use in patients with COPD (38). A previous study indicated that symptoms of COPD were relieved through the improvement of inflammation by bronchodilators, including β_2 -receptor agonists and anti-muscarinic agents (39). Fixed-dose drug combinations, including vilanterol/umeclidinium, indacaterol/glycopyrronium, olodaterol/tiotropium and formoterol/aclidinium, are licensed for the treatment of COPD worldwide (39). Schunemann (40) identified that formoterol combined with tiotropium improved airflow obstruction in COPD and improved dyspnea, asthma and chest distress. These reports suggest that formoterol is an effective drug for the treatment of COPD.

Previous studies have reported that comprehensive pharmacological treatments have improved the overall survival of patients with COPD (16,41-43). In the current study, the efficacy of a fixed-dose combination of formoterol and salmeterol was evaluated in a mouse model of COPD. The experimental data suggested that this fixed-dose combination of formoterol and salmeterol decreased levels of proinflammatory factors, and was more effective compared with either single treatment. Notably, it was observed that a fixed-dose combination of formoterol and salmeterol contributed to the repair of alveolar structure and a reduction of COPD symptoms compared with either single treatment or the control group.

In conclusion, repeated treatment with a fixed-dose combination of formoterol and salmeterol by inhalation led to an improvement in the symptoms and characteristics of COPD in the present study. This suggests that that co-treatment with salmeterol and formoterol may be an effective approach for COPD therapy.

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