Ecotoxicology and Environmental Safety 228 (2021) 112918

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effective-component compatibility of Bufei Yishen formula protects COPD rats against PM2.5-induced oxidative stress via miR-155/FOXO3a pathway

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ARTICLE INFO

Edited by: Professor Bing Yan

Keywords:
PM2.5
Chronic obstructive pulmonary disease
Oxidative stress
Bufei Yishen formula

ABSTRACT

Ambient particulate matter <2.5 μm (PM2.5) has been identified as a critical risk factor in chronic obstructive pulmonary disease (COPD) exacerbation, but therapies for this condition are limited. Effective-component compatibility of Bufei Yishen formula (ECC-BYF) exhibits beneficial efficacy on COPD rats. However, its effect on PM2.5-aggravated COPD rats are considered to be uncertain. In this study, we used an established PM2.5-aggravated COPD rat model in vivo to evaluate the protective effect of ECC-BYF, and focused on its anti-oxidative role in PM2.5-stimulated bronchial epithelial cells via regulating microRNA (miR)−155/ forkhead box class O3a (FOXO3a) pathway. As expected, PM2.5-aggravated COPD rats showed a reduction of lung function, persistent lung inflammation, and remodeling of lung tissue. In comparison, ECC-BYF administration significantly enhanced lung function, alleviated alveolar destruction, inflammatory cell infiltration, mucus hypersecretion, and collagen deposition, along with diminishing inflammatory cytokine production and oxidative stress. Furthermore, ECC-BYF pretreatment markedly decreased the fluorescence intensity of reactive oxygen species (ROS) in PM2.5-induced human bronchial epithelial (Beas-2B) cells and primary mouse tracheal epithelial cells (MTECs), as well as reversing the imbalance between oxidants and antioxidants in Beas-2B. Meanwhile, ECC-BYF elevated FOXO3a while inhibiting miR-155 expression dose -dependently. In vitro transfection of miR-155 mimic into Beas-2B significantly decreased FOXO3a protein expression, accompanied by the reduced superoxide dismutase 2 (SOD2) and catalase (CAT) expressions, thus eliminating the protective effect of ECC-BYF on PM2.5-evoked oxidative stress. Nonethless, FOXO3a overexpression could partially restore the antioxidative effect of ECC-BYF. In conclusion, ECC-BYF can protect pre-existing COPD against PM2.5 contamination by exerting a profound antioxidative influence via regulating miR-155/FOXO3a signaling.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major global burden, affecting more than 300 million people worldwide and accounting for approximately 3.2 million deaths annually (GBD, 2017; Causes of Death Collaborators, 2018). It is well known that the initiation of COPD is a consequence of the genetically susceptible individual exposed to sufficient environmental exposure (Global Initiative for Chronic Obstructive Lung Disease, 2021). Ambient air pollution, especially the particulate matter (PM) in the respiratory range, has been verified as an attractive environmental contributor for the increased morbidity and mortality of COPD (Andersen et al., 2011; Doiron et al., 2019). Inhalation of ultrafine particles with an aerodynamic diameter less than 2.5 μm (PM2.5) can induce both bronchial and systematic inflammation by penetrating deep into alveoli or even blood-stream, causing reduced lung function and damage to a number of organs and tissues (Falcon-Rodriguez et al., 2016). Our previous study has demonstrated that PM2.5 exposure could markedly accelerate lung

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https://doi.org/10.1016/j.ecoenv.2021.112918
Received 6 May 2021; Received in revised form 2 October 2021; Accepted 16 October 2021
Available online 10 November 2021

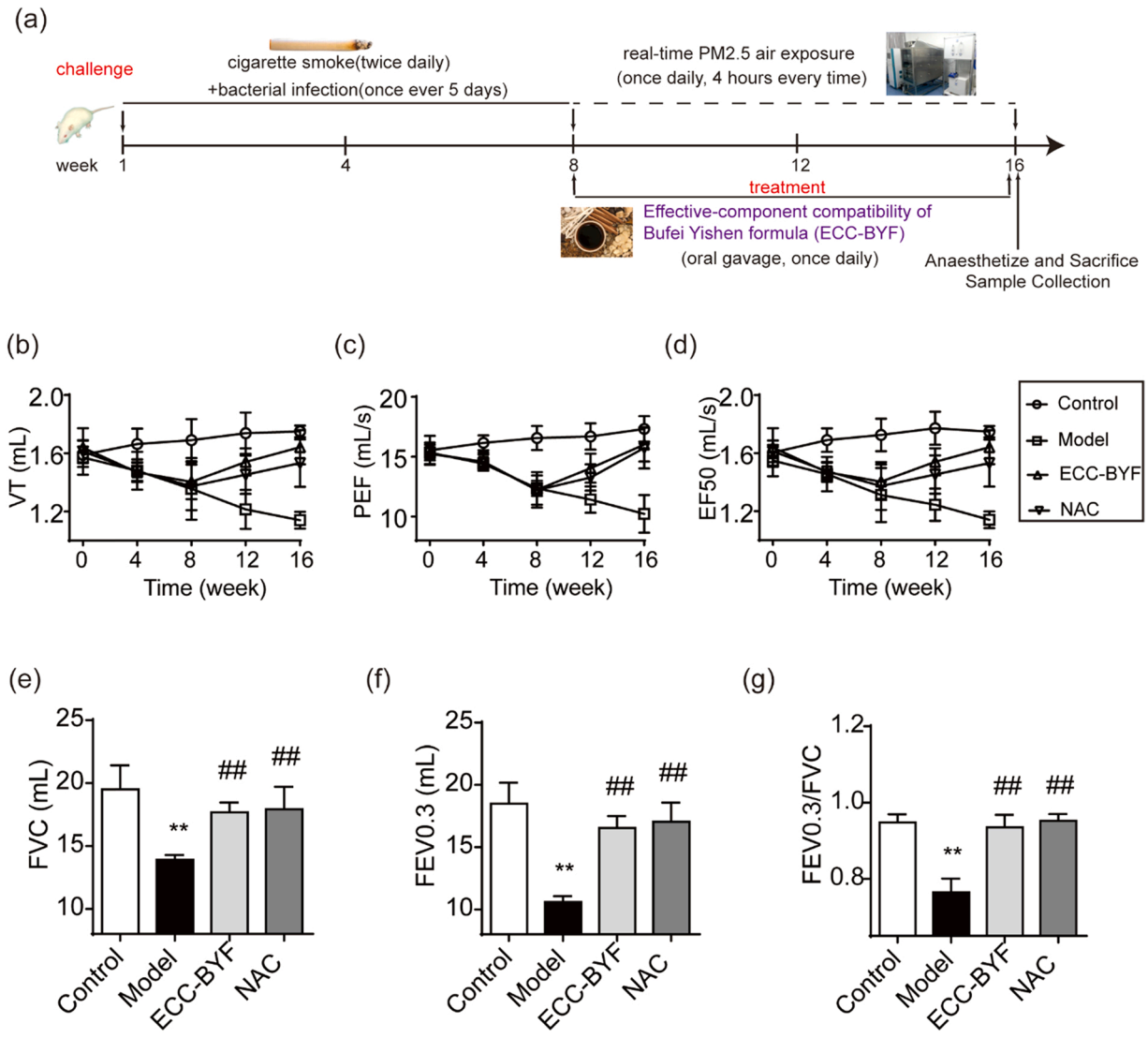

Fig. 1. ECC-BYF enhances pulmonary function in PM2.5-aggravated COPD rats. (a) Schematic model of ECC-BYF-treated rats: with repeated cigarette smoke exposure and bacterial infection on weeks 1–8, following real-time concentrated PM2.5 atmosphere exposure concomitant with ECC-BYF treatment on weeks 8–16, and sacrifice on week 17. (b-d) Changes of the noninvasive pulmonary function (V_T, PEF, EF50) in each group (n = 8). (e-g) Changes of the invasive pulmonary function (FVC, FEV0.3, FEV0.3/FVC) in each group (n = 8). Control=healthy control rats; Model=PM2.5-aggravated COPD rats; ECC-BYF=ECC-BYF- treated rats; NAC=N-acetylcysteine-treated rats. All data are presented as the mean ± S. $^{**}P < 0.01$, versus Control group. $^{\#\#}P < 0.01$, versus Model group.

Table 1
The primer sequences used for real-time PCR assay in the present work.

Gene	Primer (5′ to 3′)
Rat CAT	Forward: TCACCTGAAGGACCCTGACA
	Reverse: TCCATCTGGAATCCCTCGGT
Rat SOD2	Forward: AGGGCGTCATTCACTTCGAG
	Reverse: CCTCTCTTCATCCGCTGGAC
Rat FOXO3a	Forward: CTACGCTGACCTGATCACCC
	Reverse: TTCGTTCTGAACCCGCATGA
Rat GAPDH	Forward: ACAGCAACAGGGTGGTGGAC
	Reverse: TTTGAGGGTGCAGCGAACTT
Human CAT	Forward: TGGAGCTGGTAACCCAGTAGG
	Reverse: CCTTTGCCTTGGAGTATTTGGTA
Human SOD2	Forward: GGTGGGCCAAAGGATGAAGAG
	Reverse: CCACAAGCCAAACGACTTCC
Human miR-155–5p	Forward: AACACGCTTAATGCTAATCGTGA
	Reverse: GTCGTATCCAGTGCAGGGT
Human U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT
Human GAPDH	Forward: GGAGCGAGATCCCTCCAAAAT
	Reverse: GGCTGTTGTCATACTTCTCATGG

function decline, amplify oxidative stress, inflammatory response, mucus hypersecretion, and airflow obstruction in rats with pre-existing COPD (Wang et al., 2020). However, information regarding the molecular mechanisms linking PM2.5 exposure and exacerbation of COPD is lacking, and new therapies for this condition have yet to be explored.

It is well known oxidative stress plays a critical role in COPD pathogenic processes. PM2.5, like tobacco smoke, exhibits a potent redox potential and chronic elevation of reactive oxygen species (ROS) is a crucial factor that results in PM2.5-elicited lung injury (Reche et al., 2012; Niu et al., 2020). Recent studies indicate that bronchial epithelial cells from COPD patients may be vulnerable to diesel emission exposure due to reduced antioxidant capacity and that the antioxidant N-acetylcysteine (NAC) pretreatment appear to offer protection (Vaughan et al., 2019). Our previous data also demonstrated a further reduction of several antioxidant factors upon PM2.5 exposure in pre-existing COPD rats, including total superoxide dismutase (T-SOD), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) (Wang et al., 2020). These observations prompted us to explore the potential molecular mechanisms by which PM2.5 trigger oxidative stress and exacerbate lung injury in COPD, in order to propose new effective

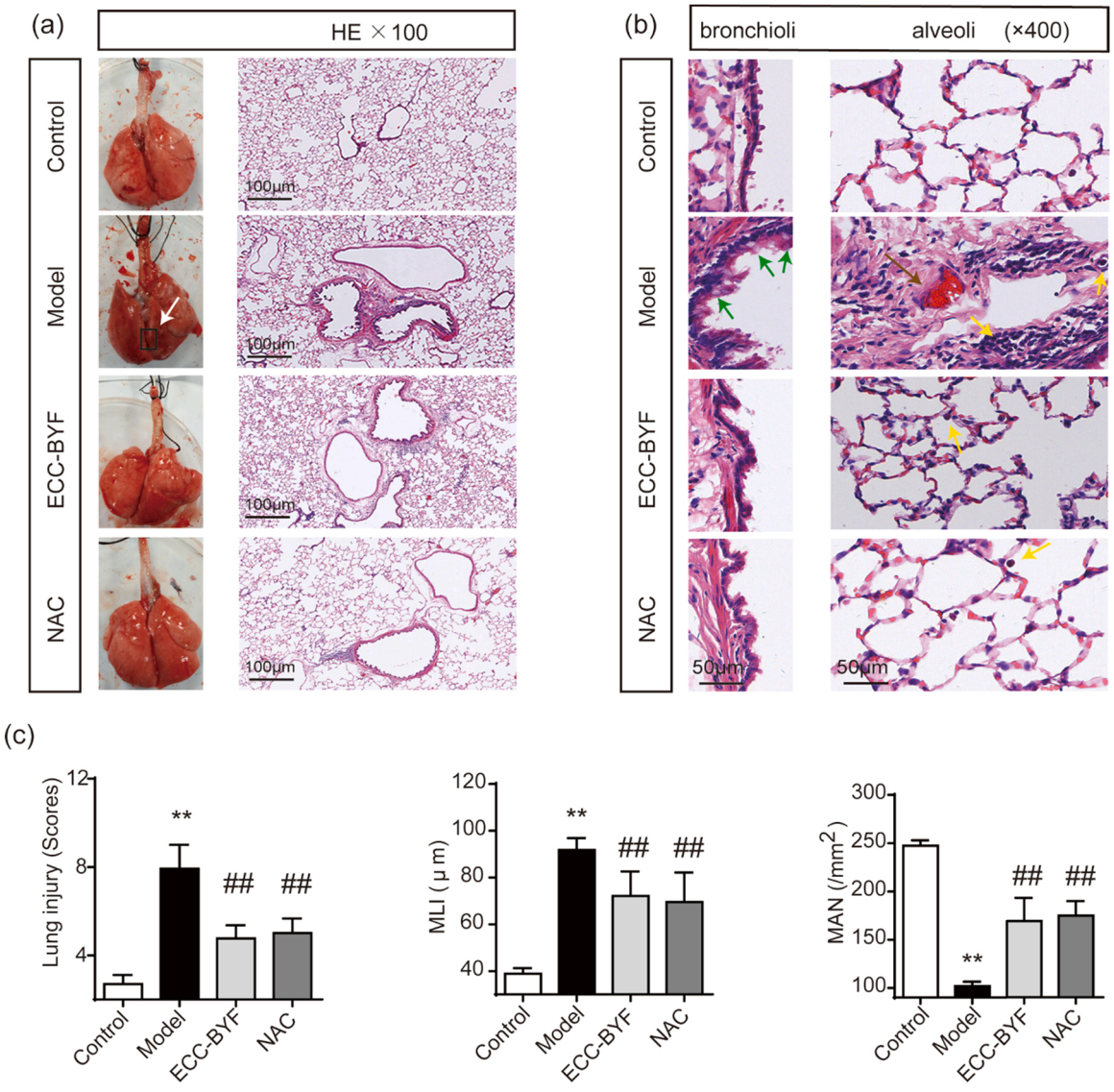

Fig. 2. ECC-BYF alleviated the lung injury in PM2.5-aggravated COPD rats. (a) Representative gross lesion and histology of lungs from control and all experimental rats. In photomacrographs, the black box indicates pulmonary hemorrhage and the white pustular nodules, the white arrow indicates black discoloration. The scale bar of H&E-stained lung section is 100 μm. (b) Representative images of bronchiolar epithelium and alveoli in lung tissues of rats (scale bar: 50 μm). The green arrows indicate goblet cells, yellow arrows indicate lymphocyte infiltration, and brown arrows indicate extravasated red blood cells. (c) Histomorphometric analysis for the lung sections in each group (n = 6–8). All data are presented as the mean ± S. $^{**}P < 0.01$, versus Control group. $^{\#\#}P < 0.01$, versus Model group.

therapeutic strategies.

Forkhead transcription factor class O (FOXO)3a is a member of the Fork frame transcription factors subfamily. It has been extensively studied in recent years because of its critical role in controlling the expression of genes involved in oxidative stress responses, DNA damage repair, inflammation and cellular metabolism (Kops et al., 2002; Du et al., 2017; White et al., 2020). Its inhibition can lead to increased susceptibility to cigarette-induced lung inflammatory response and airspace enlargement resulting in the development of COPD (Hwang et al., 2011). FOXO3a expression has been found decreased by cigarette smoke in bronchial epithelial cells and this may be due to the cigarette smoke-activated oxidative stress associated with mitochondrial damage (Di Vincenzo et al., 2021).

FOXO3a is modulated by multiple regulatory factors with recent observations manifest a crucial role played by microRNAs (miRNAs). MiR-155 has been found to directly bind to 3′-untranslated region (3′-UTR) of FOXO3a mRNA, thus inhibiting its function (Li et al., 2020a, 2020b, 2020c, 2020d). A prominent upregulation of miR-155 was detected to be induced by PM2.5, cigarette smoke or biomass combustion particles in human, animals and bronchial epithelial cells (Ruiz-Vera et al., 2019; Xiao et al., 2019; Li et al., 2020a, 2020b, 2020c, 2020d). Aberrant expression of miR-155 was closely associated with oxidative stress and inflammatory response (Yu et al., 2018). miR-155 deficiency could strongly inhibit ROS expression and down-regulate inflammation (Qiu et al., 2018). Overexpression of miR-155 could decrease major antioxidants including SOD2 and catalase, and promote ROS generation and oxidative damage partially via downregulating FOXO3a expression (Wang et al., 2015). Therefore, we speculated that the miR-155/FOXO3a pathway might play an essential role in regulating oxidative stress induced by PM2.5, thereby affecting COPD.

Bufei Yishen formula (BYF) is a traditional Chinese herbal formula that is widely used in COPD treatment. It has been confirmed efficacious in preventing COPD by easing symptoms, reducing the risk of exacerbations, and improving exercise endurance and life quality (Li et al., 2012a, 2012b, 2013; Zhao et al., 2018). Effective-component compatibility of *Bufei Yishen* formula (*ECC-BYF*) is a combination of five bioactive equivalent components derived from BYF, contained *ginsenoside Rh1*, *astragaloside IV*, *icariin*, *nobiletin*, and *paeonol*. With equal

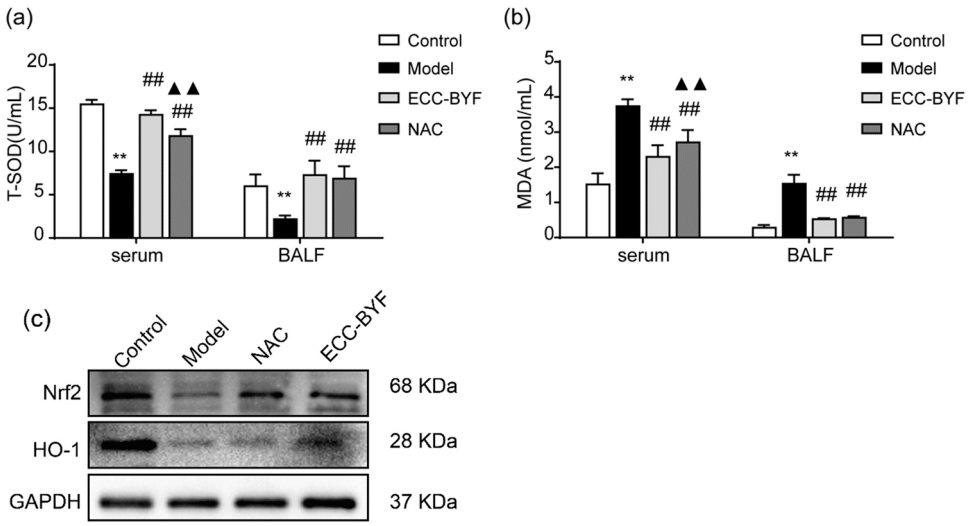

Fig. 3. ECC-BYF lessens the oxidative stress in PM2.5-aggravated COPD rats. (a) Levels of antioxidant T-SOD in serum and BALF from rats in each group. (b) Levels of oxidant MDA in serum and BALF from rats in each group. (c) Representative immunoblots of Nrf2 and HO-1 in rats' lung tissues. All data are presented as the mean ± S. $^{**}P < 0.01$, versus Control group. $^{\#\#}P < 0.01$, versus Model group. $^{\blacktriangle\blacktriangle}P < 0.01$, versus ECC-BYF group.

efficacy to BYF, *ECC-BYF* has exhibited beneficial effects on COPD model rats by enhancing lung function and attenuating inflammatory response, oxidative stress, and airway remodeling (Li et al., 2020a, 2020b, 2020c, 2021, 2020d). However, it has not yet any studies characterizing the preventive potential of *ECC-BYF* for the COPD exacerbated by exposure to PM2.5.

In the current study, we focused on evaluating the effect of *ECC-BYF* on COPD in the present of PM2.5 pollution by using the PM2.5-aggravated COPD rats model that established previously (Wang et al., 2020), and explored its antioxidative mechanism via the modulation of miR-155/FOXO3a signaling, thereby further laying a foundation for its clinical application on COPD.

2. Materials and methods

2.1. Preparation of drugs

ECC-BYF is composed of 20-S-ginsenoside Rh1 (6.25 mg/mL), astragaloside IV (1.25 mg/mL), icariin (25 mg/mL), nobiletin (1 mg/mL), and paeonol (1.5625 mg/mL). The purity of these compounds was > 99%. N-acetylcysteine (D1118A01, 200 mg/tablet) was purchased from Hainan Zambon Pharmaceutical Co. Ltd (Hainan, China).

2.2. Animal treatment and sample collection

All animal experiment protocols were approved by the Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Chinese Medicine (YFYDW2017013). 32 SD rats (male, 6–9 wk, Experimental Animal Center of Shandong Province, Jinan, China) were randomized into four groups. After 8-week repeated cigarette smoke inhalation (twice daily, 30 min each time) and intranasal instillation of Klebsiella pneumonia suspension (once every five days) (Li et al., 2012a, 2012b), rats in the PM2.5-aggravated COPD model group were exposed to concentrated PM2.5 atmosphere in the whole-body exposure chamber (1.2 m^3) for additional 8 weeks (4 h /day, average daily concentration: 739.97 μg/m^3). The successful establishment of PM2.5-aggravated rats model was evaluated by further declined lung function and worsened lung tissue pathology compared with that of the previous 8 weeks, which was in correspondence with our previous study (Wang et al., 2020). Simultaneously, rats in the ECC-BYF group and NAC group were intragastrical administered with ECC-BYF (5.5 mg/kg·d) and N-acetylcysteine (108 mg/kg·d) before the daily PM2.5 exposure. The control rats were maintained in filtered air (PM2.5 concentration < 10 μg/m^3) and received gavage with distilled water (2 mL per rat).

Finally, the animals were anesthetized with 3% pentobarbital sodium (35 mg/kg, ip), and pulmonary function was measured as previously described (Wang et al., 2020). Lung tissues were collected for hematoxylin-eosin (H&E) staining. Serum and bronchoalveolar lavage fluid (BALF) were collected for determining malondialdehyde (MDA), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) levels using matched kits according to the manufacturer's protocol (Nanjing Jiancheng Co., Jiangsu, China). Fig. 1a shows the flow chart of the experiment design.

2.3. Preparation of PM2.5 suspension

Daily PM2.5 samples were gathered on polytetrafluoroethylene (PTEE) filter membrane using a high-volume PM2.5 sampler (1050 L/min, HY-1000 BL) set on a building rooftop (14 m above the ground), located closely to the busiest roads and surrounded by residences. Sampling was performed from 1st December, 2018–31 st January, 2019. These collected filters were cut into pieces and sonicated in sterilized water. PM2.5 suspensions were then assembled using eight layers of filter gauze, then completely frozen and lyophilized using a vacuum freeze-dryer. PM2.5 powders were then collected and stored at − 80 °C. The chemical analysis and distribution of particle size of these collected PM2.5 samples were described in Supplementary Table S1 and Fig. S1. Before delivery to cells, PM2.5 powders were resuspended in DMSO at a 100 mg/mL concentration, sonicated for 30 min, and stored at − 20 °C.

2.4. Cell culture and treatment

Human bronchial epithelial cells (Beas-2B) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Beas-2B were grown in Dulbecco's modified Eagle medium (DMEM, Solarbio Co., Beijing, China) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS, Lonsera science SRL, Uruguay) in a humidified atmosphere of 5% CO2 at 37 ℃.

Primary mouse tracheal epithelial cells (MTECs) were isolated from the tracheas of C57BL/6 mice (male, 6–8 wk, Charles River, Beijing, China). Of note, sixteen mice were randomly divided into two groups (n = 8) and exposed to either filtered air or the cigarette smoke accompanied by intranasal instillation of Klebsiella pneumonia

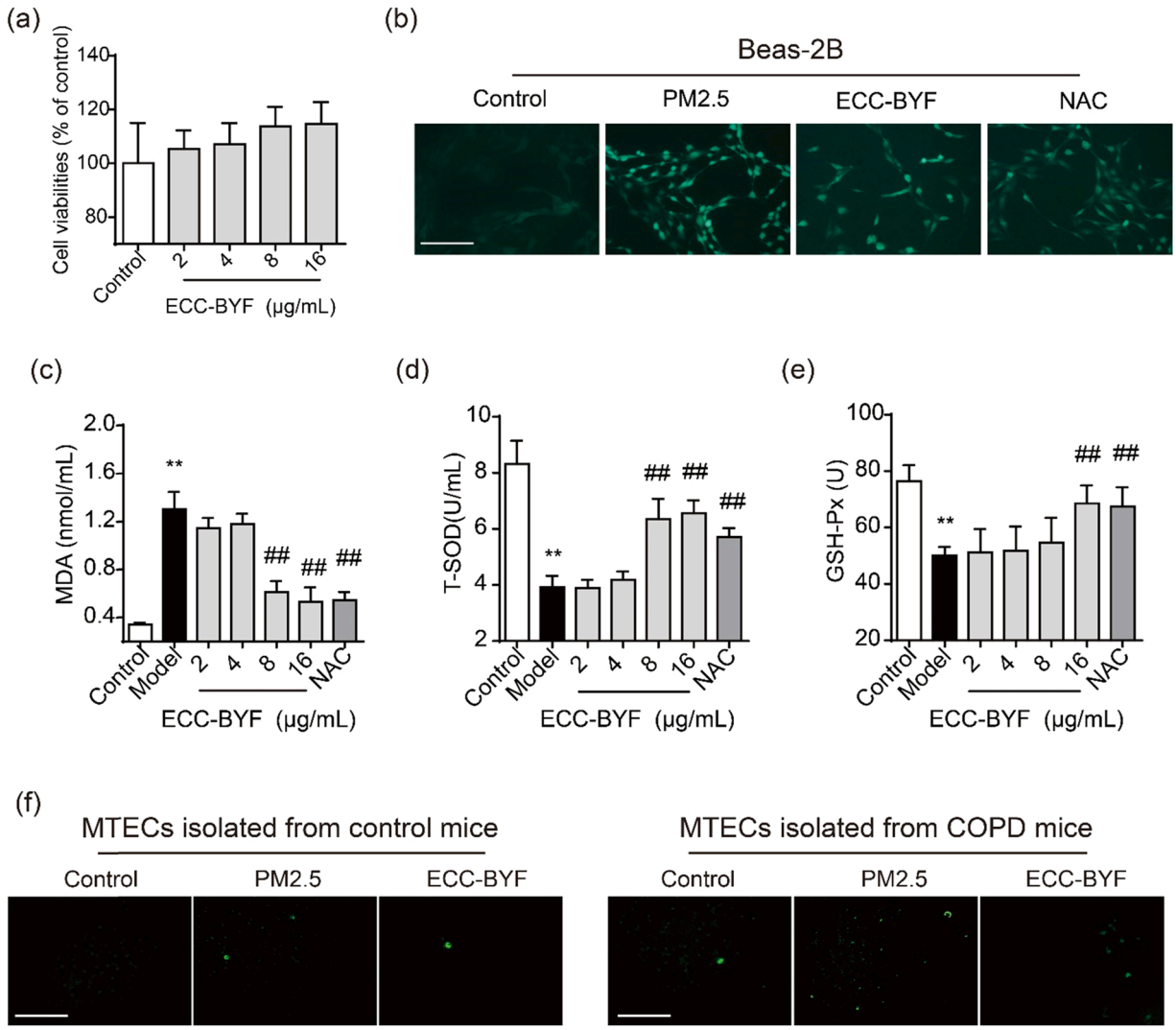

Fig. 4. ECC-BYF attenuates PM2.5-induced oxidative stress in human bronchial epithelial (Beas-2B) cells and primary mouse tracheal epithelial cells (MTECs). (a) The effect of ECC-BYF on the viability of Beas-2B. (b) Representative images of immunofluorescent labeling of ROS (scale bar: 100 μm) in Beas-2B pre-treated with 16 μg/mL of ECC-BYF, 500 μM of NAC or DMSO, for 24 h, and then exposed to 100 μg/mL of PM2.5 solution for 24 h. (c-e) Levels of MDA, T-SOD, and GSH-Px in Beas-2B that were pretreated with different concentrations (2, 4, 8, and 16 μg/mL) of ECC-BYF solution, 500 μM of NAC or DMSO, for 24 h, and then exposed to 100 μg/mL of PM2.5 solution for 24 h. (f) Representative images of immunofluorescent labeling of ROS (scale bar: 200 μm) in MTECs. All data are presented as the mean ± S. $^{**}P < 0.01$, $^{*}P < 0.05$.

suspension for 8 weeks as previously described (Li et al., 2012a, 2012b). MTECs were plated on transwell plate in proliferation medium and upon reaching confluency, and cultured at ALI in differentiation medium according to the method of Lam HC (Lam et al., 2011). Table S2 lists the culture media and supplements.

Beas-2B and MTECs were pretreated with ECC-BYF (16 μg/mL) before they were challenged with PM2.5 suspension (100 μg/mL) for an additional 24 h. Cell viability was evaluated by a cell counting kit 8 (CCK8) assay (Solarbio, Beijing, China). Intracellular reactive oxygen species (ROS) was detected by a 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) probe.

2.5. Cell transfection

Beas-2B cells were seeded in six-well plates (60%~70% confluent) in a serum-free medium and transfected with the pcDNA-FOXO3a plasmid (14 μg), miR-155 mimic (50 nM), or related negative controls (Guangzhou RiboBio Co., Ltd., China) mixed with Lipofectamine 2000 (Invitrogen Biotechnology Co., Ltd., USA) according to the manufacturer's instructions. After 24 h, Beas-2B were used for ECC-BYF treatment and PM2.5 exposure experiments.

2.6. Quantitative real-time PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen, 15596026) from lung tissues or cultured cells and reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, 15596026). Then, the expression of genes was measured by quantitative real-time PCR (qPCR), which performed on an 7500 real-time PCR system (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems, 00743571). The relative expression was analyzed using the $2^{-\Delta\Delta CT}$ method. Of note, the relative amount of miR-155–5p was normalized to U6 snRNA, while FOXO3a, SOD2, and CAT mRNA were normalized to GAPDH. Table 1 lists the primer sequences (JinRuiSi, Nanjing, China).

2.7. Western blotting

Protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and probing with the Nrf2 (16396–1-AP,

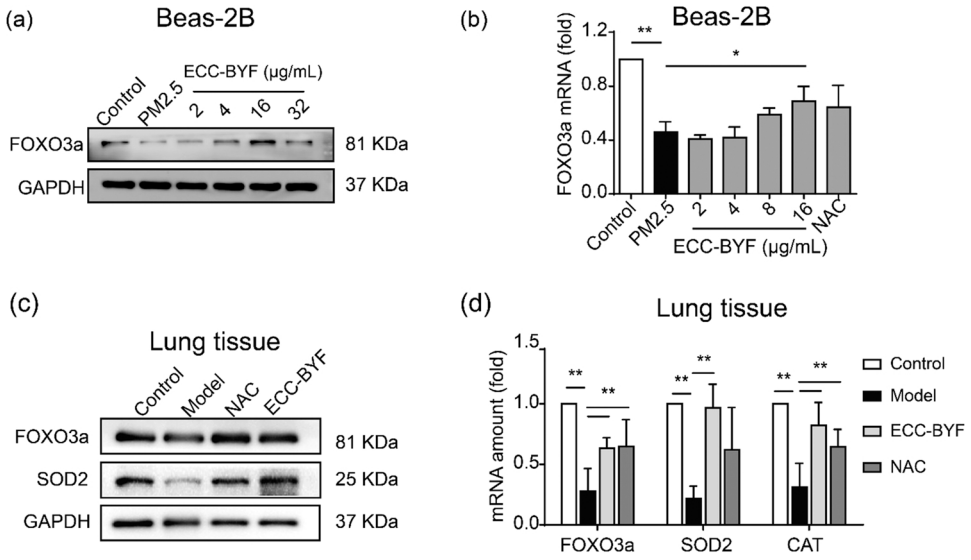

Fig. 5. ECC-BYF activates FOXO3a in PM2.5-induced Beas-2B cells and lung tissues. (a) Representative immunoblots of FOXO3a, and (b) qPCR analysis of FOXO3a mRNA levels in Beas-2B pre-treated with different concentrations (2, 4, 8, 16, and 32 μg/mL) of ECC-BYF, for 24 h, and then exposed to 100 μg/mL of PM2.5 solution for 24 h. (c) Representative protein band of FOXO3a and SOD2 in lung tissues. (d) FOXO3a, SOD2 and CAT mRNA levels in lung tissues of rats. Model=PM2.5-aggravated COPD rats. All data are presented as the mean ± S. $^{**}P < 0.01$, $^{*}P < 0.05$.

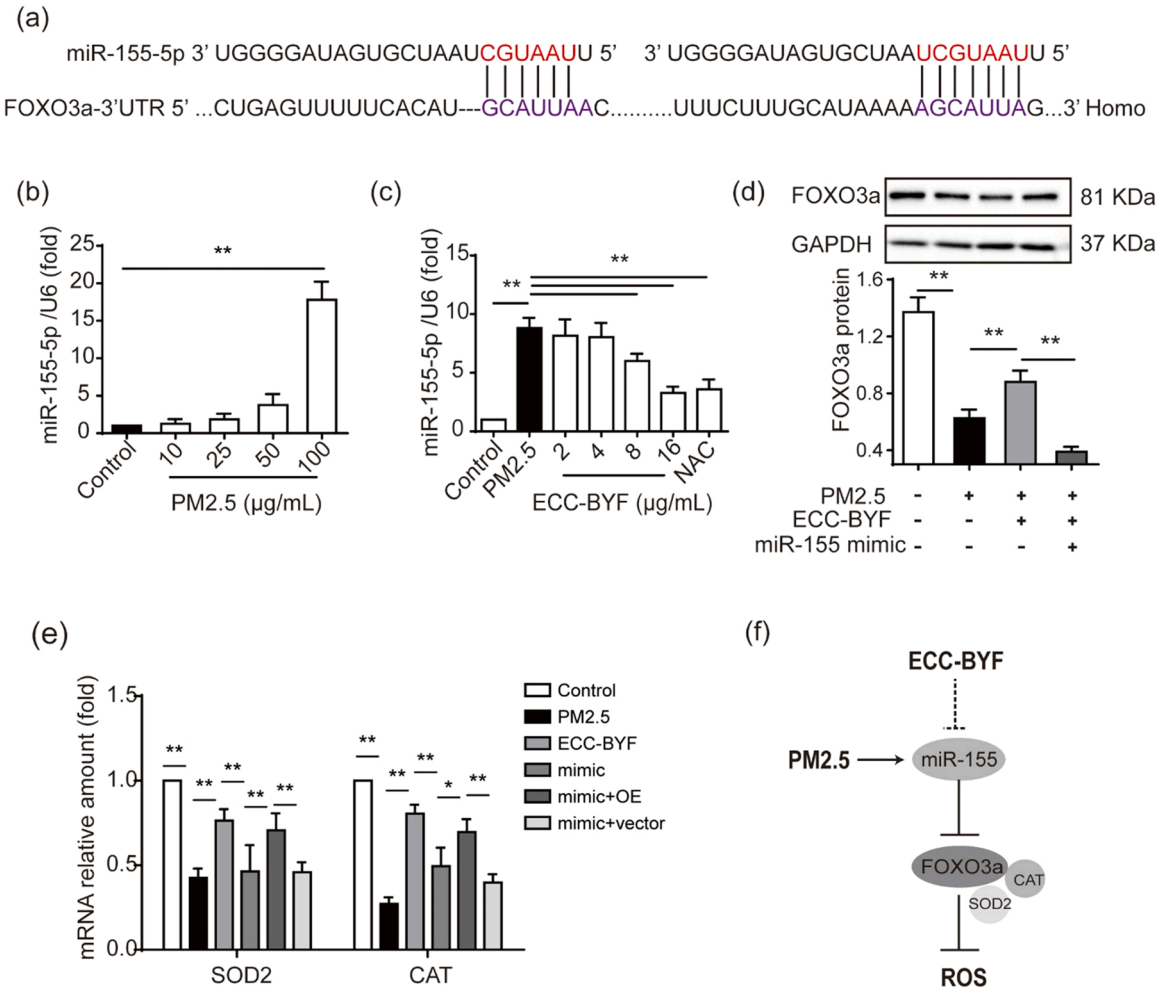

Fig. 6. ECC-BYF partially rescues PM2.5-induced oxidative stress via miR-155/FOXO3a pathway. (a) The predicted miR-155 target sequences in 3′-UTR of FOXO3a in human. (b) qPCR analysis of miR-155–5p expression in Beas-2B treated with different concentrations of PM2.5 solution (10, 25, 50 and 100 μg/mL) for 24 h. (c) qPCR analysis of miR-155–5p expression in Beas-2B pre-treated with different concentrations (2, 4, 8, and 16 μg/mL) of ECC-BYF, for 24 h, and then exposed to 100 μg/mL of PM2.5 solution for 24 h. (d) Western blot analysis of FOXO3a protein expression in miR-155-overexpressing Beas-2B with or without ECC-BYF pre-treatment upon PM2.5 induction. (e) qPCR analysis of SOD2 and CAT mRNA levels in miR-155-mimic-transfected Beas-2B with or without FOXO3a overexpression (OE) that pre-treated with 16 μg/mL of ECC-BYF, for 24 h, and then exposed to 100 μg/mL of PM2.5 for 24 h. (f) A proposed working model of ECC-BYF protects Beas-2B against oxidative stress induced by PM2.5 through regulating the miR-155/FOXO3a signaling. All data are presented as the mean ± S. $^{**}P < 0.01$, $^{*}P < 0.05$.

Proteintech), HO-1 (GTX101147, GeneTex), FOXO3a (GTX100277, GeneTex) and SOD2 (GTX116093, GeneTex) antibody (all 1:1000) were performed as described (Wang et al., 2020). GAPDH was used as an internal control.

2.8. Statistics

Data are presented as mean ± standard deviation. Differences between experimental groups were assessed for significance using one-way ANOVA and unpaired two-tailed Student's *t*-test. Statistical significance between time-based measurements (lung function) within each group was determined by analysis of variance for repeated measurements. A *P* value of less than 0.05 was considered to be statistically significant. Analyses were performed using SPSS software, version 23.0, for Windows (IBM, Armonk, NY, USA).

3. Results

3.1. ECC-BYF enhances pulmonary function in PM2.5-aggravated COPD rats

To evaluate the therapeutic effects of ECC-BYF on PM2.5-aggravated COPD rats, unrestrained lung function was detected by the whole-body plethysmography every fourth week. Tidal volume (V_T), peak expiratory flow (PEF), and expiratory flow at 50% tidal volume (EF50) continuously decreased from week 1 to week 16 in the model group, while significantly increased after ECC-BYF administration (Fig. 1b to d). Given forced expiratory volume in 0.3 s/ forced vital volume (FEV0.3/FVC) is widely accepted as a diagnostic biomarker for COPD, we also measured FVC, FEV0.3, and FEV0.3/FVC at week 17 utilizing an invasive test system (PFT). As expected, these three indexes in the model group markedly decreased compared with the control group but increased upon ECC-BYF and NAC administration (Fig. 1e to g).

3.2. ECC-BYF alleviated the pathological injury in lung tissues of PM2.5-aggravated COPD rats

The pathology of lung tissues is another critical evidence for estimating the development of COPD. Obvious pulmonary hemorrhage, white pustular nodules, black discoloration, and old petechial were observed on lung tissue surface from the model rats. Correspondingly, their light microscopic examination by H&E staining showed evident interalveolar septa thickening, numerous inflammatory cell infiltration, narrowed airway lumen, and goblet cells hyperplasia in the airway epithelium. Nonetheless, ECC-BYF and NAC-treated rats revealed ameliorated macroscopic and histopathological changes that appeared more or less similar to that of the control (Fig. 2a and b). Regarding the quantification of emphysematous lesions, the mean linear intercepts of alveolar (MLI), as well as the total lung injury score, was significantly elevated, whereas the mean alveolar number (MAN) was declined in model rats. Inversely, these characteristics were significantly improved upon treatment with ECC-BYF or NAC (Fig. 2c).

3.3. ECC-BYF mitigates oxidative stress in PM2.5-aggravated COPD rats

To determine the effect of ECC-BYF on oxidative stress in PM2.5-aggravated COPD rats, we detected both antioxidant T-SOD and oxidant MDA in serum and BALF. As shown in Fig. 3a and b, T-SOD significantly decreased, whereas MDA increased in the model group compared with that in control, indicating an imbalance between oxidants and antioxidants appeared in PM2.5-aggravated COPD rats. In contrast, ECC-BYF and NAC treatment reversed the imbalance by elevating T-SOD while diminishing MDA. Besides, a significantly higher T-SOD while lower MDA in the serum from ECC-BYF-treated rats was observed than that of NAC-treated rats. Additionally, compared to the model group, increased Nrf2 and HO-1 in the ECC-BYF group, two major regulators of antioxidant response, revealed by western blot further validated the antioxidant effect of ECC-BYF on PM2.5-aggravated COPD (Fig. 3c).

3.4. ECC-BYF attenuates PM2.5-induced oxidative stress in human bronchial epithelial (Beas-2B) cells and primary mouse tracheal epithelial cells (MTECs)

Airway epithelium forms the first barrier for the defense against airborne particles and pathogens. Specifically, the injured epithelium is known to release free radicals and inflammatory factors. Thus, we used the Beas-2B and MTECs to further address the protection of ECC-BYF on PM2.5-induced oxidative stress in vitro. As shown in Fig. 4a, the viability of Beas-2B changed slightly compared with that of the control group by ECC-BYF treatment, suggesting 2–16 μg/mL of ECC-BYF is non-toxic to Beas-2B. ECC-BYF pretreated-cells expressed a significant reduction of ROS fluorescence in comparison with that of the model cells (Fig. 4b). We also observed a dose-dependent increase in T-SOD and GSH-Px, while a decrease in MDA upon ECC-BYF pretreatment for 24 h compared with those of model group (Fig. 4c to e), demonstrating an antioxidative effect of ECC-BYF in response to PM2.5. We next determined whether PM2.5 altered the level of oxidative stress in the MTECs of COPD model mice. We found that indeed intracellular ROS was increased in the MTECs isolated from both normal and COPD mice when exposed to PM2.5 suspension, in agreement with our previous in vivo study showing that PM2.5 exposure elevated oxidative stress in pre-existing COPD (Wang et al., 2020). In contrast, ECC-BYF pretreatment protected MTECs from PM2.5-induced oxidative stress as reflected by decreased ROS in ECC-BYF- treated MTECs compared to that of PM2.5-induced cells (Fig. 4f).

3.5. ECC-BYF partially rescues PM2.5-induced oxidative stress via miR-155/FOXO3a pathway

Protein level of FOXO3a in Beas-2B by western blot revealed that ECC-BYF significantly elevated FOXO3a expression in a dose-dependent fashion (Fig. 5a). The up-regulated FOXO3a mRNA expression was also proved in Beas-2B through qPCR (Fig. 5b). Concomitantly, both protein and mRNA expressions of FOXO3a, together with its downstream SOD2 and CAT, were markedly decreased in the lung tissues from PM2.5-aggravated COPD model group, while up-regulating by ECC-BYF or NAC administration.

MiR-155 has been found to directly bind to the 3′-UTR of FOXO3a mRNA, which subsequently inhibits its function (Fig. 6a, data from TargetScan database). We observed significant increase of miR-155 in Beas-2B after PM2.5 stimulation in the present study (Fig. 6b). Nonetheless, dose-dependent decrease of miR-155 was found in response to ECC-BYF treatment in PM2.5-induced Beas-2B (Fig. 6c). To further investigate whether the antioxidative effect of ECC-BYF in response to PM2.5 was due to the modulation of the miR-155/FOXO3a pathway, miR-155 overexpressing Beas-2B cells were established. Compared to the ECC-BYF-treated Beas-2B, miR-155 mimic-pretreated cells expressed a significant decrease of FOXO3a (Fig. 6d) along with reduced SOD2 and CAT mRNA expressions (Fig. 6e) in response to PM2.5 stimulation. Furthermore, compared to the miR-155 mimic pretreated cells, FOXO3a plasmid co-transfected cells exhibited a significant increase in SOD2 and CAT mRNA expressions. Collectively, suppressed miR-155 under ECC-BYF treatment may result in up-regulation of FOXO3a, which subsequently attenuates the oxidative stress induced by PM2.5 (Fig. 6f).

4. Discussion

In this study, we demonstrated that ECC-BYF treatment could significantly protect pre-existing COPD rats against PM2.5 exposure by alleviating the decrease of lung function, pathological injury in lung tissues, and oxidative stress. Moreover, miR-155–5p, which upregulated

in response to PM2.5 and negatively regulated FOXO3a, could sensitize Beas-2B cells to oxidative stress. While by downregulating miR-155, ECC-BYF enhanced the expression of FOXO3a and ultimately alleviated PM2.5-stimulated oxidative stress, suggesting that the antioxidative mechanism of ECC-BYF might be related to the miR-155/FOXO3a signaling pathway.

PM2.5 absorbs abundant polycyclic aromatic hydrocarbons (PAHs), transition metals, and reactive gases, leading to various adverse health outcomes such as inflammatory response and oxidative stress (Falcon--Rodriguez et al., 2016). COPD subjects are more susceptible to the damaging effects of particle pollutants due to the preexisting redox stress, chronic airway inflammation, and deficient clearance ability (Sint et al., 2008). In the present study, we utilized a whole-body PM2.5 online enrichment system to mimic real-world COPD sufferers living with environmental pollution conditions, and examined the rats' pulmonary function and pathological changes in the lung tissue to verify the replication of PM2.5-aggravated COPD model (Wang et al., 2020). The lung function (V_T, PEF, EF50) in the model group declined with time from week 1 to week 8, and further decreased from week 9 to week 16 caused by PM2.5 exposure. By the end of week 16, both noninvasive (V_T, PEF, EF50) and invasive (FVC, FEV0.3, FEV0.3/FVC) lung function parameters in model group were significantly lower than those in the control, indicating an irreversible airflow limitation in model rats. The pathological results indicated that rats in model group developed more lung tissue injury than controls, including alveolar wall thickening, alveolar cavity enlargement, inflammatory cells infiltration and small conducting airways thickening, suggesting the successful establishment of PM2.5-aggravated COPD rat model.

ECC-BYF administration showed a beneficial efficacy in COPD aggravation caused by PM2.5 exposure for improving pulmonary function and alleviating histological alterations in lung tissues including alveolar destruction, inflammatory cell infiltration, and mucus hypersecretion. As PM2.5 exhibits potent free-radical activity, it is widely accepted that oxidative stress plays a critical role in the progression of COPD caused by PM2.5 exposure (Wang et al., 2020). In this study, elevated expression of ROS after PM2.5 induction seen in MTECs isolated from COPD mice further support this view. Nonetheless, we demonstrated that ECC-BYF pre-treatment strongly scavenged free radicals and restored the activity of antioxidants in both Beas-2B and MTECs, exerting an equal antioxidative property to N-acetylcysteine (NAC), a well-known antioxidant recommended for COPD treatment in the guideline, and consistent with the improved imbalance between oxidants and aitioxidants after ECC-BYF administration in the serum and BALF from the PM2.5-aggravated COPD rats. These results suggest ECC-BYF could protect pre-existing COPD rats against PM2.5 pollution by suppressing oxidative stress. FOXO3a is a member of the FOXOs transcription factors involved in activating the coding gene for antioxidant enzymes and thus protects cells from oxidative stress (Kops et al., 2002). Literatures had verified that imparied activity of FOXO3a is one of the intracellular responses activated by cigarette smoke (Di Vincenzo et al., 2021). What's more, recent researches showed lung tissues from COPD patients displaying FOXO3a suppression compared with controls (Gu et al., 2017), while reactivation of FOXO3a was an effective novel strategy for COPD by counteracting oxidative stress-induced cellular damage (Hwang et al., 2011). Herein, we demonstrated that PM2.5 statistically decreased FOXO3a expression accompanied with the antioxidants including SOD2 and CAT in vivo and in vitro. Whereas enhanced expressions of these mRNAs and proteins were observed when ECC-BYF pre-treatment, indicating that elevated FOXO3a as a consequence of ECC-BYF treatment might attenuate PM2.5-induced oxidative stress via up-regulating the transcription of antioxidant enzymes.

Previous evidences indicated that aberrant miRNAs targeting gene expression was closely associated with the pathogenesis of COPD (Sundar et al., 2019), suggesting a diagnostic, prognostic, and therapeutic potential of miRNAs. Pulmonary expression of miR-155 was reported to be enhanced in smokers and was further amplified in patients with severe COPD, and miR-155 deficiency could markedly attenuate cigarette smoke-induced pulmonary inflammation (De Smet et al., 2020). Besides, epidemiological study has suggested that long-term exposure to traffic-derived air particles correlate with increase serum miR-155 expression in truck drivers (Hou et al., 2016). In agreement with the previous data, we observed that PM2.5 could induce markedly miR-155 increase in Beas-2B cells. Then our data showed a dose-dependent decrease of miR-155–5p expression in ECC-BYF pre--treated Beas-2B cells, suggesting that miR-155 was sensitive to ECC-BYF and might be a downstream factor mediating the protective effect of ECC-BYF on PM2.5-induced lung injury. It's demonstrated that miR-155 could directly target the FOXO3a 3′-UTR and suppress its expression, thereafter regulating varieties of biological processes (Li et al., 2020a, 2020b, 2020c, 2020d). In the present study, miR-155 negatively linked to the antioxidative effect of ECC-BYF reflected by reduced expressions of SOD2 and CAT in miR-155 mimic pre-treated Beas-2B cells, whereas FOXO3a overexpression was potent to re-evoke the antioxidative activity of ECC-BYF. Thus we speculated that the antioxidative effects of ECC-BYF on PM2.5-induced lung injury might be related to an upregulated activity of FOXO3a via inhibiting miR-155.

In summary, we primarily demonstrate a significant efficacy of ECC-BYF on PM2.5-aggravated COPD rats by enhancing lung function and alleviating airway inflammation, oxidative stress, and airway remodeling. Furthermore, we describe herein that the upregulation of miR-155, which directly targets and inhibits antioxidant FOXO3a, plays a crucial role in the oxidative stress stimulated by PM2.5. In comparison, ECC-BYF exerts an antioxidative effect on PM2.5-induced lung injury, at least in part, via regulating the miR-155/FOXO3a axis. Our findings are the first to establish a novel therapeutic intervention for COPD sufferers living with air particle contamination and provide essential insights into the interaction of traditional Chinese medicine effective components compatibility (ECC) in gene regulation and their contribution to COPD treatment.

5. Conclusion

This study data indicate a protective effect of ECC-BYF on COPD aggravation induced by PM2.5 exposure by inhibiting oxidative stress via regulating miR-155/FOXO3a signaling.

CRediT authorship contribution statement

Jiansheng Li participated in project design, providing funding for the project, writing and proofreading of the manuscript. Jing Wang participated in data analysis, in vivo and in vitro experiments, writing and proofreading of the manuscript. Ya Li, Peng Zhao and Yange Tian participated in project design and proofreading of the manuscript. Xuefang Liu provided study materials, reagents, and instrumentation. Huihui He and Rui Jia helped with animal experiment, qRT-PCR, western blot, and immunohistochemical analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81973822).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112918.

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