Tongxinluo Boosts the Effect of Atorvastatin in Treatment of Atherosclerosis With COPD by Maintaining the Pulmonary Microvascular Barrier

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Abstract

Background: Atherosclerosis (AS) is a common comorbidity of chronic obstructive pulmonary disease (COPD) and the main cause of death in patients with COPD. Systemic inflammation is a significant mechanism of COPD with AS. The dysfunction of pulmonary microvascular barrier is involved in the formation of chronic inflammation in COPD, and its functional disruption will induce systemic inflammation. Atorvastatin (Ato) is a common medicine for the treatment of AS; however, the effect is not ideal for COPD combined with AS. Tongxinluo (TXL) improves the function of vascular endothelial cells. This study aims to prove that the impairment of pulmonary microvascular barrier function participated in the process of COPD aggravating AS and investigated whether TXL enhances the therapeutic effect of Ato on COPD with AS by protecting the pulmonary microvascular endothelial barrier function.

Methods: In vivo, the COPD with AS model of ApoE^{-/-} mice was established by cigarette smoke combined with a high-fat diet. The animals were administered TXL (1.5 g/kg/day), Ato (10 mg/kg/day), and TXL+Ato once a day for 20 weeks. Then, lung function, lung microvascular permeability, lung inflammation, systemic inflammation, serum lipid level, atheromatous plaque formation, and the biomarker of endothelial damage were measured. In vitro, human pulmonary microvascular endothelial cells (HPMECs) were pretreated with TXL for 6 h and incubated with cigarette smoke extract (CSE) for 24 h to establish a model of CSE-induced pulmonary microvascular barrier dysfunction. The permeability of the endothelial monolayer, inflammatory cytokines, endothelial damage biomarkers, tight junction proteins were determined.

Results: Cigarette smoking significantly exacerbated, which induced by high fat diet, the pulmonary function decline, pulmonary microvascular endothelial barrier dysfunction, pulmonary and systemic inflammation, and atherosclerotic plaque. These changes were reversed by TXL-Ato combination; the combination therapeutic effect was better than that of Ato alone. In addition, TXL protected the function of HPMEC barrier and inhibited the inflammation in CSE-induced HPMECs.

Conclusions: COPD aggravates AS, the mechanism may be the destruction of pulmonary microvascular barrier function and thus lung inflammation triggers systemic inflammation. In treating COPD with AS, TXL enhances the anti-AS effect of Ato protecting the pulmonary microvascular barrier.

Background

Chronic obstructive pulmonary disease (COPD) is characterized by incomplete reversible airflow restriction, which gradually worsening and is associated with abnormal inflammatory responses to harmful gases or particles in lungs, such as smoke from tobacco and biomass combustion [1, 2]. Over 300 million people suffer from COPD. Currently, it is the fourth cause of deaths worldwide and is predicted to be third by 2030 [3]. In addition to pulmonary symptoms, COPD also accompany with extrapulmonary comorbidities, such as cardiovascular disease, metabolic syndrome, and diabetes [4, 5].

Cardiovascular disease (CVD) is a common comorbidity and a major cause of death in patients with COPD [6, 7], so their epidemiological link has been established [8, 9]. Accumulating references show that systemic inflammatory is associated with COPD, which may be the mechanism of the increased risk to COPD combined with AS [10–13].

Previous studies have shown that increased pulmonary microvascular permeability may be a key factor for airway obstruction in patients with COPD [14]. The increased permeability of pulmonary microvascular endothelial cells (PMECs) causes neutrophil accumulation and subsequent activation in the lungs. These cytokines stimulate additional inflammatory cells and facilitate the release of inflammatory mediators, which in turn trigger inflammatory cascades and amplify injury signals to cause systemic inflammation. Importantly, systemic inflammation plays a crucial role in the pathogenesis of AS [15]. Therefore, maintaining the integrity of pulmonary microvascular endothelial barrier and inhibiting pulmonary inflammation from entering systemic inflammation is a key link in the treatment of COPD combined with AS. However, the role of pulmonary microvascular barrier function in the progress of COPD with AS has not yet been elucidated due to the lack of relevant studies. The treatment regimen for patients with COPD combined with AS involves treatment of symptoms for a single disease, including inhalation of bronchodilator and anti-inflammatory therapy for COPD [16], lipid-lowering treatment (for example, statins), antihypertensive agents, or β -blockers for AS [17]. The symptoms of both diseases are treated as independent modalities and the interactions between COPD and AS are not considered. Therefore, new treatment regimens for COPD combined with AS are required.

Tongxinluo (TXL) is a Chinese herbal compound that has been widely used in the treatment of CVDs and cerebrovascular diseases in China [18, 19]. Hitherto, about 6 million patients with CVDs and cerebrovascular diseases have been treated with TXL for its beneficial effects against vascular diseases. Our Previous studies showed TXL can not only relieve the symptoms of chest tightness and chest pain, reduce the levels of cardiac markers N-terminal pro-brain natriuretic peptide (NT-Pro-BNP) and cardiac troponin I (cTnI), but also relieve the lung symptoms of dyspnea, significantly increase 6MWD (6-minute walk distance) and the level of lung marker clara cell secretory protein-16 (CC-16)[20] in patients of COPD with CVD. Previous studies showed that TXL protects human cardiac microvascular endothelial cells from hypoxia/reoxygenation injury and alleviates cerebral microcirculatory disturbances against ischemic injury. Furthermore, TXL ameliorates renal structure and function of diabetic nephropathy [21, 22, 23]. It can be seen that TXL-mediated improvement of microvascular blood flow perfusion is a common mechanism in the treatment of major diseases of heart, brain, and diabetic nephropathy, and its target is mainly microvascular endothelial cells. However, the role of TXL in pulmonary microvascular endothelial cells has not been reported. The anti-AS effects and concomitant lipid-lowering effects of Atorvastatin (Ato) have been demonstrated in previous studies [24]. The present study aimed to determine whether the key mechanism of COPD exacerbation of AS is the pulmonary microvascular barrier dysfunction, lung inflammation triggers systemic inflammation, and whether TXL can enhance the anti-AS effect of Ato in COPD combined with AS through pulmonary microvascular barrier protection.

Materials And Methods

Ethics Statement.

Adult male wild-type (WT) mice of genetic background C57BL/6N and ApoE^{-/-} mice [25,26] (20-25g; 8-10 weeks old) were used in this study. All animals were kept at the Key Laboratory of State Administration of Traditional Chinese Medicine (Cardio-Cerebral Vessel Collateral Disease) (Shijiazhuang, China). All procedures with animals were conducted under the guidelines on the Care and Use of Laboratory Animals for biomedical research published by National Institutes of Health (No.85-23, revised 1996) and according to the ethical guidelines of the Ethics Committee of Hebei Yiling Pharmaceutical Research Institute (No. N2020051).

Animals and treatments

All mice were housed in an animal center under a 12-h light-dark cycle for 7 days to acclimatize to the environment prior to experimentation. The animals had free access to standard rodent diet and water at all times. Wild type C57BL/6N mice were divided into control group, and ApoE^{-/-} mice were divided random into five groups (n=10/group): 1) AS; 2) COPD+AS; 3) TXL; 4) Ato; 5) TXL+Ato. According to the preliminary experiments, the dose of TXL was determined to be 1.5 g/kg/day (about 2 times of human dose), and atorvastatin was 10 mg/kg/d[27]. As previously described [28,29], the smoke was pumped into a plastic box, measuring 42 cm (length) × 28 cm (width) × 27 cm (height), containing the animals that passively inhaled the cigarette smoke. The AS was induced in ApoE^{-/-} mice fed a high fat diet (SCXK2019-0003, Beijing Keao Xieli Feed Co., Ltd. Beijing, China) for 20 weeks. Briefly, mice were exposed to smoke twice daily, burning 20 cigarettes each time for an hour, 5 days a week for up to 20 weeks. The control group mice were fed a normal chow diet and exposed to an airy room. The AS group mice were fed a high-fat diet for 20 weeks. The COPD+AS group, TXL group, Ato group, and TXL+Ato group mice were exposed to cigarette smoke combined with a high-fat diet for 20 weeks. TXL group, Ato group, and TXL+Ato group mice were given TXL (1.5 g/kg/day), Ato (10 mg/kg/day), and TXL+Ato once a day.

Mouse pulmonary functions

Mice were anesthetized with 50 mg/kg sodium pentobarbital (Sigama–Aldrich, USA) via intraperitoneal injection, and then tracheostomized and placed in a forced pulmonary maneuver system (Buxco Research Systems). To acquire the lung function of mice, the Boyle's low functional residual capacity (FRC), quasistatic PV, and fast flow volume maneuver were performed using the Buxco system [30]. The FRC was determined using the Boyle's law FRC maneuver; the dynamic compliance (cdyn) was acquired by the quasistatic PV maneuver; the forced vital capacity (FVC), forced expiratory volume (FEV) at 50 ms (FEV50), and inspiratory resistances (RI) were recorded with the fast flow volume maneuver. Each maneuver was repeated at least three times.

Lung microvascular permeability

Lung microvascular permeability was determined by the Evans Blue dye method [31]. According to, 20 mg/kg body weight, 1% of Evans blue dye (Sigma-Aldrich, USA) was solubilized in 250 μ L phosphate-buffered saline (PBS) and injected into the tail vein of the mice. After 2 h, the mice were anesthetized with pentobarbital sodium and perfused with 10 mL of 0.9% sodium chloride solution to remove the blood. Then, the lung tissue was weighed, incubated, and homogenized with formamide (Sigma–Aldrich, USA) for 48 h at 37 °C. The concentration of the dye (μ g/mg tissue) was determined in the supernatant using a spectrophotometer at 620 nm against a standard curve.

Tissue collection and morphological analysis

Mouse left lung without lavage and the total length of aortic arch were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4), embedded in paraffin, and sliced into 8- μ m-thick sections that were stained with hematoxylin and eosin (HE) solution. The alveolar enlargement was determined by the average linear intercept and the ratio of the total length of alveoli to the number of alveoli per field.

For the quantification of atherosclerotic lesions, Oil Red O-positive lesion surface areas on en face preparation of whole aorta were measured. Briefly, the aorta from root to the abdominal area was dissected and fixed with formalin, followed by removal of the connective tissues carefully, and longitudinal opening with the intima towards the outside. Then, the tissues were stained in prewarmed Oil Red O solution for 10 min and differentiated in 85% propylene glycol for 5 min at room temperature. The extent of atherosclerotic lesion development was defined as the percentage of total Oil Red O-positive lesion area over the total surface area.

For detection of VE-cadherin (anti-VE-cadherin, sc-9989, 1:50, Santa Cruz, Texas, USA), frozen sections were stained with 8 μ M dihydroetidium (DHE; Wako) for 30 min, followed by counterstaining and sealing with mounting medium, antifading (with DAPI) (Solarbio, Beijing, China). The fluorescence images were captured under a ZEISS confocal microscope (Oberkochen, Germany).

Transmission electron microscopy (TEM)

The ERCs were centrifuged at 2,000 $\times$ g for 5 min and the pellets were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in PBS buffer (pH 7.2) at room temperature for 2 h. After several rinses with PBS, the cells were post-fixed with 1% osmium tetroxide (Sigma-Aldrich) for 1 h at 4°C, dehydrated in a graded series of acetone, and embedded in Epon 812. Ultrathin sections were cut (60–80 nm thick), and then double-stained with uranyl acetate and lead citrate (both from Amresco, Solon, OH, USA) prior to examination under a transmission electron microscope (H-7700; Hitachi, Tokyo, Japan).

Serum lipid analysis

Serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels were determined using enzymatic kits from Jiuqiang Biotechnology (Beijing, China) according to the manufacturer's protocols.

Cell culture and treatment

Human pulmonary microvascular endothelial cells (HPMECs) were obtained from Yaji Biological (Shanghai, China). The cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, New York, USA) and 1% antibiotic-antimycotic in a humidified incubator under 5% CO₂ at 37 °C. All cells between passages 3-4 were used for subsequent experiments.

The HPMECs were divided into five groups: (i) Control: HPMECs were incubated in regular cell culture conditions; (ii) cigarette smoke extract (CSE): HPMECs were incubated with diluted CSE for 24 h to establish CSE-induced pulmonary microvascular barrier dysfunction; (iii) CSE + 200 µg/mL TXL: HPMECs were pretreated with TXL (200 µg/mL) for 6 h and underincubated with diluted CSE for 24 h; (iv) CSE + 400 µg/mL TXL: HPMECs were pretreated with TXL (400 µg/mL) for 6 h and underincubated with diluted CSE for 24 h; (v) CSE + 800 µg/mL TXL: HPMECs were pretreated with TXL (800 µg/mL) for 6 h and underincubated with diluted CSE for 24 h. All experiments were performed in triplicate.

Preparation of CSE

CSE was generated as described previously [32]. A syringe-driven apparatus device was designed and operated to allow a stream of smoke to flow into a tube-shaped trap. The smoke then entered a flask submerged in liquid nitrogen. The amount of smoke obtained was calculated by the increase in the weight inside the flask. The smoke particulates were collected in dimethylsulfoxide (DMSO) at a concentration of 40 mg/mL, filtered through a 0.22-µm-pore filter, and stored at -80 °C for subsequent use.

Preparation of TXL

TXL ultrafine powder (Shijiazhuang Yiling Pharmaceutical Co., Shijiazhuang, China) was solubilized in TXL in serum-free DMEM and sonicated. The pellet was collected by centrifugation at 10000 ×g for 10 min. Subsequently, the precipitate was dried at 60 °C, and the supernatant was filtered (0.22 µm pore size, Costar, Cambridge,

Massachusetts, USA) to calculate an accurate weight of the dissolved ingredients [33]. According to the previous reports[34,35] and MTS experiments, the doses of TXL were determined to be 200, 400 and 800 µg/ml in HPMECs.

HPMECs monolayer permeability assays

Test of the permeability to FITC-dextran

Cells were grown on Transwell compartments. Tracer experiments with fluorescent

dextran were performed using the 12-well Transwell system (0.4-µm-pore size, 12-mm diameter, transparent, Costar, Cambridge, Massachusetts, USA). HPMECs (1×10⁵ cells/well) were plated in the

Transwell chambers that were supplemented with 500 and 1500 μ L culture media in the upper and lower compartments, respectively. The cells were grown for 5 days and replaced with

serum-free medium for 1 h before the treatment. The permeability was measured by the addition of FITC-dextran (40 kDa; Sigma–Aldrich, New York, USA) for 1 h.

Measurement of transendothelial electrical resistance

The cellular barrier properties were assessed by measuring the resistance values across confluent HPMECs using Millicell-ERS (MERS00002, Millipore, Molsheim, France) [36]. The 12-well Transwell system was inserted into the 12-well plate.

HPMECs were plated in Transwell chambers. The electrode tips were inserted into the upper and lower compartments, respectively, and the resistance (R sample) was measured. The value for the blank well represented the blank resistance (R blank). The TER value was calculated based on the resistance per unit area as $(R \text{ sample} - R \text{ blank}) \times 1.33 \text{ cm}^2$ (the area of a 12-well Transwell chamber is 1.33 cm^2) [37].

Immunofluorescence staining

After pretreated HPMECs reached 70% confluency, the cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, and permeabilized for 5 min with the buffer containing 0.1% Triton X-100, followed by blocking with goat serum for 1 h at room temperature. The cells were further incubated with primary antibodies

(VE-cadherin, 1:200, SC9989, Santa Cruz, Texas, USA; β -catenin, 1:200, ab32572, San Francisco, USA) at 4 °C overnight. After washing, the cells were incubated with secondary antibodies (goat anti-mouse, SA00013-3, 1:200; goat anti-rabbit

SA00013-2, 1:200). Finally, the cell nuclei were stained using DAPI for an additional 15 min and visualized under a ZEISS confocal microscope (Oberkochen, Germany) [38].

ELISA for inflammatory cytokines

Lungs were homogenized in a saline solution (0.9% NaCl), the mice serum and the cell culture supernatants were collected via centrifugation (1200 rpm, 10 min, 4 °C) to remove cell debris and were stored at -80°C until subsequent analyses. The levels of inflammatory cytokines were quantified using enzyme linked immunosorbent assays (ELISA). The kits were purchased from Proteintech (mouse IL-1 β ELISA kit, cat.

No.KE10003, Rosemont, USA; Mouse IFN- γ ELISA kit, cat. No.KE10001; Mouse TNF- α ELISA kit, cat. No.KE10002; Human IL-6 ELISA kit, cat. No. KE00139; Human IL-1 β ELISA kit, cat. No.KE20005) and Zcibio (Human hsCRP ELISA kit, cat. No. ZC-M6088, Shanghai, China). The operation steps were strictly carried out according to the instructions of manufacturer.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from mouse pulmonary and aorta tissue and HPMECs by Eastep Super Total RNA extraction kit and measured by Nanodrop2000 (Thermo Scientific, Waltham, MA, USA). Subsequently, reverse-transcription was performed using a Prime-Script™ RT Reagent kit, according to the manufacturer’s instructions. Quantitative PCR was carried out on a 7900 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). GAPDH was used as an internal reference, and the primer sequences are shown in Table 1.

Table 1: Primer sequences used for RT-PCR

Genes	Forward(5'→3')	Reverse(5'→3')
VE-cadherin (Mouse)	TGACAACACCGCCAACATC	TGACCAACTGCTCGTGAATC
β-catenin (Mouse)	CCATCTGTGCTCTTCGTCATC	ACTGCTGCTGCGTTCCA
IL-1β (Mouse)	ATCTCGCAGCAGCACATCA	CCAGCACGGTTATCATCATCAT
TNF-α (Mouse)	AAGCAAGCAGCCAACCAG	CCACAAGCAGGAATGAGAAGA
VCAM-1 (Mouse)	CCAGGACTCAGAATGACTTC	CGACCATCTTCACAGGCATT
ICAM-1 (Mouse)	CAGTGAGGAGGTGAATGTAT	AGGATGTGGAGGAGCAGAGA
GAPDH (Mouse)	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG
VE-cadherin (Human)	GATGTGTCGTGCTCAACTC	CCAGTCGTTAAGGAAGTCGTA
β-catenin (Human)	CCGACACCAAGAAGAAGCA	GCACGAACAAGCAACTGAACT
Claudin5 (Human)	TGGTAAGGCTGGTGAGGTC	GGAGTGGAGTATGGAGTTGGA
VCAM-1 (Human)	CTTAAAATGCCTGGGAAGAT	GTCAATGAGACGGAGTCACCA
ICAM-1 (Human)	TCATTGACTTGCAGCACCAC	TGCACAGGTAAGAGTGTTCGT
GAPDH (Human)	CGACCATTGTCAAGCTCA	AGGGGTCTACATGGCAACTG

Western blotting analysis

Protein concentration of mice lung tissue and HPMECs was determined using the BCA protein assay kit before SDS-PAGE(Genscript, Nanjing, China). Then, the proteins were transferred to nitrocellulose blotting membrane (Life Sciences, Mexico).

After blocking with Odyssey® blocking Buffer (LI-COR, Lincoln, USA) for 1 h at 37 °C, the membranes were incubated with primary antibodies (VE-cadherin, SC9989, Santa Cruz, Texas, USA; β -catenin, ab32572, San Francisco, USA; Claudin5, ab131259, San Francisco, USA; NF- κ B, CST8242s, MA, USA; β -actin, GB11001, Servicebio, Wuhan, China) at 4 °C overnight, followed by incubation with secondary antibodies at 37 °C for 1 h. Finally, the membranes were rinsed with TBST before scanning by Odyssey two-color infrared imaging system (Li-Cor, Lincoln, NE, USA). The β -actin was used as an internal control.

Statistical analysis

Statistical significance of differences was assessed using one-way ANOVA or repeated measures analysis, followed by post-hoc analysis using Fisher's LSD multiple comparison test. Differences at $P < 0.05$ were regarded as statistically significant. Data are presented as means $\pm$ SEM.

Results

- **Cigarette smoke aggravates high-fat diet-induced lung function decline in ApoE^{-/-} mice, and TXL-Ato combination improves the lung function in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice.**

To ensure that the cigarette smoking treatment of ApoE^{-/-} mice using our apparatus can reproduce the pathologic features of COPD, we assessed their lung functions by a forced pulmonary maneuver system. The results showed that cigarette smoke exposure aggravates emphysema-like phenotype (FRC and cdyn) and the airflow-limited phenotype (FEV₅₀/FVC and RI) induced by high-fat diet in mice. A significant increase in FRC (Fig. 1A) and a significant decrease in cdyn (Fig. 1B) and FEV₅₀/FVC (Fig. 1C) was observed in the AS and COPD + AS groups compared to the control group. Especially, a significant increase was noted in FRC and RI (Fig. 1D), and a significant decrease in cdyn and FEV₅₀/FVC in the COPD + AS group compared to the AS group. Compared to the COPD + AS group, FRC and RI decreased significantly and FEV₅₀/FVC and cdyn increased significantly in the TXL and TXL + Ato groups. Compared to the COPD + AS group, the significant decrease in FRC and significant increase was observed in FEV₅₀/FVC and cdyn in the Ato group.

Nonetheless, Ato group had no significant effect on RI compared to COPD + AS group. Compared to the TXL group, a significant decrease was noted in FRC and RI and a significant increase in FEV₅₀/FVC was observed in the TXL + Ato group; however, cdyn in the TXL group did not show a significant difference in the TXL + Ato group.

Compared with Ato group, a significantly decreased in FRC and RI and a significantly increased in FEV₅₀/FVC and cdyn was observed in TXL + Ato group.

These data indicated that cigarette smoke exposure reduced lung function induced by high-fat diet in mice. The TXL and Ato combination improved the lung function than either drug alone.

- **Cigarette smoke aggravates high-fat diet-induced pulmonary microvascular endothelial barrier dysfunction in ApoE^{-/-} mice, and TXL-Ato combination protects pulmonary microvascular endothelial barrier function in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice.**

Evans Blue content in the lung tissue was measured in each group to assess the pulmonary microvascular permeability, which plays a pivotal role in the pulmonary inflammatory response and acts as a therapeutic target for the treatment of COPD. To address whether TXL-Ato combination modulates pulmonary microvascular permeability in vivo, pulmonary microvascular leak was assessed. In addition, Transmission electron microscopy (TEM) was used to examine the ultrastructural changes in lung tissues and VE-cadherin, β -catenin were determined by RT-qPCR, and the expression of VE-cadherin was determined by immunofluorescent staining. The current results showed that the increase in Evans Blue content in lung tissue (Fig. 2B) in AS and COPD + AS groups compared to the control group, and a significant increase in the Evans Blue content in the COPD + AS group compared to the AS group. Compared to the COPD + AS group, TXL group and TXL + Ato group significantly decreased the Evans Blue content in the lung tissue, but Ato group had no effect. However, TXL-Ato combination significantly decreases the Evans Blue content compared to the TXL and Ato groups. The mRNA level of VE-cadherin and β -catenin (Fig. 2C and D) in mice was significantly reduced in the AS and COPD + AS groups compared to the control group. Additionally, a significant decrease in VE-cadherin and β -catenin was detected in the COPD + AS group compared to the AS group. However, VE-cadherin and β -catenin mRNA expression was significantly increased in the TXL, Ato, and TXL-Ato groups compared to the COPD + AS group. Compared to the TXL group, the mRNA level of VE-cadherin and β -catenin significantly increased in the TXL-Ato group. Compared to the Ato group, the mRNA level of VE-cadherin and β -catenin was significantly increased in the TXL-Ato group. Immunofluorescence staining of VE-cadherin in lung tissues demonstrated that COPD + AS group diminished the expression of VE-cadherin, while TXL-Ato combination treatment restored VE-cadherin level on the cell-cell junction (Fig. 2E).

Type I epithelial cell cytoplasm (I), vascular basement membrane (BM), and endothelial cell cytoplasm (En) together form gas-blood barrier. The ultrastructure was observed by TEM, the endothelial cells lining the lumen of the pulmonary microvasculature and the Tj proteins between adjacent endothelial cells maintain the integrity of the pulmonary barrier (Fig. 2A). In the control group, the structure of the air-blood barrier was complete, the endothelial cells showed slight edema, the Tjs were dense, and the BM was smooth. The COPD + AS group's capillary En has moderate edema, the cell membrane is incomplete, the Tj between the cells is blurred and nearly disappeared, and the BM is blurred and discontinuous. The BM in the air-blood barrier of the TXL group is fuzzy and discontinuous, and the Tj is fuzzy. The air-blood barrier in the Ato group showed edema, the BM was blurred, and the Tj disappeared partially. The air-blood barrier damage in the TXL + Ato group was slightly less than that in the TXL group, and the Tj was also slightly better than that in the TXL group.

These data indicated that cigarette smoke aggravates high-fat diet-induced pulmonary microvascular endothelial barrier dysfunction in ApoE^{-/-} mice. The TXL-Ato combination significantly attenuates pulmonary microvascular permeability under cigarette smoke combined with high-fat diet. In the

treatment of COPD, TXL may have more targets than Ato, such as the protective effect of the pulmonary microvascular barrier.

- **Cigarette smoke aggravates high-fat diet-induced pulmonary inflammation in ApoE^{-/-} mice, and TXL-Ato combination inhibits pulmonary inflammation in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice.**

Lung histopathology, the gene expression level in lung tissues, and the supernatant of lung homogenates further assessed the airway inflammation and airway remodeling. In normal lung tissue, the contour of bronchial tube was clear and only slight inflammatory cell infiltration was observed around the bronchial submucosa. There was perivascular and peribronchial inflammatory cell infiltration in the AS group. Compared to the AS group, the COPD + AS group mice showed profound peribronchial and/or perivascular inflammatory infiltration and mucus secretion. The TXL and Ato groups had perivascular and peribronchial inflammatory infiltration and mild inflammation in the alveolar septa, predominantly composed of mononuclear cells. The TXL + Ato group showed the presence of few inflammatory cells in the peribronchial space and mild infiltration in the alveolar septa (Fig. 3A).

Mean linear intercept (MLI) analysis also suggested an enlargement of alveolar in the COPD + AS group (Fig. 3B) compared to the AS group. Compared to the COPD + AS group, the MLI in the three treatment groups was significantly decreased, and TXL + Ato group significantly decreased MLI compared to the TXL and Ato groups. The expression of inflammatory factors in mice lung tissue was measured using RT-qPCR (Fig. 3C and D), significantly elevating the IL-1 β and TNF- α expression in COPD + AS group compared to the AS group. Furthermore, TXL-Ato combination significantly decreased the expression of TNF- α compared to the TXL and Ato groups. However, TXL-Ato group showed a significant decrease in IL-1 β mRNA level compared to the Ato group, but no significant difference in the TXL group. Enzyme-linked immunosorbent assay (ELISA) results showed that (Fig. 3E and F), the IL-1 β and TNF- α level in the COPD + AS group significantly increased compared to the AS group, and TXL + Ato group showed significantly decreased IL-1 β and TNF- α levels compared to TXL and Ato groups.

- These data together with lung function data suggested that cigarette smoke aggravates high-fat diet-induced pulmonary inflammation in ApoE^{-/-} mice. TXL-Ato combination inhibits pulmonary inflammation in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice, and TXL-Ato combination exerted a marked anti-inflammatory effect than TXL and Ato.
- **Cigarette smoke aggravates high-fat diet-induced systemic inflammation in ApoE^{-/-} mice, and TXL-Ato combination attenuates systemic inflammation in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice.**

To test the potential effect of cigarette smoke combined with high-fat diet on circulatory inflammation, we assessed the levels of different cytokines from the serum of the animal model. Figure 4 shows significantly increased levels of IL-1 β (Fig. 4A), IFN- γ (Fig. 4B), and TNF- α (Fig. 4C) in the COPD + AS group compared to the AS group. TXL, Ato, and TXL-Ato combination significantly decreased the expression of

IL-1 β , IFN- γ , and TNF- α compared to the COPD + AS group, while TXL-Ato combination significantly decreased the expression of IFN- γ and TNF- α compared to the Ato group. However, compared with TXL group and Ato group, there was no significant difference in IL-1 β in TXL + Ato group.

These findings indicated that with the destruction of pulmonary microvascular barrier function, pulmonary inflammation in COPD aggravates systemic inflammation. The TXL-Ato combination was more effective in reducing circulatory inflammation than either drug alone.

- **Cigarette smoke aggravates high-fat diet-induced AS lesion in ApoE^{-/-} mice, and TXL-Ato combination attenuated AS lesion in cigarette smoke combined with high-fat diet-induced ApoE^{-/-} mice.**

Figure 5A and B showed that COPD + AS group significantly increased the marked formation of atherosclerotic plaques compared to the AS group. The plaques showed cholesterol-rich lipid clefts within the intimal layer containing distinct, large macrophage-derived foam cells (Fig. 5C). However, compared to the COPD + AS group, TXL, Ato, TXL + Ato groups significantly decreased the marked formation of atherosclerotic plaques. TXL-Ato combination significantly decreased atherosclerotic lesions compared to the Ato group (Fig. 5B).

Compared to the control group, TC, TG, and LDL-C levels were significantly increased and the HDL was significantly decreased in the COPD + AS and AS groups. However, the levels of TC, TG, LDL-C, and HDL-C in the COPD + AS group did not differ significantly from those in the AS group. The administration of TXL, Ato, and TXL-Ato combination reversed the serum lipid levels compared to the COPD + AS group. The TXL-Ato combination significantly increased the HDL-C level and significantly decreased the LDL level compared to that in the TXL group, and TC and TG did not differ significantly between TXL + Ato and TXL groups. Compared to the Ato group, TC, TG, and LDL-C, HDL-C showed no significant difference in the TXL + Ato group (Fig. 5D–G).

RT-qPCR analysis revealed that VCAM-1 and ICAM-1 mRNAs (Fig. 5H and I) were highly expressed in the aortic tissue of the AS and COPD + AS groups in contrast to the control group. Compared to the AS group, the VCAM-1 and ICAM-1 mRNAs were significantly increased in the COPD + AS group. Nevertheless, TXL, Ato, and TXL-Ato groups markedly reduced VCAM-1 and ICAM-1 mRNAs compared to the COPD + AS group. Compared to the TXL group, VCAM-1 and ICAM-1 mRNAs were significantly reduced in the TXL + Ato group. Compared to the Ato group, the mRNA level of ICAM-1 and VCAM-1 was significantly reduced in the TXL + Ato group.

These data highlighted that subjecting ApoE^{-/-} mice to cigarette smoke aggravates high-fat diet-induced AS lesion in ApoE^{-/-} mice and the combined use of TXL with Ato enhances the anti-AS effect of Ato.

- **TXL protects the CSE-induced pulmonary microvascular endothelial barrier dysfunction in HPMECs.**

Herein, we demonstrated that pulmonary microvascular barrier dysfunction is critical in COPD exacerbation of AS in vivo. Next, we verified whether the key mechanism of COPD aggravates AS is

pulmonary microvascular barrier dysfunction through in vitro experiments and whether TXL can protect the function of pulmonary microvascular barrier. CSE was applied to HPMECs for 24 h to mimic the pulmonary microvascular endothelial barrier dysfunction conditions in vitro. The permeability of the endothelial monolayer was tested by measuring the influx of FITC-dextran and the TER across cells. CSE induced the endothelial barrier dysfunction, as represented by increased FITC-dextran leakage (Fig. 6A) and decreased TER (Fig. 6B). The influx of FITC-dextran was significantly decreased and the TER across cells was significantly increased by TXL (Fig. 6B). Immunofluorescence staining revealed that the CSE downregulated the expression of VE-cadherin and β -catenin in HPMECs (Fig. 6H), which was improved by TXL.

We also tested the changes in the expression of Tj proteins, the relative expression levels of VE-cadherin and β -catenin, as determined by Western blot (Fig. 6C and D), indicating that CSE significantly decreased VE-cadherin and β -catenin protein level and TXL significantly increased the β -catenin protein level. TXL 800 μ g/mL upregulated the level of VE-cadherin; however, 400 and 200 μ g/mL TXL did not show any significant effect on VE-cadherin. Similarly, RT-qPCR (Fig. 6E–G) results showed that VE-cadherin, β -catenin, and Claudin5 mRNA levels were downregulated in the CSE group, and TXL reversed this effect.

TXL against pulmonary microvascular barrier dysfunction induced by CSE, was consistent with the results that TXL attenuates pulmonary microvascular hyperpermeability under cigarette smoke combined with high-fat diet in vivo.

- **TXL against CSE-induced HPMECs' inflammation**

Next, we investigated the correlation between inflammatory signaling and increased permeability of pulmonary microvascular barrier induced by CSE. ELISA revealed that CSE exposure elevated the levels of IL-6, IL-1 β and high sensitivity C-reactive protein (hsCRP) in the media supernatant of the cells (Fig. 7A–C), while TXL could inhibit the levels of IL-6, IL-1 β and hsCRP. Western blot analyzed NF- κ B level (Fig. 7D). The protein expression of NF- κ B was increased in the CSE group compared to the control group, and 200 and 800 μ g/mL TXL significantly reduced the level of NF- κ B compared to the CSE group. However, 400 μ g/mL TXL did not exert a significant effect on NF- κ B compared to the CSE group. Collectively, TXL suppressed CSE-mediated inflammation, which was consistent with the results of TXL-inhibited systemic inflammation in cigarette smoke combined with high-fat diet in ApoE $^{-/-}$ mice.

- **TXL protects HPMECs from endothelial damage**

A previous study demonstrated that CSE mediates endothelial damage under various insults. Furthermore, TXL inhibits endothelial damage in HPMECs. Therefore, the present study evaluated the effects of TXL measuring the expression of *ICAM-1* and *VCAM-1* mRNAs. The current results showed that these mRNAs were significantly upregulated after HPMECs were exposed to CSE, and pretreatment with TXL significantly counteracted this CSE-induced effect (Fig. 8A and B).

Discussion

The present study elucidated the effects of TXL on COPD combined with AS in vivo and in vitro models. Several observations were summarized as follows: (i) The key mechanism of COPD exacerbation of AS is the pulmonary microvascular barrier dysfunction, which leads to pulmonary inflammation triggers systemic inflammation, thus exacerbating the severity of AS. (ii) TXL enhances the anti-AS effect of Ato by protecting the pulmonary microvascular barrier function in COPD combined with AS mice. (iii) TXL suppresses the CSE-induced pulmonary microvascular barrier dysfunction and inflammation in HPMECs.

According to the Global Burden of Disease report, about 3.2 million people died of COPD in 2017 [39], accounting for 81.7% of all deaths from chronic respiratory diseases. Cardiovascular disease is the leading cause of death in patients with COPD, accounting for 25–27% of patients with mild to moderate COPD [40, 41], and the two diseases are often combined [42]. COPD is known to be an independent risk factor contributing to the development of atherosclerotic vascular disease [43]. Some studies demonstrated that in addition to inflammation in the lungs, COPD patients experience a significant increase in systemic inflammatory markers (CRP, IL-6, and TNF- α) [44], which can interact with endothelial cells and deposit locally in AS lesions, inducing the expression of adhesion factors and chemokines in endothelial cells, promoting its dysfunction and the formation of atherosclerotic plaques [45]. It is suggested that the potential mechanism of COPD combined with AS is systemic inflammation [43]. Smoking may lead to the secretion of inflammatory mediators (such as IL-6, TNF- α , and IL-1 β) in the peripheral airway of COPD, thus leading to the upregulation of airway microvascular permeability [46]. The early occurrence of microvascular disease may be the driving factor for the development of COPD chronic airway inflammatory disease. PMVECs are one of the most abundant cells in lung tissue and form the innermost layer of pulmonary microcirculation exchange vessels through intercellular junction interaction and connection with extracellular matrix. The functional and structural integrity of PMVECs is crucial for maintaining normal lung physiological functions [47]. In pulmonary inflammation, PMVECs are one of the first effector cells attacked by inflammatory factors. The destruction of the structural and functional barriers maintained by PMVECs and the resulting increase in cell permeability underlies the pathology of various pulmonary diseases [48]. PMVECs act under the action of inflammatory factors; endothelium cells cause edema. On the other hand, a large number of inflammatory cells are “impound” in the lung, and the aggregated inflammatory cells are activated and release a large number of inflammatory mediators. These inflammatory mediators can directly damage microvascular endothelial cells and activate more inflammatory cells, which together trigger a cascade of inflammatory reactions and systemic inflammation [49]. Pulmonary microvascular barrier dysfunction is caused by pulmonary inflammation that stimulates the apoptosis of PMVECs or changes in recombinant endothelial cytoskeleton and intercellular junction proteins, resulting in abnormal cell structure and function, impaired cell barrier function, and increased cell monolayer permeability [50]. Thus, we hypothesized that pulmonary microvascular barrier dysfunction, and then pulmonary inflammation leading to systemic inflammation, is the potential mechanism of COPD aggravating AS.

Smoking is a common risk factor for both COPD and AS. Previous studies have shown that cigarette smoke causes systemic inflammation in mice, similar to that seen in COPD patients with atherosclerosis. In this study, ApoE^{-/-} mice exposed to cigarette smoke combined with a high-fat diet for 20 weeks were

used to establish a model of COPD combined with AS to explore the role of pulmonary microvascular barrier dysfunction in pulmonary inflammation leading to systemic inflammation, which leads to COPD aggravation of AS. We observed that the peripheral airway inflammatory mediators (IL-1 β and TNF- α) and lung tissue pathological analysis were more severe in ApoE $^{-/-}$ mice induced by smoking combined with a high-fat diet than in mice induced by a high-fat diet alone. In addition, smoking aggravated the decline in lung function, including an increased pulmonary resistance (FRC and RI) and a reduction of elastic recoil (cdyn and FEV $_{50}$ /FVC) in mice induced by high-fat diet. The alterations in lung function observed in this study were similar to changes in lung function in humans with emphysema. These data were consistent with the findings of the study by Bezerra et al. [51], in which mice were exposed to the same protocol of cigarette smoke. We further observed that serum systemic inflammatory markers (IL-1 β , interferon- γ , and TNF- α), aortic histopathology (hematoxylin-eosin and Oil Red O staining), serum lipid levels, and biomarkers of aortic endothelial injury (ICAM-1 and VCAM-1) were upregulated in mice induced by cigarette smoking combined with high-fat diet compared to mice induced by high-fat diet alone. These results indicated that smoking combined with high-fat diet not only induced lung inflammation (IL-1 β and TNF- α), but also aggravated systemic inflammation (IL-1 β , IFN- γ , and TNF- α) in circulation and the lesion degree of AS in mice. This phenomenon was consistent with relevant clinical studies; elevated circulating inflammatory markers (such as CRP, IL-6, IL-8, IFN- γ , TNF- α , fibrinogen, and white blood cell count) in patients with COPD directly stimulate various early atherosclerotic processes, including endothelial cell adhesion molecules (ICAM-1 and VCAM-1) increased [52] and the uptake of LDL by macrophages increased [53]. Reportedly, C-reactive protein has a direct effect on endothelial function [54, 55] and promoting the formation of atherosclerotic plaques [56]. In order to verify whether pulmonary microvascular barrier dysfunction is closely related to pulmonary inflammation leading to systemic inflammation and then COPD aggravating AS, we further detected relevant indicators of pulmonary microvascular barrier dysfunction. The pulmonary microvascular permeability (Evans Blue content, VE-cadherin, and β -catenin expression) was observed in vivo. The results showed that the pulmonary microvascular permeability of mice induced by smoking combined with high-fat diet was significantly increased, and the expression of VE-cadherin and β -catenin in lung tissue was significantly decreased compared to mice induced by high-fat diet. We further verified the same through in vitro experiments. TER and macromolecular permeability are known to reflect endothelial cell barrier function. We established a CSE-induced pulmonary barrier dysfunction model in HPMECs for 24 h in vitro and demonstrated that CSE could increase pulmonary microvascular permeability by decreased TER and increased influx of FITC-dextran of HPMEC monolayer, while reducing the expression of Tj proteins (VE-cadherin and β -catenin). NF- κ B drives the inflammatory response by inducing the expression of proinflammatory and anti-apoptotic genes [57]. Inflammatory mediators, including CRP, TNF- α , IL-6, and VCAM-1, are produced by various cells in response to stimulation by conserved tissue damage signals [58]. These mediators contribute to the development of chronic inflammation and a pro-atherogenic lipid profile [59, 60]. The current results showed that CSE significantly increased the levels of IL-6, IL-1 β , and hsCRP, as detected in the cell culture supernatant. The levels of endothelium injury markers, VCAM-1 and ICAM-1 mRNA in HPMECs were also significantly increased, which was consistent with the results of in vivo experiments. Furthermore, CSE activated the NF- κ B signaling pathway in HPMECs. These findings suggested that

microvascular barrier dysfunction, and pulmonary inflammation leading to systemic inflammation, is a critical link in COPD exacerbating AS.

TXL is a traditional Chinese medicine composed of extractions or powders from various natural products. It plays a major role in improving the function of vascular endothelial cells, reducing blood lipid content, inhibiting plaque inflammation, and enhancing the stability of vulnerable plaques. Studies have found that the BODE index and grade of patients with stable COPD treated with TXL capsule for 8 weeks are significantly lower than those before treatment and conventional treatment group, indicating that TXL capsule can better improve the prognosis of patients with stable COPD [61]. Our previous studies demonstrated that TXL protects cardiac microvascular endothelial cells (CMECs) against reperfusion injury [62], significantly increases the microvascular density of the ischemic brain tissue [63], and improves the microangiopathy of diabetic nephropathy [64], revealing that the common target of TXL in the treatment of major diseases of heart, brain, and diabetic nephropathy is microvascular endothelial cells. To the best of our knowledge, the present study, for the first time, clarified the protective effects of TXL on pulmonary microvascular endothelial cells in cigarette smoke combined with high-fat diet-induced COPD combined with AS mice. Statins are a commonly used lipid-lowering drug in clinical practice. Several studies have shown that statins also reduce inflammation [65], inhibit oxidative stress [66], protect endothelial cells [67], and stabilize plaques [68]. The results showed that TXL combined with Ato inhibited serum lipid levels and aortic endothelial injury biomarkers in COPD + AS group mice, reduced the expression of systemic inflammatory biomarkers, and inhibited the formation of atherosclerotic plaques better than Ato or TXL alone. Thus, it could be deduced that TXL can enhance the anti-atherosclerosis effect of Ato. On the anti-inflammatory side, Ato reduces the production of inflammatory mediators by reducing lipid production [69]. In this study, we observed that the pathomorphological and functional improvements were mainly due to a restoration of the decrease in the influx of inflammatory cells (macrophages and neutrophils) with a consequent reduction in the release of pulmonary inflammatory mediators (IL-1 β , TNF- α) and inhibit systemic inflammation. Another previous study showed that Ato can improve lung function in mice with emphysema induced by smoke exposure [70]. The Ato or TXL treatment groups showed improvement in the assessment of pulmonary mechanical parameters. After TXL-Ato combined treatment, the improvement of lung inflammation and lung function in mice was more significant than that of Ato, suggesting that TXL enhances the improvement of Ato on lung inflammation and lung function. Herein, we observed that TXL increases the expression of Tj protein (VE-cadherin and β -catenin) and reduced the pulmonary microvascular hyperpermeability, and Ato upregulates Tj proteins but has no significant effect on pulmonary microvascular hyperpermeability of the COPD + AS group mice. Notably, the TXL-Ato combined treatment significantly reduced the pulmonary microvascular hyperpermeability and increased the expression of Tj protein in mice caused by smoking combined with high-fat diet. Furthermore, TXL enhances the protective effect of Ato on pulmonary microvascular barrier. Therefore, it is speculated that Ato may reduce lung inflammation rather than protecting the pulmonary microvascular barrier and thus, delay the decline of lung function. TXL may inhibit pulmonary inflammation and trigger systemic inflammation through the protective effect of pulmonary microvascular barrier, thereby reducing the decline of lung function. This phenomenon was

confirmed in vitro: the cells treated with TXL significantly decreased the pulmonary microvascular permeability and showed an upregulated expression of Tj proteins. In line with the in vivo results, TXL also protected the pulmonary microvascular barrier function of HPMECs induced by CSE. Furthermore, the pre-treatment with TXL not only decreased the level of inflammatory markers (IL-6, IL-1 β , hsCRP, VCAM-1, and ICAM-1) but also reduced protein levels of NF- κ B. The results showed that TXL reduces the inflammatory response of HPMECs by protecting the function of pulmonary microvascular barrier. In conclusion, through the protective effect of pulmonary microvascular barrier, TXL inhibits pulmonary inflammation and systemic inflammation, thus enhancing the effect of Ato on COPD combined with AS.

Although the current findings were primarily obtained from high-quality studies, the deficiencies cannot be ignored: (1) a cigarette smoking group can be set up to explore the key link between high-fat diet aggravating cigarette smoking induced COPD by observing the degree of damage to the lung microvascular barrier; (2) the signaling pathway of TXL protecting the pulmonary microvascular barrier can be further explored to clarify the drug intervention target of TXL.

Conclusions

COPD aggravates AS, the mechanism is the destruction of pulmonary microvascular barrier function and thus lung inflammation triggers systemic inflammation. TXL enhances the anti-AS effect of Ato through the protective effect of pulmonary microvascular barrier in COPD combined with AS. This combined treatment regimen has prospects in clinical application.

Abbreviations

AS: Atherosclerosis; COPD: chronic obstructive pulmonary disease; Ato: Atorvastatin; TXL: Tongxinluo; HPMECs: human pulmonary microvascular endothelial cells; CSE: cigarette smoke extract; CVDs: cardiovascular diseases; NT-Pro-BNP: N-terminal pro-brain natriuretic peptide; cTnI: cardiac troponin I; 6MWD: 6-minute walk distance; CC-16: clara cell secretory protein-16; WT: wild-type; FRC: functional residual capacity; cdyn: dynamic compliance; FVC: forced vital capacity; FEV: forced expiratory volume; RI: inspiratory resistances; PBS: phosphate-buffered saline; pH: paraformaldehyde; HE: hematoxylin and eosin; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; DMEM: dulbecco's modified eagle medium; DMSO: dimethyl sulfoxide; FITC: Fluorescein Isothiocyanate; ELISA: enzyme linked immunosorbent assays; IL: Interleukin; IFN: Interferon; hsCRP: high sensitivity C-reactive protein; TEM: transmission electron microscopy; MLI: Mean linear intercept; TER: Transepithelial electrical resistance; ICAM-1: Intercellular cell adhesion molecule-1; VCAM-1: Vascular cell adhesion molecule-1.

Declarations

Authors' contributions JZH designed the experiments; WYF, LZ, and KXN participated in the experiments; LWY, YYJ, and CLP analyzed the data; HYL, WHT and LJQ provided the technical support and advices for

the study; KXN and WYF wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests The authors declare that they have no competing interests.

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Figures

Figure 1

Lung function tests data revealed that TXL-Ato combination improved the lung function of COPD+AS mice. CSE combined with high-fat diet induced a significant increase in the FRC and RI and a significant decrease in cdyn and FEV50/FVC in the COPD+AS group. The TXL-Ato combination improved the lung function, including reduced FRC, RI, and preserved cdyn and FEV50/FVC compared to the COPD+AS group. n = 8, *P < 0.05 vs. control group; ▲P < 0.05 vs. AS group; #P < 0.05 vs. COPD+AS group; △P < 0.05 vs. TXL+Ato group.

Figure 2

Effects of TXL–Ato combination on pulmonary microvascular barrier. A: Scale: 1 μ m. Transmission electron microscopy (TEM) of pulmonary microvasculature region. B: Evans blue content in lung tissue was measured in each group to assess pulmonary microvascular permeability. Gene expression levels of VE-cadherin(C), β -catenin(D) were determined by RT-qPCR. E: Immunofluorescent analysis of VE-

cadherin($\times 400$). $n = 3$, * $P < 0.05$ vs. control group; $\blacktriangle P < 0.05$ vs. AS group; $\#P < 0.05$ vs. COPD+AS group; $\triangle P < 0.05$ vs. TXL+Ato group.

Figure 3

Effects of TXL–Ato combination on pulmonary inflammation of COPD+AS mice. A: Scale: 50 μ m. Lung tissue morphology was examined by HE staining. B: Image Pro Plus was performed to measure the mean linear intercept (MLI). Each plot represents mean $\pm$ SEM from 7 different animals. The expression of IL-1 β (C), TNF- α (D) level in lung tissues was determined by RT-qPCR. The IL-1 β (E), TNF- α (F) levels in the supernatant of lung homogenates were measured according to ELISA. $n = 6$, * $P < 0.05$ vs. Control group; $\blacktriangle P < 0.05$ vs. AS group; $\#P < 0.05$ vs. COPD+AS group; $\triangle P < 0.05$ vs. TXL+Ato group.

Figure 4

Inflammatory factors (IL-1 β , IFN- γ and TNF- α) in serum were detected by ELISA. Cigarette smoke exposure combined with high-fat diet induced a significant increase in the levels of IL-1 β (A), IFN- γ (B), TNF- α (C) in the COPD+AS group, and TXL-Ato combination decreased the inflammation level compared to that in the COPD+AS group. n =6, *P <0.05 vs. control group; ▲P <0.05 vs. AS group; #P <0.05 vs. COPD+AS group; △P <0.05 vs. TXL+Ato group.

Figure 5

Cigarette smoke aggravates high-fat diet-induced AS lesions in AS and COPD+AS groups and TX-Ato combination attenuated atherosclerosis lesions compared to the COPD+AS group. A: AS lesion shown with oil red O staining; B: Quantitative analysis of plaque areas in the aortic sinus by Image Pro Plus software. C: Scale: 50 μ m. Aortic root morphology was examined by HE staining. Blood lipid levels TC (D), TG (E), HDL (F), LDL (G) of serum. RT-qPCR determines the mRNA levels of endothelial damage

biomarkers ICAM-1(H), VCAM-1(I) in the aorta tissue. n =6, *P <0.05 vs. control group; ▲P <0.05 vs. AS group; #P <0.05 vs. COPD+AS group; △P <0.05 vs. TXL+Ato group.

Figure 6

TXL protects HPMECs’ pulmonary microvascular endothelial barrier dysfunction induced by CSE. The permeability of the endothelial monolayer was tested by measuring the influx of FITC-dextran (A) and the transendothelial electrical resistance(TEER) (B). The expression of Tj proteins VE-cadherin (C), β-catenin

(D) were detected in HPMECs using Western blot. (A: Control group B: CSE group C: CSE+TXL200μg/mL D: CSE+TXL400μg/mL E: CSE+TXL800μg/mL). The expression of Tj proteins mRNA level of VE-cadherin (E), β-catenin (F) and Claudin5 (G) were detected by RT-qPCR. H: Tj proteins (VE-cadherin and β-catenin) were evaluated by immunofluorescence microscopy (×400). n =3, *P <0.05 vs. Control group; #P <0.05 vs. CSE group.

Figure 7

TXL against CSE-induced inflammation in HPMECs. The cell supernatants were assayed for hsCRP (A) , IL-6 (B), and IL-1β (C) by ELISA. D: NF-κB protein level was detected by Western blot.(A: Control group B: CSE group C: CSE+TXL200μg/mL D: CSE+TXL400μg/mL E: CSE+TXL800μg/mL). n =3, *P <0.05 vs. control group;#P <0.05 vs. CSE group.

A

B

Figure 8

TXL protects HPMECs from endothelial damage. RT-qPCR assay for endothelial damage biomarkers ICAM-1(A),VCAM-1(B) mRNA level in HPMECs. ICAM-1 and VCAM-1 mRNAs were significantly upregulated after HPMECs exposure to CSE, and pretreatment with TXL significantly counteracted CSE-induced ICAM-1 and VCAM-1 mRNA upregulation in HPMECs. n =3, *P <0.05 vs. control group; #P <0.05 vs. CSE group.