**Research Article**

**Effect of TAK242 on MCP-1 and TGF-β in COPD Rats**

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**Abstract**

**Objective:** To investigate the mechanism of MCP-1 and TGF-β regulation by TAK242 in COPD rats.

**Methods:** Thirty-six SD rats were randomly divided into normal, COPD control, and TAK242 groups. The normal group was freely fed, and the other groups used the method of fumigation plus lipopolysaccharide tracheal drip to establish an experimental animal model of COPD. After successful modeling, each experimental group received 0.9% NaCl solution and corresponding drugs by intraperitoneal injection for 7 d. After drug administration, lung function was examined; pathological changes in lung tissue were observed by light microscopy with hematoxylin-eosin staining; mRNA expression of MCP-1 and TGF-β was detected by q-PCR; and protein expression of MCP-1 and TGF-β in lung tissue was detected by Western blot and IHC, TGF-β protein expression in rat lung tissue.

**Results:** Compared with the normal group, rats in the COPD control group showed signs and symptoms of COPD, decreased lung function, and increased expression of MCP-1 and TGF-β. The TAK242 group showed decreased expression of MCP-1 and TGF-β compared to the COPD control group.

**Conclusion:** MCP-1, and TGF-β played a crucial role in the early stage of COPD fibrosis. TAK242 could ameliorate airway inflammation and inhibit the progression of COPD lung fibrosis in pre-existing rats in COPD model rats.

**Introduction**

COPD due to long-term inhalation of smoke and other pollutants, caused by inflammatory reactions, lung infections, or deposition of interstitial immune complexes in the lung and other factors, inducing the production of fibrotic cytokines, which is the main cause of the formation of chronic pulmonary fibrosis [1-3]. Pulmonary fibrosis is also a major challenge in the diagnosis and treatment of lung disease. It is therefore important to delay the progression of pulmonary fibrosis and to find effective treatments. As the pathogenesis of pulmonary fibrosis is better understood, there is increasing evidence that inflammation is also a recognized factor in the pathogenesis of pulmonary fibrosis [4]. Inflammatory responses such as immune inflammatory cells and cytokines play an important role in the development of pulmonary fibrosis. The inflammatory response in pulmonary fibrosis is caused by the increased expression of monocytes and macrophages in lung tissue; MCP-1 and TGF-β are two important causes leading to the infiltration of monocytes and macrophages into lung tissue [5,6]. MCP-1 is one of the major inflammatory factors involved in lung injury and plays a key role in exacerbating the progression of COPD pulmonary fibrosis. As a member of the inflammatory chemokine family, MCP-1 mediates the infiltration of a wide range of inflammatory cells in COPD lung fibrosis, with the number of monocytes and macrophages being directly proportional to the degree of interstitial fibrosis [5]. TGF-β is the major cytokine involved in the pathogenesis of pulmonary fibrosis in COPD. The inflammatory environment enhances oxidative stress-induced TGF-β expression [8], while TGF-β promotes cellular hypertrophy, ECM accumulation, and EMT.
the many regulators of EMT, TGF-β is considered an important factor in pulmonary fibrosis [9]. Although many studies have elucidated the formation mechanism of pulmonary fibrosis, the intrinsic influence of inflammatory mediators in the disease remains to be further investigated and confirmed. Inflammation inhibition has the characteristics of multi-pathway and multi-target, which has certain advantages in the prevention and treatment of pulmonary fibrosis, and plays an important role in the treatment of pulmonary fibrosis, with comprehensive therapeutic advantages in anti-inflammatory, antioxidant, and delaying pulmonary fibrosis [10-12]. Among them, TAK242 belongs to the toll-like receptor inhibitors, which affect the NF-κB signaling pathway, which belongs to the typical inflammatory pathway, and inhibition of which has the functions of reducing inflammation, improving the degree of fibrosis to regulate immunity and antioxidant [13,14], etc. In this study, based on previous studies and the efficacy of known drugs, we established a COPD model and observed the expression of pulmonary fibrosis factors MCP-1 and TGF-β in lung tissues to explore the therapeutic role and mechanism of TAK242 in the early stage of pulmonary fibrosis in COPD.

Materials and methods

Experimental animals

**Basic information:** Thirty-six healthy SPF-grade male SD rats with an average body mass of (180±10) g were provided by the Experimental Animal Centre of Ningxia Medical University (SYXX Ning 2020-0001), randomly divided into the COPD group and the normal group, with 13 rats in the normal group and 23 rats in the COPD group, all housed in a clean SPF grade environment. At the time of modeling, 3 rats each from the normal and COPD groups were randomly selected to verify the success of the model, and then the COPD group was randomly divided into a COPD control group (10 rats) and a TAK242 group (10 rats).

**Reagents and instruments:** Lipopolysaccharide (Sigma: L2880), TAK242(Solarbio), Taishan brand cigarettes (Shandong China Tobacco Company), TIANGEN RNA extraction kit (DP431), reverse transcription kit (Takara: RR036A), Q-PCR kit (Takara: RR820A), primers (Shanghai Sangong Co., Ltd.); homemade staining boxes (70 cm×55 cm×35 cm), and a homemade staining box (70 cm×55 cm×35 cm), smoke generator (Buxco, USA), small animal lung function meter (Buxco, USA), UV-visible spectrophotometer (Thermo, USA), fluorescence quantitative PCR (Bio-Rad, USA).

Methods

**COPD rat model preparation:** Lipopolysaccharide (200 μg/time) was injected into the trachea of rats in the COPD group on days 1, 14, 28 and 42; the rats were placed in a home-made poisoned box for passive smoking on days 2-13, 15-27 and 29-41 (2 times a day, each time for 60 min, with an interval of > 8 h between the two times, and each time for 10 cigarettes); the modeling was performed for a total of 42 d [15-17]. The rats in the normal group received no special treatments. At the end of the modeling, 3 rats from each of the COPD and normal groups were randomly selected and the airflow limitation of the COPD group rats was assessed using a small animal spirometer; The rats were anesthetized with sodium pentobarbital, and then the chest was opened after execution under anesthesia, and the lung tissues were collected for HE staining, which verified that the lung function and histopathological changes of the COPD group rats were consistent with the characteristics of COPD, and confirmed that the COPD rat model was successfully established. The rat model of COPD was successfully established.

**Intervention program:** 1mg/ml Lipopolysaccharide solution: Prepare according to the following recipe, dispense, and place in a 4 °C refrigerator.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide</td>
<td>10 mg</td>
</tr>
<tr>
<td>0.9% saline</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

**1 mL TAK242 working solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Dose</th>
</tr>
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<tbody>
<tr>
<td>TAK242</td>
<td>5mg</td>
</tr>
<tr>
<td>DMSO</td>
<td>50 μL</td>
</tr>
<tr>
<td>PEG300</td>
<td>400 μL</td>
</tr>
<tr>
<td>Tween80</td>
<td>50 μL</td>
</tr>
<tr>
<td>ddH2O</td>
<td>500 μL</td>
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</tbody>
</table>

**Marginal notes:** Take 50 μL of 102 mg/ml of clarified DMSO stock solution and add it to 400 μL of PEG300, mix well to make it clarified; add 50 μL of Tween80 to the above system, mix well to make it clarified; and then continue to add 500 μL of ddH2O to fix the volume to 1 mL.

**Normal group:** free feeding, no intervention.

**COPD control group:** COPD rats were injected intraperitoneally with the same volume of saline once a day for 7 consecutive days.

**TAK242 group:** TAK242 was administered to COPD rats by intraperitoneal injection at 6 mg/kg once a day for 7 consecutive days.

**Pulmonary function measurements in rats:** Rats were anesthetized with 2% pentobarbital (50 mg/kg) by intraperitoneal injection, the trachea was incised in the middle of the neck, and the tracheal tube was inserted and placed in the body box. Total lung capacity (TLC), 200 ms rate (FEV200/FVC), and 100 ms rate (FEV100/FVC) were measured using a small animal spirometer.
Rat lung tissue sampling and pathological observation: Rats were killed by air embolization, and the left lung was removed from the open chest and immersed in 14% paraformaldehyde fixative for 24 h. The lung was rinsed with phosphate buffer solution and then serially sectioned by conventional paraffin embedding and stained with HE. The upper lobe of the right lung of rats was placed in a freezing tube, frozen in liquid nitrogen and then stored in a refrigerator at -80 °C.

Determination of MCP-1 and TGF-β mRNA in rat lung tissues: Total RNA was extracted from frozen right lung tissues according to the instructions of the TIANGEN RNA Kit, and the OD260/280 was measured by UV spectrophotometer at 1.8-2.0. cDNA was synthesized according to the instructions of the Reverse Transcription Kit, and the mRNA concentration was determined using a qPCR kit. The reaction system consisted of 20 μL, 10 μL TB Green Premix, 0.8 μL forward primer, 0.8 μL reverse primer, 2 μL DNA template, and 6.4 μL of sterilized water, and the reaction conditions were as follows: pre-denaturation at 95 ℃ for 30 s, and cycling for 40 times according to the following procedure: denaturation at 95 ℃ for 5 s, annealing at 57 ℃ for 30 s, and extension at 72 ℃ for 1 min. The relative expression of MCP-1 and TGF-β was determined by the 2-ΔΔCT method using the release curve of the instrument and ACTB as an internal reference.

Western blot detection of MCP-1, TGF-β protein expression: The rats in normal, COPD, and TAK242 groups were killed under anesthesia, then the chest was opened, the right lung tissues were quickly taken out and put into liquid nitrogen, the rat lung tissues were taken out and ground, and the protein immunoblotting method was applied for protein quantification of rat lung tissues for MCP-1 and TGF-β. Total tissue protein was extracted according to the instructions of the kit, and protein quantification was performed by the BCA method. Take 40μg of protein and add the sample buffer to denature for use. Preparation of SDS-PAGE gel, concentrated gel 100V voltage electrophoresis 40min, separated gel 120V voltage electrophoresis 1h, 200mA constant current wet rotation 80min, the protein will be transferred to the PVDF membrane, Li Chun red staining to observe the bands, TBST wash the membrane, 5% skimmed milk TBST closure of 1. 5h, add a primary antibody incubated at room temperature for 2h, TBST wash the membrane, add a secondary antibody reaction of 1h, TBST wash the membrane, ECL luminous reagent luminescence, film exposure development and fixation. TBST washed membrane, ECL luminous reagent luminescence, film exposure, development, and fixation. The images were captured and the results were scanned for absorbance using gel image analysis software, and the ratio of absorbance of MCP-1 and TGF-β protein bands to GADPH was used as the amount of MCP-1 and TGF-β protein expression. The differences in MCP-1 and TGF-β protein expression in rat lung tissues of the two groups were compared.

Immunohistochemical staining: After paraffin embedding and sectioning, tissue sections were routinely deparaffinized and dehydrated, 3% hydrogen peroxide (H2O2) deionized water was closed to peroxidase, the reaction was carried out for 10 minutes, and rinsed in distilled water for 5 minutes × 3 times. In citrate buffer, microwave heat repair, boiling twice, PBS rinse, 5 min × 3 times. Reagent A (blocking solution, blue liquid) was added dropwise, incubated for 20 min at room temperature, decanted, do not wash. Add the corresponding primary antibody dropwise, overnight at 4 ℃, 30 min at room temperature, and rinse in PBS, 5 min × 3 times. Drops of reagent B (the corresponding secondary antibody working solution, yellow liquid), room temperature incubation for 15 minutes, PBS rinse, 5 minutes × 3 times, drops of reagent C (horseradish enzyme-labeled streptavidin working solution, orange liquid), room temperature incubation for 20 minutes, PBS rinse, 5 minutes × 3 times. DAB color development (microscopic observation of the color of the color to terminate the color development), the tap water sufficiently washed, and hematoxylin restaining 30 seconds. Dewatering, transparency, sealing. The positive cells were determined by the presence of brownish-yellow granular deposits in the cytoplasm or nucleus. Comparison of the semiquantitative and localized expression of MCP-1 and TGF-β proteins in the lung tissues of four groups of rats.

Statistical methods

The experimental data were expressed by the mean standard deviation (x ± S), they were processed by SPSS statistical software. One-way ANOVA was used to compare multiple samples. With a difference of p < 0.05 being statistically significant.

Results

Significant effect on lung function in COPD rats

In order to verify the effect of COPD on the lung function of rats, we tested FEV100/FVC and FEV200/FVC parameters, and the results showed that there was a significant difference in FEV100/FVC and FEV200/FVC between rats in the control group and rats in the COPD group (p < 0.05), but there was no difference in the improvement of lung function of rats in the TAK242 group (p > 0.05) (Figure 1).

Different interventions have significant effects on the general condition and histopathological changes in the lungs of COPD rats

The rats in the normal group had normal activity and diet throughout the modeling period, shiny hair, good mood, and normal respiration; HE of lung tissue showed that the bronchial walls were regular without inflammatory cell infiltration and the alveolar structure was normal without pathological expansion (Figure 2A). Compared with the normal group, rats in the COPD group gradually reduced their activities and food consumption with the prolongation of the exposure time to LPS+ smoke, their fur became dull and pale yellow, and their mood was depressed, accompanied
by congestion of the eyeballs and the appearance of nasal secretions; HE of lung tissues showed mucus secretion and inflammatory cell infiltration of bronchial tubular lumen, submucosal glandular hyperplasia, disorganization of alveolar structure, and enlargement of alveolar lumen coalescing into pulmonary pustules (Figure 2B). Compared with the COPD control group, the TAK242 group showed a significant reduction in bronchiolar luminal mucus and inflammatory cell infiltration, and the rats' activity and diet improved significantly, with bright fur color and good mood. The HE of the lung tissue showed significantly less mucus secretion and inflammatory cell infiltration in the bronchial lumen, and the changes in the alveoli and glands were not obvious (Figure 2C).

**Different interventions regulate the expression of MCP-1 and TGF-β in lung tissue**

We used IHC to detect MCP-1 and TGF-β protein expression in rat lung tissues, and the results showed that the expression of each protein index was high in the COPD group and lowest in the normal group. Among them, the expression of MCP-1 protein in the lung tissue of rats in the COPD control group was significantly higher than that in the normal group; the expression of TGF-β protein in the lung tissue of rats in the COPD group was significantly higher than that in the control group; \( p < 0.05, \) Figure 3).

**Changes in MCP-1 and TGF-β protein levels in lung tissues of different intervention groups**

We used WB to semiquantitatively detect the results of the above IHC experiments, and the results showed that the expression trend of MCP-1 and TGF-β was consistent with the results of the IHC experiments, and the statistical analysis of the grey value suggested that the expression of MCP-1 and TGF-β was high in the COPD group, and the expression level of the normal group was low \( p < 0.05, \) Figure 4).

**Different interventions regulate the relative expression of MCP-1 and TGF-β mRNA in rat lung tissue**

There were differences in the levels of MCP-1 and TGF-β in the lung tissues of rats in each group. The levels of MCP-1 and TGF-β in the lung tissues of rats in the COPD control group were significantly higher than those in the normal group \( p < 0.05, \) (Figure 5).

**Discussion**

Pulmonary fibrosis is one of the complications of COPD [1,18], with mild clinical manifestations in the early stage and the following pathological features: early lesions are mainly confined to the alveolar wall, the alveolar lumen is filled with fluid and cellular exudates, and the structure of the alveoli is blurred and disappears; then there is the destruction of fibrous tissue, disorganization of the lung structure, and the gradual development of interstitial fibrosis. Numerous studies have shown the presence of inflammatory cell infiltration, elevated cytokines, and increased levels of inflammatory mediators in pulmonary fibrosis [19] and in the early stage of pulmonary fibrosis, monocyte/macrophage infiltration is present in all lung tissues [20]. Infilirating monocytes/macrophages destroy lung structures by releasing cytokines, inflammatory mediators, and oxygen free radicals, accelerating the process of lung tissue sclerosis. In this study, after the success of the COPD model, MCP-1 and TGF-β were found to be elevated.
in rat lung tissue, but the HE of lung tissue did not show signs of fibrosis, but the elevation of MCP-1 and TGF-β was positively proportional to chronic lung fibrosis, confirming that there is an obvious causal relationship between COPD and lung fibrosis. Our use of TAK242 to inhibit toll-like receptors resulted in a significant decrease in MCP-1 and TGF-β and had the function of delaying lung fibrosis. In our study, we found that TAK242 did not significantly improve lung function in rats, suggesting that inhibition of this inflammatory pathway was not able to reverse alveolar damage. Other studies have shown that inflammation is a key factor in the pathogenesis of lung fibrosis [21]. Although COPD is a recurrent inflammatory process, many cytokines are involved in the inflammatory response, such as interleukin-6 (IL-6), TGF-β, MCP-1, etc [7,22,23]. They play different roles and form a complex cytokine network. Among them, MCP-1 and TGF-β are closely associated with the progression of fibrosis. MCP-1, as a member of the inflammatory chemokine superfamily, can be released by a wide range of cells and is low expressed under physiological conditions. In contrast, under pathological conditions such as hyperglycemia and oxidized lipoproteins, thylakoid and endothelial cells can stimulate MCP-1 gene expression. On the one hand, high levels of MCP-1 an autocrine or paracrine stimulate the expression of IL-1 and TGF-β produced by alveolar cells and exacerbate endothelial cell injury. On the other hand, alveolar cells can be directly activated to produce myofibrillar proteins, leading to interstitial fibrosis.
synergizing with MCP-1 can in turn stimulate the production
in organisms, MCP-1 levels increase, while macrophages
Studies have shown that when recombinant TGF-β is present
TGF-β, which promotes fibrosis and leads to lung lesions [28].
released by activated monocytes/macrophages to produce
MAPK signaling pathway via inflammatory response factors
Meanwhile, high expression of MCP-1 can activate the p38-
nuclear transcription factor-κB (NF-κB) signaling pathway.
and enhances the transcription of MCP-1 through the
Smad signaling pathway, which promotes ECM aggregation
prolonged hyperglycemia [26]. TGF-β mediates the TGF-β/
associated with oxidative stress, which is increased by
Failure of the protective effect of TAK242 on COPD lung fibrosis
inflammatory situation in rats in vivo. In terms of mechanism
stance, in recent years, a growing body of new evidence has demonstrated that TAK242 has
broad therapeutic efficacy and comprehensive therapeutic
value with fewer side effects. Given these advantages,
TAK242 is a promising chemical agent. There are some possible limitations to this study. First, this experiment was
limited to animals only, which may lead to bias in clinical
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Conclusion
The present study demonstrated that TAK242 has a
promising protective effect against COPD-induced lung fibrosis. These beneficial effects were closely associated with the
downregulation of MCP-1 and TGF-β expression, which is important for the development of new strategies for the
prevention and treatment of pulmonary fibrosis in COPD.

Compliance statement
This research has been conducted in accordance with
ethical standards and regulatory guidelines, and has received full approval from the Laboratory Animal Ethical and Welfare Committee of the Laboratory Animal Center at Ningxia Medical University. The Laboratory Animal Ethical and Welfare Committee of Laboratory Animal Center has reviewed and approved the research protocols, ensuring the protection and welfare of any human participants, animals, or data involved. The study adheres to the principles outlined in relevant regulations. Approval for the study was granted under the protocol or project license/approval number IACUC-NYLAC-2022-067, with the approved date March 2022.

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Author contributions

All authors contributed to researching the data, discussing the content, writing the text, and reviewing or editing the manuscript before submission.

References


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