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Quercetin improves ESAT-6-induced pleural mesothelial cell fibrosis by activating the NRF2/HO-1 pathway

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ABSTRACT

Objective(s): This study investigated the mechanism by which quercetin suppresses oxidative stress and improves fibrosis in human pleural mesothelial cells (HPMCs) induced by the Mycobacterium tuberculosis-specific antigen early secretory antigen target protein-6 (ESAT-6) by activating the Nrf2/HO-1 signaling pathway, thereby suppressing oxidative stress.

Materials and Methods: An in vitro model of ESAT-6-induced HPMC fibrosis was established. The effects of various concentrations of quercetin on HPMCs were assessed using the CCK-8 assay. Markers of oxidative stress, such as superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH), were assessed. Immunofluorescence was utilized to detect levels of nuclear factor erythroid 2-related factor 2 (Nrf2), and western blot analysis was conducted to evaluate the protein levels of Nrf2, heme oxygenase-1 (HO-1), E-cadherin (E-cad), and α-smooth muscle actin (α-SMA).

Results: Quercetin significantly improved ESAT-6-induced HPMC proliferation, reduced the oxidative stress marker MDA, and decreased the fibrosis marker α-SMA levels. It also promoted the translocation of Nrf2 into the nucleus in ESAT-6-induced pleural mesothelial cell fibrosis. Furthermore, quercetin enhanced the enzymatic activity of antioxidants, particularly GSH and SOD, and increased the expression levels of HO-1, Nrf2, and E-cad.

Conclusion: The findings indicate that quercetin can inhibit oxidative stress by modulating the Nrf2 pathway and up-regulating HO-1 activity, thereby improving ESAT-6-induced pleural mesothelial cell fibrosis.

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Introduction

Tuberculous pleuritis, the most common form of extrapulmonary tuberculosis, is characterized by an inflammatory response primarily driven by immune and hypersensitivity reactions to *Mycobacterium tuberculosis* and its metabolic products (1). This condition frequently results in pleural fibrosis due to fibrin exudation and the proliferation of fibrous tissue following infection of the pleural cavity, leading to pleural thickening, adhesions, and encapsulated pleural effusion. If left unchecked, tuberculous pleuritis can progress and cause restrictive ventilatory dysfunction, significantly diminishing patients' quality of life (2). Therefore, understanding the pathogenesis of tuberculous pleural fibrosis and identifying effective intervention strategies are both valuable and necessary.

The process of epithelial-mesenchymal transition (EMT) is essential in the progression of tuberculous pleural fibrosis. Previous studies have demonstrated that excessive production of extracellular matrix (ECM) in this condition leads to abnormal deposition and fibrotic changes. Oxidative stress can induce abnormal expression of collagen and related factors, triggering EMT, which is significant in the context of fibrotic diseases (3). Nrf2, a key regulator of endogenous antioxidant defense, promotes the production of various active enzymes that provide notable antioxidant and anti-apoptotic cellular protection (4). Studies have shown that activation of Nrf2 can significantly improve pulmonary fibrosis (5, 6).

HO-1 is a key antioxidant enzyme that participates in both acute and chronic oxidative stress injuries, serving as a downstream target of Nrf2.Nrf2 promotes the synthesis of multiple anti-oxidant genes, including HO-1 (7). The

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Nrf2/HO-1 pathway plays a critical role in maintaining redox balance and mitigating fibrosis by alleviating oxidative stress(8). Therefore, therapeutic strategies targeting this pathway may significantly reduce tuberculous pleural fibrosis.

Quercetin, one of the most abundant flavonoids in the human diet, is found in various vegetables, fruits, and tea. Studies have demonstrated that quercetin possesses antioxidant and anti-inflammatory properties and can improve both pulmonary and hepatic fibrosis(9, 10). Additionally, research indicates that quercetin can activate Nrf2 to ameliorate myocardial fibrosis (11). ESAT-6 is a specific antigen of the Mycobacterium tuberculosis complex and also serves as a key virulence factor (12).The objective of this study is to elucidate the inhibitory impacts of quercetin on ESAT-6 induced pleural mesothelial cell fibrosis and explore the potential mechanisms of oxidative stress modulation through Nrf2 activation.

Materials and Methods

Reagents

Quercetin (with a purity of 97%) was sourced from Shanghai Macklin Biochemical Technology Co., Ltd. (Shandong, China). Primary antibodies, including rabbit anti-Nrf2(Proteintech), rabbit anti-HO-1, and rabbit anti-α-SMA, were purchased from Shanghai Beyotime Biotech Inc. (Shanghai, China), while mouse anti-E-cadherin and mouse anti-β-tubulin were sourced from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). Secondary antibodies were obtained from Proteintech™ Biotechnology Co. (Wuhan, China). ML385, an Nrf2-specific inhibitor that inhibits the activity of the Nrf2 transcription factor by binding to Neh1 (13, 14), was acquired from MedChemExpress (Shanghai, China).M199 and fetal bovine serum (FBS) were obtained from Pricella Biotechnology Co., Ltd. (Wuhan, China).

Cell culture and experimental protocol

The MeT-5A is an epithelial cell line from the mesothelium that was isolated from the pleural fluids of non-cancerous individuals (The MeT-5A cell line notes—https://www.atcc.org/products/crl-9444). MeT-5A cells were obtained from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Wuhan, China). The cells were grown in M199 medium enriched with 10% fetal bovine serum (FBS), 0.5% ITS-G, 3.3 nM epidermal growth factor (EGF), 400 nM hydrocortisone, and 1% penicillin-streptomycin (P/S), and were kept at 37 °C in an incubator with 5% CO_2.To determine the appropriate quercetin dose, MeT-5A cells were exposed to quercetin at concentrations of 2.5 μM, 5 μM, and 10 μM for 24 hr and 48 hr (15-17). Dissolve 5 mg of quercetin in 1.65 mL of DMSO to prepare a 10 mM stock solution. A model of cellular injury was established using ESAT-6 (5, 10, 20, 40 μg/ml), a specific antigen of *M. tuberculosis* (18-20). The MeT-5A cells were divided into six groups: control, ESAT-6 (10 μg/ml), ESAT-6 (10 μg/ml) + quercetin (2.5 μM), ESAT-6 (10 μg/ml) + quercetin (10 μM), ESAT-6 (10 μg/ml) + ML385 (20 μM), ESAT-6 (10 μg/ml) + ML385 (20 μM) + quercetin (10 μM). ML385 was added to the MeT-5A cells for 1 hour prior to the administration of quercetin and ESAT-6.

Cell viability assay

Cell viability was assessed using the CCK-8 assay (Beyotime, Shanghai, China). MeT-5A cells were seeded in 96-well plates at a density of 1×10^5 cells per well. After completing the experimental procedures, the original culture medium was substituted with 100 μl of serum-free medium, and then 10 μl of CCK-8 solution was added to each well. Absorbance readings were taken at 450 nm after a 1-hr incubation.

Immunofluorescence assay

Immunofluorescence staining was performed to detect the localization of Nrf2 in MeT-5A cells. The MeT-5A cells were cultured in 12-well plates and treated with ESAT-6 and quercetin for 24 hr. Following two washes with pre-cooled PBS, the cells were fixed at room temperature with 4% paraformaldehyde. Next the cells were treated with 0.1% Triton X-100 for permeabilization and incubated with the Nrf2 antibody at 4 °C. The nuclei were then stained using DAPI. Images were observed and captured using a microscope (VMF20A, MicroDemo, USA).

Measurement of MDA, SOD, and GSH activities

MeT-5A cells were lysed in phosphate-buffered saline and then centrifuged at 10,000 × g for 10 min, and assayed for oxidative stress markers (Beyotime, Shanghai, China). Thiobarbituric acid (TBA) method: MDA reacts with TBA under acidic heating conditions to form a red product. DTNB Colorimetric Assay: Reduced glutathione (GSH) reacts with DTNB reagent to form yellow TNB anion. Nitroblue Tetrazolium (NBT) Reduction Assay: SOD inhibits the reaction where superoxide anion reduces NBT to blue methanesulfonate. Enzyme activity is calculated based on the inhibition rate.

Western blot analysis

Protein extraction from MeT-5A cells was performed using RIPA lysis buffer from Booster (Wuhan, China). The protein concentration was measured using a BCA protein assay kit obtained from Beyotime (Shanghai, China). Proteins were resolved using 8-10% SDS-PAGE (Solarbio, China) and subsequently transferred to PVDF membranes (Millipore, USA). The membranes were incubated at room temperature for one hour with 5% skimmed milk for blocking, and then they were stored overnight at 4 °C with different primary antibodies (Nrf2 1:1500, catalog number:16396-1-AP; HO-1 1:3000, catalog number:AG2181; α-SMA 1:1500, catalog number:AG8004; E-cadherin 1:1500, catalog number:GB11082).After incubation, the membranes were washed three times with TBST and then exposed to a secondary antibody diluted to 1:6000 for one hour at a temperature of approximately 20–25 °C. β-Tubulin served as the internal standard. Target proteins were identified with an Enhanced Chemiluminescence (ECL) system, and the intensities of the bands were quantitatively analyzed using ImageJ software.

Statistical analysis

Data analysis was conducted using SPSS version 20.0, with results expressed as mean ± SD. Group differences were evaluated using one-way ANOVA, followed by Tukey's multiple comparisons test. When applicable, non-parametric tests such as the Kruskal-Wallis test and Dunn's multiple comparisons test were utilized. A *P*-value of less than 0.05 was regarded as statistically significant.

Results

Effect of quercetin on the activity of pleural mesothelial cells at different concentrations and times

The CCK-8 assay was utilized to assess changes in cell viability following treatment with quercetin for 24 and 48

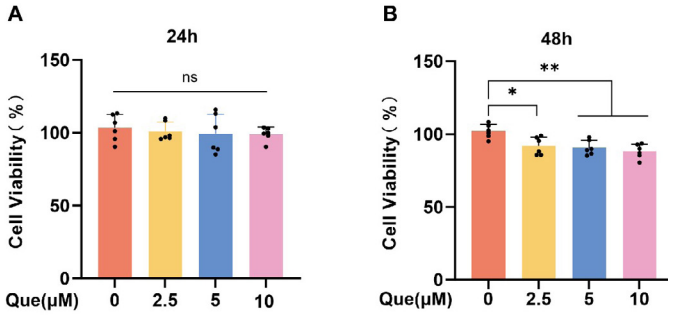

Figure 1. Effect of Quercetin on the activity of MeT-5A cells at different concentrations and times
Cell viability was assessed using CCK-8 assay (n=6). Data are expressed as means±SD. Intergroup comparisons were performed using one-way analysis of variance (ANOVA). * P<0.05, ** P<0.01.

hr. As depicted in Figure 1, at 24 hr, cell viability remained stable across the 2.5 μM, 5 μM, and 10 μM quercetin concentrations, showing no significant changes compared to the control group. However, at 48 hr, quercetin treatment inhibited cell proliferation in comparison to the control group. These findings suggest that quercetin at doses of 2.5 μM, 5 μM, and 10 μM exhibits no toxicity to pleural mesothelial cells within 24 hr.

Quercetin reduced the proliferation of pleural mesothelial cells induced by ESAT-6

A cell injury model of tuberculous pleuritis was established using the Mycobacterium tuberculosis-specific antigen ESAT-6. As shown in Figure 2A, at 24 hr, both 5 μg/ml and 10 μg/ml concentrations of ESAT-6 promoted cell proliferation, using the 10 μg/ml concentration having the most significant effect. Conversely, 20 μg/ml of ESAT-6 inhibited cell activity, while 40 μg/ml exhibited some toxicity. Figure 2B illustrates that quercetin at 2.5 μM and 10 μM significantly inhibited cell proliferation compared to the ESAT-6 model group, with 10 μM quercetin yielding the most pronounced effect. These results demonstrate that quercetin effectively inhibits ESAT-6-induced cell proliferation.

Quercetin reduced oxidative stress in esat-6-induced pleural mesothelial cell fibrosis

Quercetin exhibited antioxidant properties in the cell injury model in Figure 3. In the model group, MDA

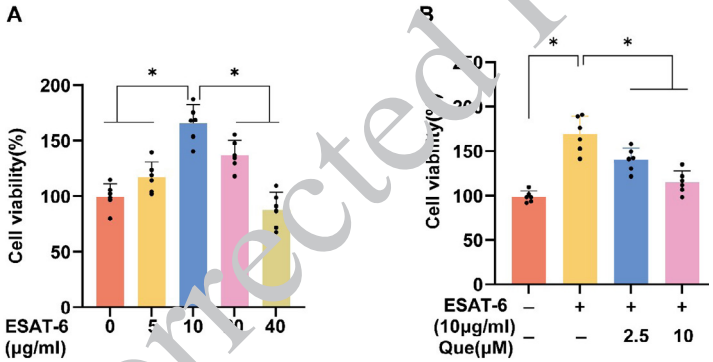

Figure 2. Quercetin reduced the proliferation of MeT-5A cells induced by ESAT-6
(A) Proliferation of MeT-5A cells during treatment with various concentrations of ESAT-6 for 24 hr. (B) Quercetin inhibited cell proliferation at 2.5 μM and 10 μM (n=6). Data are expressed as means±SD. Intergroup comparisons were performed using one-way analysis of variance (ANOVA). *P<0.05. ESAT-6: Early secretory antigen target protein-6

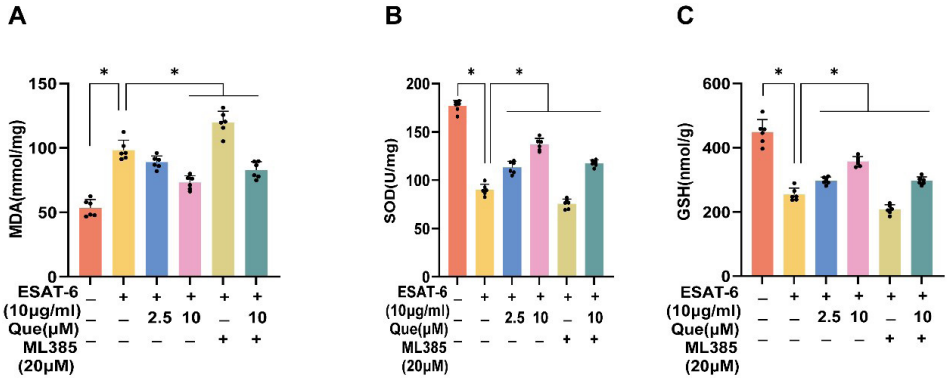

Figure 3. Quercetin reduced oxidative stress in ESAT-6 induced MeT-5A cells fibrosis (A:MDA,B:SOD,C:GSH) (n=6)
Data are expressed as means±SD. Intergroup comparisons were performed using one-way analysis of variance (ANOVA). *P<0.05.
ESAT-6: Early secretory antigen target protein-6; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione peroxidase

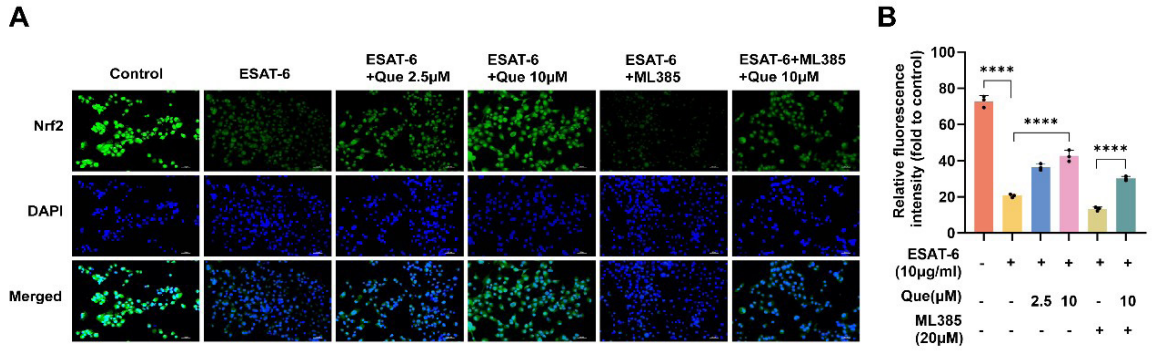

Figure 4. Quercetin promoted Nrf2 translocation into the nucleus in ESAT-6-induced MeT-5A cells fibrosis (n=3)
Data are expressed as means±SD. Intergroup comparisons were performed using one-way analysis of variance (ANOVA). ****P<0.0001. Nrf2: Nuclear factor erythroid 2-related factor 2; ESAT-6: Early secretory antigen target protein-6

concentrations were significantly elevated, while the levels of SOD and GSH were markedly reduced compared to the normal control group. Quercetin treatment led to elevated levels of SOD and GSH, along with a reduction in MDA concentrations, with the effects being more pronounced at 10 μM. The group receiving both quercetin and ML385 showed decreased SOD and GSH levels and increased MDA concentrations compared to the quercetin monotherapy group. These data illustrate that quercetin effectively reduces oxidative stress.

Quercetin promoted Nrf2 translocation into the nucleus in ESAT-6-induced pleural mesothelial cell fibrosis

We evaluated the impact of quercetin on the nuclear translocation of Nrf2 in pleural mesothelial cell fibrosis induced by ESAT-6. As shown in Figure 4, ESAT-6 treatment down-regulated Nrf2 expression in pleural mesothelial cells compared to the control group. This down-regulation could be reversed by treatment with 2.5 μM and 10 μM quercetin, with 10 μM proving to be more effective. Additionally, the expression of Nrf2 significantly decreased in the presence of the Nrf2-specific blocker ML385, while the addition of 10 μM quercetin improved Nrf2 levels. These results suggest that quercetin may enhance Nrf2 nuclear translocation in ESAT-6-treated pleural mesothelial cells by activating the Nrf2 signaling pathway.

Quercetin alleviated ESAT-6-Induced pleural mesothelial cell fibrosis

We assessed the effect of quercetin on ESAT-6-induced pleural mesothelial cell fibrosis. As shown in Figure 5, compared to the control group, the epithelial marker protein E-cadherin was reduced in the model group but improved with treatment at 2.5 μM and 10 μM quercetin, significantly at 10 μM. In contrast, the reduction of E-cadherin was more pronounced with the addition of ML385, although 10 μM quercetin showed improvement. Conversely, the mesothelial marker protein α-SMA increased in the model group, with a more significant rise following ML385 treatment. Quercetin treatment resulted in a reduction of α-SMA expression. These experimental results indicate that quercetin can mitigate fibrosis caused by ESAT-6.

Quercetin up-regulated the levels of Nrf2 and HO-1 expression in ESAT-6-induced pleural mesothelial cell fibrosis

As depicted in Figure 6, the expressions of Nrf2 and its downstream protein HO-1 were lower in the model group compared to the control group. However, quercetin intervention improved the expressions of Nrf2 and HO-1, with the effects being more pronounced at 10 μM. In contrast to the model group, the introduction of ML385 led to a more pronounced decrease in Nrf2 and HO-1 levels, while the addition of 10 μM quercetin demonstrated an improvement. These results suggest that quercetin may alleviate ESAT-6-induced pleural mesothelial cell fibrosis through the activation of the Nrf2/HO-1 pathway.

Discussion

Tuberculous pleuritis often occurs as a complication

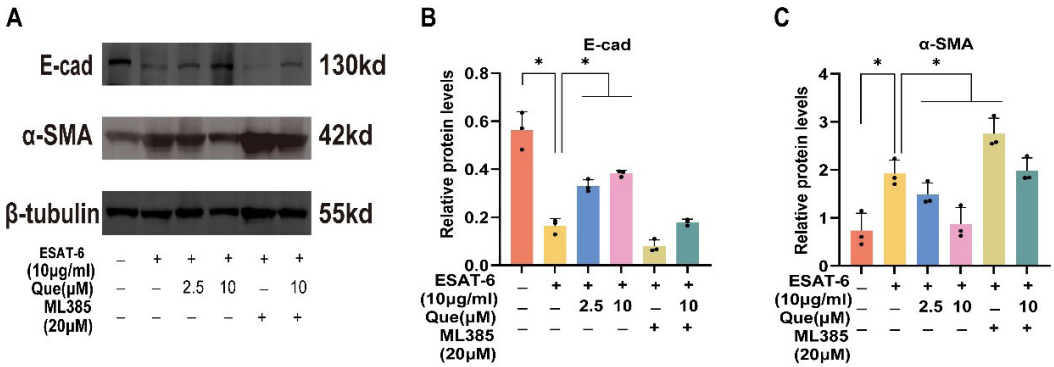

Figure 5. Quercetin alleviated ESAT-6-induced MeT-5A cells fibrosis (n=3)
Data are expressed as means±SD. Intergroup comparisons were performed using one-way analysis of variance (ANOVA). *P<0.05. ESAT-6: Early secretory antigen target protein-6

of pulmonary tuberculosis, leading to exudative pleural effusion and pleural fibrosis. The development of pleural fibrosis is particularly concerning, as delayed diagnosis or treatment can result in severe fibrosis, thoracic deformity, collapse, and ultimately chronic respiratory failure. Even among patients who successfully complete treatment, half may experience pleural adhesion thickening and fibrin deposition (21). Thus, the search for novel therapeutic agents to treat pleural fibrosis remains a critical clinical challenge. This study investigates the beneficial effects of quercetin on pleural mesothelial cell fibrosis induced by the Mycobacterium tuberculosis-specific antigen ESAT-6. Following quercetin intervention, oxidative stress markers and fibrosis-related protein expressions improved compared to the ESAT-6 group, highlighting quercetin's protective role against tuberculous pleural fibrosis. Additionally, in the quercetin-treated group, levels of Nrf2 and HO-1 proteins significantly increased, whereas these levels were inhibited upon the addition of the Nrf2 inhibitor ML385. Thus, quercetin may alleviate ESAT-6-induced pleural mesothelial cell fibrosis through the activation of the Nrf2/HO-1 pathway.

When Mycobacterium tuberculosis and its metabolites enter the pleural cavity, they can induce a hypersensitive state of the pleura, leading to increased pleural effusion and elevated peroxidase activity. Pleural mesothelial cells are essential in modulating the fibrinolytic and pro-coagulant activities associated with pleural effusion, acting as initiators of the cascade reaction. Recent studies indicate that pleural mesothelial cells are pivotal in the progression of pleural fibrosis (22). Our findings demonstrate that the *M. tuberculosis*-specific antigen ESAT-6 induces abnormal proliferation and fibrosis markers in pleural mesothelial cells, consistent with existing literature. Oxidative stress is closely related to fibrotic diseases, with established links to idiopathic pulmonary fibrosis and renal fibrosis (23). Inhibiting oxidative stress-induced activation and proliferation of hepatic stellate cells is a key strategy in anti-hepatic fibrosis treatment (24). Activation of oxidative pathways can lead to abnormal oxidation of lipid molecules and nucleic acids, triggering signaling abnormalities, disrupting cell membrane integrity, mitochondrial dysfunction, and DNA strand breaks ultimately resulting in myocardial cell fibrosis. However, the impact of oxidative stress on the progression of tuberculous pleural fibrosis has not been previously reported. Our study found that ESAT-6 induces increased levels of the oxidative stress marker MDA and decreases the activity of antioxidant enzymes SOD and GSH in pleural mesothelial cells. Additionall7y, there was a loss of E-cadherin expression and up-regulation of the mesenchymal marker α-SMA, indicating a dysfunction in oxidative stress and a diminished antioxidant capacity contributing to the progression of tuberculous pleural fibrosis. Therefore, exploring the impact of oxidative stress pathways in tuberculous pleural fibrosis is of significant practical importance.

Nrf2 is a key molecule regulating intracellular redox balance and controlling multiple signaling pathways. It acts as a sensor for antioxidant enzyme genes such as SOD and GSH. Upon activation, Nrf2 migrates to the nucleus and engages with antioxidant response elements (AREs), initiating the transcription of defense proteins like HO-1, thereby reducing ROS production (25). The Nrf2/HO-1 pathway is crucial for managing endogenous oxidative stress and has shown efficacy in mitigating oxidative damage. Numerous studies have demonstrated that activating Nrf2 and its related pathways provides protective effects against pulmonary, myocardial, renal, and oral mucosal fibrosis (26, 27). However, the role of the Nrf2/HO-1 pathway in the development of tuberculous pleural fibrosis has not been explored. Our study found that ESAT-6 inhibits the levels of Nrf2 and HO-1 proteins in pleural mesothelial cells, suggesting the potential for alleviating tuberculous pleural fibrosis by activating the Nrf2/HO-1 pathway. Quercetin, a major bioflavonoid, is a natural anti-inflammatory and antioxidant agent. Studies have shown that quercetin can improve pulmonary fibrosis by activating the Nrf2 pathway (28). Our research also confirmed that quercetin inhibits ESAT-6-induced proliferation of pleural mesothelial cells, up-regulates Nrf2 and HO-1 protein expression, activates antioxidant enzymes SOD and GSH, reduces oxidative stress marker MDA levels, improves E-cadherin expression, and down-regulates α-SMA expression. This indicates that quercetin may alleviate ESAT-6-induced pleural fibrosis by modulating the Nrf2/HO-1 pathway.

Conclusion

The results of this study suggest that quercetin may act as a modulator of the Nrf2/HO-1 signaling pathway, which could explain its protective effects against ESAT-6-induced pleural fibrosis.

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Authors' Contributions

N H and H L conceived the study; L C and Z Y performed literature search and wrote the initial draft; L C, Z Z, B T, and Z H performed experiments. Z Y, N H, and Y C revised the draft and prepared the final version of the manuscript.

Conflicts of Interest

The authors state that they have no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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