



## Curcumin ameliorates pulmonary fibrosis progression by inhibiting the fibrotic process of lung fibroblasts

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### ABSTRACT

The objective of this study was to analyze the effect of curcumin (Cur) on pulmonary fibrosis (PF), so as to provide new clinical evidence for future PF treatment. To achieve these goals, the researchers set up bought human lung fibroblasts MRC-5 as a control group without treatment, a model group for PF cell modeling, and an intervention group for Cur intervention after PF modeling. Cell proliferation capacity and cellular TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I, Collagen III, Bax, N-cadherin and E-cadherin protein expression were determined. The results show that markedly enhanced cell proliferation capacity and TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I and Collagen III protein levels were observed in the model group, while the cell activity and fibrosis degree in the intervention group were significantly decreased compared with the model group ( $P < 0.05$ ). In addition, the intervention group exhibited lower N-cadherin and Bax with higher E-cadherin than the model group ( $P < 0.05$ ). In addition, the team found that the inflammatory response and oxidative stress were also more significantly improved in the intervention group ( $P < 0.05$ ). These experimental results tell us that Cur can ameliorate the fibrotic process of PF by inhibiting the activity of MRC-5.

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### Introduction

Pulmonary fibrosis (PF) is an end-stage change of a major category of lung diseases characterized by fibroblast (FB) proliferation and massive aggregation of extracellular matrix (ECM), accompanied by inflammatory damage and tissue structure destruction, that is, normal alveolar tissue is damaged and abnormally repaired, resulting in structural abnormalities (scarring) (1). Men aged 40-50 are predisposed to PF, a condition that may only be initially manifested as dyspnea during strenuous activity, resulting in the fact that it is often ignored or misdiagnosed as other diseases. As PF progresses, dyspnea also occurs at rest, and severe PF can also present with progressive dyspnea (2-4). According to statistics, the disease has shown an increasing trend in recent years, with an average annual increase of PF cases exceeding 300,000 globally (5). Currently, there is no specific clinical drug for PF. For advanced PF patients, their mean survival time is merely 2.8 years, with a mortality even exceeding that of some malignancies (6). Therefore, finding new effective treatment options for PF is the focus and difficulty of current clinical research.

Curcumin (Cur), as a diketone compound, has shown excellent application results in the fields of anti-inflammation, antioxidation, and scavenging oxygen-free radicals in recent years, without inducing obvious toxic and side effects (7, 8). Moreover, Cur has been proven to be able to blunt the epithelial-mesenchymal transition (EMT) of hepatocytes, alleviate liver fibrosis progression (9), and inhibit autophagy and activate mTOR to reduce cardiac hypertrophy and fibrosis (10). This speaks volumes for the

potentially important application value of Cur for future treatment of PF, but so far few studies have confirmed our views.

Consequently, this study discusses the influencing mechanism of Cur in the fibrotic process of MRC-5 through in vitro experiments to confirm the role of Cur in PF, so as to lay a reliable foundation for the future clinical use of Cur in PF.

### Materials and Methods

#### Cells data

Human lung fibroblasts MRC-5 were purchased from ATCC and cultured in a 37°C, 5% CO<sub>2</sub> cell culture incubator using DMEM complete medium containing 10% fetal bovine serum, 100 mg/L penicillin and streptomycin.

#### Grouping and treatment

MRC-5 was divided into three groups: one group was regarded as a control group without any treatment; another group was set as a model group by establishment of in vitro PF cell model, and the third group was set as the intervention group by culturing PF cells with Cur adjusted to 25  $\mu$ mol/L by dimethyl sulfoxide after PF cell model establishment. All the above cultures lasted for 24 hours.

#### PF cell model establishment

The PF cell model was constructed using hypoxia-induced MRC-5 (11), and the cells were inoculated in 96-well plates and incubated in an incubator at 1% O<sub>2</sub>, 94% N<sub>2</sub>, 5% CO<sub>2</sub>, and 37°C for 48 h.

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### Cell proliferation assay

Cells grown to the logarithmic (Log) phase were digested with 0.25% trypsin and inoculated into the wells of a 96-well plate (100  $\mu$ L/well). Each group was equipped with 4 duplicate wells, and 10  $\mu$ L MTT solution was dripped into one well every 24 hours. The absorbance (490 nm) was detected by a microplate reader. Cells were also taken and inoculated in 12-well plates (300/well) and cultured for 14 d. The medium was changed every 3 d. Subsequently, the cells were stained with 2% crystal violet at 37°C for 30 min, and the cloned cells were counted under a light microscope to calculate the clonogenic rate.

### Detection of fibrosis, epithelial-mesenchymal transition (EMT) and apoptosis progression

Log-growth-phase cells were inoculated into the wells of a 6-well plate and quantified after RIPA lysis. Proteins were then separated by SDS-PAGE (10%) electrophoresis, after which 20  $\mu$ g of the total protein was sealed with TBS containing 5% skim milk for 1 h. The protein sample was then immersed in TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I, Collagen III, N-cadherin, E-cadherin, Bax and  $\beta$ -actin primary antibodies to incubate overnight with the temperature maintained at 4°C. After 3 TBS washes, a secondary antibody was added for room temperature incubation (2 h). The proteins were developed by chemiluminescence and quantified using Image J software.

### ELISA

0.25% trypsin lysis of cells. and the concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$ /6 (IL-1 $\beta$ /6), superoxide dismutase (SOD) and malondialdehyde (MDA) were detected according to the instructions of ELISA kits, all of which were purchased from Beijing TransGen Biotech.

### Statistical analysis

Data were statistically analyzed using SPSS25.0 software. All the results of this study were expressed by ( $\bar{x}\pm s$ ), and the chi-square test and Bonferroni post hoc test were used for comparison among groups, with  $P<0.05$  indicating the presence of statistical significance.

### Results

#### Effect of Cur on the growth capacity of PF cells

The cell growth was determined to be the highest in the model group and the lowest in the control group, with that in the intervention group in between ( $P<0.05$ , Fig. 1A). In the cell cloning experiment, it was seen that the model group had a cell cloning rate of (70.47 $\pm$ 4.69 %), which was the highest among the 3 groups, while the intervention group had a cell cloning rate of (46.20 $\pm$ 3.71 %), which was lower than the model group and higher than the control group ( $P<0.05$ , Fig. 1B).

#### Effect of Cur on apoptotic proteins in PF cells

As detected, it was seen that Bax protein expression in the model group was the highest and lowest among the three groups ( $P<0.05$ ), while Bax protein expression in the intervention group was (0.81 $\pm$ 0.04), which was lower than the model group and higher than the control group ( $P<0.05$ , Fig. 2).

#### Effect of Cur on PF cell fiber processes

Protein testing results revealed the most significantly elevated TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I and Collagen III levels in the model group among the three groups, confirming the presence of obvious fibrosis ( $P<0.05$ ); and comparatively, TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I and Collagen III protein levels in the intervention group were markedly lower, but were still higher compared with the control group ( $P<0.05$ , Fig. 3).

#### Effect of Cur on EMT of PF cells

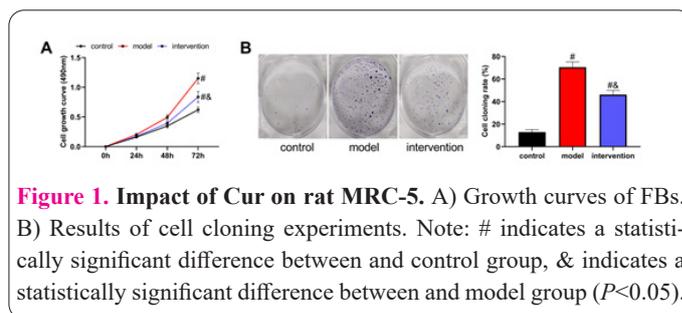
The results of EMT-associated protein detection showed that N-cadherin was the highest and E-cadherin was the lowest in the model group, while the opposite was true in control rats ( $P<0.05$ , Fig. 4).

#### Effect of Cur on the inflammatory response of PF cells

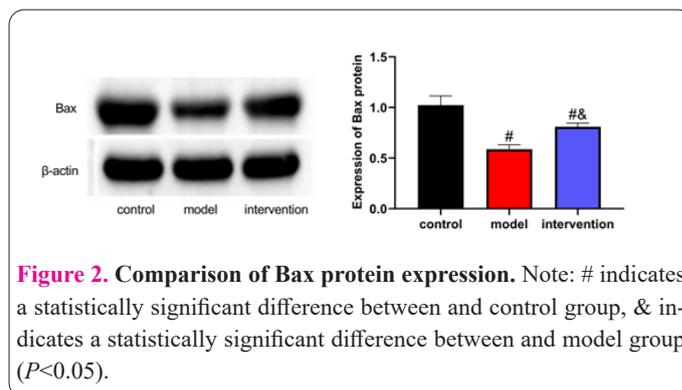
The results of inflammatory reaction tests showed that the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the intervention group were (37.44 $\pm$ 2.53 pg/L), (86.68 $\pm$ 3.46 pg/L) and (193.32 $\pm$ 6.65 pg/L), respectively, which were reduced compared with the model group but increased than the control group ( $P<0.05$ , Fig. 5).

#### Effect of Cur on oxidative stress response in PF cells

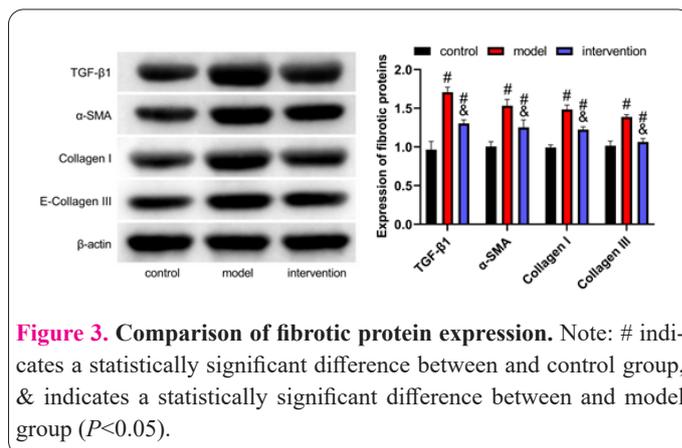
Finally, the results of oxidative stress markers in the 3



**Figure 1. Impact of Cur on rat MRC-5.** A) Growth curves of FBs. B) Results of cell cloning experiments. Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and model group ( $P<0.05$ ).



**Figure 2. Comparison of Bax protein expression.** Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and model group ( $P<0.05$ ).



**Figure 3. Comparison of fibrotic protein expression.** Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and model group ( $P<0.05$ ).

groups of cells showed that the model group had the lowest SOD and the lowest MDA among the 3 groups ( $P < 0.05$ ). The SOD of the intervention group was even lower than that of the control group, while MDA was higher than that of the control group ( $P < 0.05$ , Fig. 6).

## Discussion

The main manifestations of PF are diffuse pulmonary interstitial fibrosis with mild inflammation, the coexistence of FBs and normal lung tissue, and massive proliferation and deposition of ECM, leading to the formation of honeycomb tissue (12). It typically presents with cough and expectoration, as well as progressive dyspnea in the later stage, with recurrent attacks (13). In recent years, the excellent application effect and safety of traditional Chinese medicine in a variety of pulmonary diseases also provided a new research direction for future treatment of PF (14). By exploring the influence of Cur on PF, this study is undoubtedly of important reference significance for subsequent research.

Cur is a chemical component extracted from the roots of some Zingiberaceae and Araceae plants that contain about 3-6% of Cur, which is a rare pigment with diketones in the plant kingdom and is a diketone compound (15). With the deepening of research on Cur, it has been found

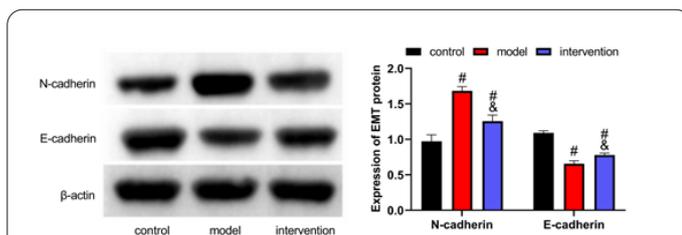
to possess a wide range of pharmacological activities such as anti-inflammatory, anti-oxidation, lipid-regulating, anti-virus, anti-infection, anti-tumor, anti-fibrosis and anti-atherosclerosis actions (16). In this study, we first established an in vitro model of PF by MRC-5. Cell proliferation capacity and TGF- $\beta$ 1,  $\alpha$ -SMA, Collagen I and Collagen III protein levels were found to be markedly elevated in the model group, suggesting enhanced activity of PF-modeled cells and obvious fibrotic process. While Cur-treated cells exhibited higher activity and fibrosis degree than control cells, they are significantly lower compared with the model group, suggesting that Cur can effectively inhibit the fibrotic process of MRC-5. Previous research has also reported the role of Cur in effectively inhibiting cardiac FB activity and lung fibrotic process (17), consistent with our experimental results, initially confirming that Cur has excellent anti-fibrosis effects. As we all know, the fibrotic process is closely related to cell EMT. When cells undergo EMT, epithelial cells lose their polarity, the cell adhesion system disappears, the cytoskeleton changes, and the motor structure of cells generates, which enhances epithelial cell deformation, migration, motor ability and anti-apoptosis ability and promotes the aggregation of FBs in large quantities, thus inducing fibrosis (18). Reviewing previous studies, we also found that Cur has the ability to regulate EMT in renal cells and breast cancer cells (19, 20). Thus, we also detected changes in EMT marker proteins in each group. The results identified notably reduced N-cadherin and enhanced E-cadherin in the intervention group compared with the model group, confirming the effective inhibitory effect of Cur on the EMT process in MRC-5, which may be the basis for Cur to ameliorate PF.

Subsequently, to further confirm the influence of Cur on PF, we also examined the inflammatory response and oxidative stress of cells in each group. The results showed that IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and MDA were significantly reduced in the intervention group, while SOD was elevated, again supporting that Cur has excellent therapeutic effects on PF. Previous pathological studies on Cur have also repeatedly confirmed its inhibitory effect on tissue inflammation and oxidative stress (21, 22), which can also validate the accuracy of the experimental results.

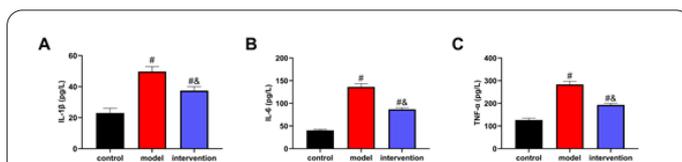
However, it has also been mentioned in previous studies that Cur can interfere with EMT in pancreatic cancer cells through the IL-6/ERK/NF- $\kappa$ B axis (23) and induce EMT and vascular fibrosis by regulating the c-Met-dependent PI3K/Akt/mTOR pathway in lung cancer (24). Yet, the pathway by which Cur affects MRC-5 remains unclear, warranting a more detailed analysis of its action pathway in subsequent studies.

In addition, in this study, we did not conduct more detailed tests on the biological behavior of MRC-5, such as cell apoptosis and cycle change, which needs to be supplemented and confirmed by subsequent analysis. Moreover, different doses of Cur were not designed to intervene in rats and cells, so the Cur dosage remains to be confirmed. Finally, we need to carry out clinical trials as soon as possible to confirm the medicinal value of Cur.

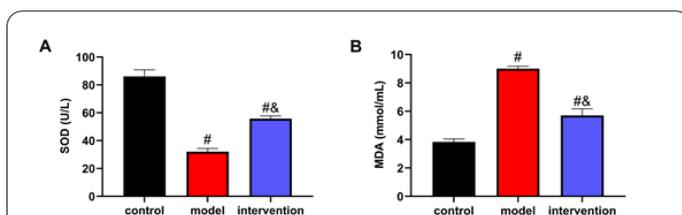
Cur can effectively inhibit the activity of MRC-5 and ameliorate the fibrotic process, inflammatory reaction and oxidative stress of PF, which is expected to be a new choice that may provide a more reliable guarantee for the health and life safety of PF patients in the future.



**Figure 4. Comparison of EMT protein expression.** Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and model group ( $P < 0.05$ ).



**Figure 5. Effect of Cur on the inflammatory response of PF cells.** A) Comparison of IL-1 $\beta$ . B) Comparison of IL-6. C) Comparison of TNF- $\alpha$ . Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and the model group ( $P < 0.05$ ).



**Figure 6. Effect of Cur on oxidative stress response in PF cells.** A) Comparison of SOD. B) Comparison of MDA. Note: # indicates a statistically significant difference between and control group, & indicates a statistically significant difference between and the model group ( $P < 0.05$ ).

**Ethical approval**

Not applicable.

**Competing interests**

The authors report no conflict of interest.

**Author contributions**

MQ designed the study and drafted the manuscript. DSL, and YH, analyzed the data. QMC, HC, XQL, and QSP conducted experiments, SYL and YJ, collected data. YW, revised manuscript. All authors read and approved the final draft of the manuscript.

**Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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