

# Baicalin Alleviates Silica-Induced Lung Inflammation and Fibrosis by Inhibiting TLR4/NF- $\kappa$ B Pathway in Rats

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## Summary

Silicosis is an occupational lung disease caused by inhaling silica dust. The disease is characterized by early lung inflammation and late irreversible pulmonary fibrosis. Here we report the effect of Baicalin, a main flavonoid compound from the roots of Chinese herbal medicine Huang Qin on silicosis in a rat model. Results showed Baicalin (50 or 100 mg/kg/day) can mitigate the silica-induced lung inflammation and reduce the harm of alveolar structure and the blue region of collagen fibers in rat lung at 28 days after administration. At the same time, Baicalin also diminished the level of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in lung tissues. The protein expression of collagen I (Col-1),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and vimentin were down-regulated while E-cadherin (E-cad) was increased in Baicalin-treated rats. In addition, the Toll Like Receptor 4 (TLR4)/nuclear factor kappaB (NF- $\kappa$ B) pathway was enabled at 28 days after silica infusion, and the treatment of Baicalin diminished the expression of TLR4 and NF- $\kappa$ B in the lungs of rat with silicosis. These results suggested that Baicalin inhibited the pulmonary inflammatory and fibrosis in a rat model of silicosis, which could be attributed to inhibition of the TLR4/NF- $\kappa$ B pathway.

## Key words

Baicalin • EMT • Pulmonary fibrosis • Silica • TLR4 • NF- $\kappa$ B

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## Introduction

Silicosis is an important occupational pneumoconiosis aroused by the inhalation of silica in occupational environment over the world, and once diagnosed, fibrotic lesions in lung tissue are hard to cure and reverse [1-2]. Silicosis is a chronic, progressive lung disease characterized by early inflammation, silicon nodules and formation of diffuse pulmonary fibrosis [3]. It is well accepted that inflammation, oxidative stress, apoptosis and epithelial-mesenchymal transition (EMT) are involved in development of silicosis. However, the pathogenesis of silicosis is very complex and has not been clarified, and there is no desirable drug to treat silicosis [4]. Therefore, it's critical to find secure and valid novel drugs for the healing of silicosis.

Inflammation is highly correlated with silicosis. It is well known that inhalation of silica in lung firstly stimulates alveolar macrophages to secrete and release inflammatory factors, which mediated inflammatory signaling pathways and promote the progress of pulmonary fibrosis. Among inflammatory factors and inflammatory signaling pathways, the inflammatory

factors of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, TGF- $\beta$ 1 and the inflammatory signaling pathways of inflammasome and NF- $\kappa$ B are associated to the development of lung fibrosis [5]. It has been reported IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and TGF- $\beta$ 1 promote the aggregation and proliferation of fibroblasts, resulting in extracellular matrix (ECM) deposition and information of pulmonary fibrosis [5]. Moreover, EMT is one of the vital progresses in the pathogenesis of silicosis, and TGF- $\beta$ 1 was considered a key mediator to induce EMT process [6]. More and more studies have shown that the EMT is involved in development of progressive pulmonary fibrosis in silicosis and inhibition of it could be ameliorated silica-induced pulmonary fibrosis [7-8]. At the molecular level, important features of EMT are the loss of epithelial surface markers especially E-cad and the increase of mesenchymal markers, covering vimentin and  $\alpha$ -SMA [9], resulting in ECM deposition and promoting fibrosis.

TLR4 belongs to the pattern recognition receptor family and acts as lipopolysaccharide sensor playing an important role in the fibrosis disease [10-11]. NF- $\kappa$ B is a multi-protein complex that can regulate various genes essential for immunity and inflammation [12], and it is also a downriver key factor of the TLR4 signaling, which plays a crucial role in activating inflammatory response and inducing EMT [13]. Meanwhile, some studies have found that the NF- $\kappa$ B target gene is significantly up-regulated during EMT process [14]. Furtherly, studies have shown that mesenchymal stem cells from human umbilical cord played an anti-inflammatory role by restraining the activation of TLR4/NF- $\kappa$ B signaling pathway, thus suppressing the process of EMT and renal fibrosis [15]. However, there are few studies on the relationship between TLR4/NF- $\kappa$ B signaling pathway and the EMT process in silicosis fibrosis.

Baicalin (5,6-dihydroxyflavone 7-O- $\beta$ -D-glucuronide; molecular weight = 446.36) is a major flavonoid compound separated from the roots of the Chinese herb named Huangqin (*Scutellaria baicalensis* Georgi) [16]. It is reported that Baicalin exerts various effects such as anti-apoptosis and anti-oxidation, especially in anti-inflammatory [17-18]. Previous studies have also confirmed that Baicalin inhibited microglia-induced neuroinflammation by suppressing NLRP3 inflammasome and TLR4/NF- $\kappa$ B signaling pathway activation [19]. Baicalin weakened inflammatory factors and regulated the TLR4 signaling pathways to inhibit the production of inflammatory factors which oxygen-

glucose deprivation-induced in microglial cells [20]. In addition, other studies have confirmed that Baicalin could alleviate bleomycin-induced pulmonary fibrosis and fibroblast proliferation in rats [21], indicating that Baicalin may have anti-fibrosis effects. However, it is unclear whether Baicalin has a protective effect on lung inflammation and fibrosis caused by silica through inhibiting TLR4/NF- $\kappa$ B signaling pathway. In this study, we aimed to investigate the potential effect and underlying molecular mechanism of Baicalin on silicosis rats.

## Materials and Methods

### *Preparation of reagents*

Silica powder was purchased from Sigma Aldrich (S5631, Shanghai, China). TAK-242, a Toll-like receptor 4 (TLR4)-specific signaling inhibitor [22], was obtained from MedChem Express (Princeton, US). Baicalin was purchased from Shanghai Yuanye Biological Limited Company (Shanghai, China). The silica powder prepared into a 50 mg/ml silica suspension with sterile saline, and then autoclaved it for 30 minutes before tracheal perfusion.

### *Animals and treatments*

Forty-two male Wistar rats, weighting 190-210 g, were purchased from Jinan Pengyue Experimental Animal Breeding Limited Company (Jinan, China). Before the experiment, the rats were placed in an air-conditioned room with temperature of 23 $\pm$ 3  $^{\circ}$ C, humidity (40-70 %), conventional light/dark cycle of 12 h, and adapted for 1 week. The rats were allowed free access to food and water. After experimental silicosis induction, all rats were randomly divided into 6 groups: control group, model group, TAK-242 group, Baicalin group (50 or 100 mg/kg/day) and Baicalin control group, with 7 rats in each group. Rats in the model, the TAK-242 and Baicalin groups received a single intratracheal instillation of 1.0 ml silica solution (50mg/ml) to induce silicosis as previously described [23]. Rats in control groups and Baicalin control groups were administered an intratracheal injection of 1.0 ml saline instead of silica. The rats of TAK-242 group were injected with TAK-242 (10 mg/kg) by tail vein at 30 minutes before tracheal instillation of silica. From the second day after silica exposure, Baicalin was administered once daily by oral gavage at 50 or 100 mg/kg/d to the rats in the Baicalin group, and rats in the control, model and the TAK-242

groups were treated with 0.9 % sodium chloride solution similarly. Baicalin control group was treated with 100 mg/kg/d Baicalin. After 28 days intratracheal instillation of silica or saline, all animals in each group were sacrificed by intraperitoneal injection of 3 % pentobarbital sodium and collected lungs for further studies. All animal experiments were conducted at Shandong Institute of Occupational Health and Occupational Medicine and approved by its Medical Ethics Committee (Protocol No: 20190004).

#### *Histopathological examination*

The lung tissues were separated from rats, cleaned with normal saline, dried with filter paper. The left upper lungs fixed in 4 % paraformaldehyde solution and then dehydrated and embedded in paraffin, were cut into 4 $\mu$ m and stained with hematoxylin and eosin (H&E) and Masson. Inflammatory cell infiltration and collagen deposition were observed under an optical microscope (Nikon Corporation, Japan). Meanwhile, Szapiel's method was used to evaluate the extent of alveolar inflammation and pulmonary fibrosis [24].

#### *ELISA analysis*

Right lung was homogenized on ice and the supernatant was carried out according to the ELISA kit instructions (Nanjing Jiancheng Bioengineering Institute, China), and the absorbance value of each well was measured at the wavelength of 450nm with a microplate reader. According to the formula provided in the instructions, the level of TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$  and IL-6 in each group was obtained. The level of each cytokine in lung homogenate supernatant was expressed as pg cytokine/ml of protein.

#### *Immunohistochemical analysis*

Firstly, the paraffin was removed from the tissue section and the antigen was recovered by citrate buffer. The parts were hatched with H<sub>2</sub>O<sub>2</sub> for 25 minutes, and then the sections were blocked with 3 % bovine serum albumin (BSA) for 30 minutes. The portions were hatched with primary antibodies against TLR4 (Wuhan servicebio Technology Limited Company, Wuhan, China),  $\alpha$ -SMA and Col-I (Abcam, USA) overnight at 4 °C, after that the horseradish peroxidase (HRP) marked secondary antibody incubated it at 37 °C for 50 minutes. With the hematoxylin counterstained the slices for 3 minutes and an image was obtained through a microscope for analysis.

#### *Western blot*

The right lungs in each group rats were homogenized with RIPA and PMSF, and centrifuged at 12,000 rpm for 10 min to gather the supernatant. The BCA protein assay kit (Wuhan Servicebio Technology Limited Company, Wuhan, China) was used to calculate the supernatant protein concentration. Proteins of equal weight (20 $\mu$ g) were separated through SDS-PAGE and transferred to PVDF membrane (Millipore, MA, USA, 200 mA, 1 h). The membranes were sealed with 5 % skim milk powder for 1h, washed with PBST for 3 times and then incubated with different antibodies NF- $\kappa$ B p65 (Santa Cruz Biotechnology, Inc, Europe), myeloid differentiation factor 88 (MyD88), p-NF- $\kappa$ Bp65, inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ ), p-I $\kappa$ B $\alpha$  and vimentin (Cell Signaling Technology, USA), TLR4, Col-I, E-cad, and  $\alpha$ -SMA (Abcam, USA), and  $\beta$ -actin (Santa Cruz, USA) overnight at 4 °C. After the membranes were washed with PBST for 3 times, the secondary antibodies (Wuhan Servicebio Technology Limited Company, Wuhan, China) were used to incubated it for 1 hours at room temperature. Afterwards, the membranes were visualized with Li-Cor and analyzed with Image Studio software.

#### *Quantitative real-time PCR (qRT-PCR)*

According to the TRIzol reagent (Gibco, USA) instruction extracted total RNA from rat lung tissue. The RNA concentration was calculated by a NanoDrop (Thermo Nanodrop 2000, America) and made the final concentration at 100-500 ng/ $\mu$ L level. Inactivate the reverse transcriptase on a PCR machine (ABI Stepone plus, America), and establish a Real-Time PCR reaction system with ACTIN as an internal reference. The data were analyzed using the method of relative gene expression changes ( $2^{-\Delta\Delta Ct}$ ). Performed each sample in triplicate, and the results are expressed as relative mRNA levels. The primer sequences are shown in Table 1.

#### *Statistical analysis*

In this study, all data was analyzed with the SPSS 22.0 software. Measurement data is tested for normality with K-S and which obeys normal distribution are represented by means  $\pm$  standard deviation. One-way ANOVA was used to compare the means among multiple groups. When comparing the means between groups, if the variance is uniform, the LSD t-test was used. If not, the Dunnett-T3 test was used, and the test level is  $\alpha=0.05$  (two-sided).

**Table 1.** PCR primer sequences used

Primer name	Primer sequences (5'-3')
R- $\beta$ -Actin-S	TGCTATGTTGCCCTAGACTTCG
R- $\beta$ -Actin-A	GTTGGCATAGAGGTCTTTACGG
R-TLR4-S	CCAGGTGTGAAATTGAGACAATTG
R-TLR4-A	AAGCTGTCCAATATGGAAACCC
R-MYD88-S	AAGGTGTCGTCGCATGGTG
R-MYD88-A	TTGGTGCAAGGGTTGGTATAGT
R-NF- $\kappa$ Bp65-S	AGAACAGCAAGGCAGCACTCC
R-NF- $\kappa$ Bp65-A	AGGTGTCGTCATCGTAGGT
R-I $\kappa$ B $\alpha$ -S	CACCAACTACAACGGCCACAC
R-I $\kappa$ B $\alpha$ -A	ACTTCAACAGGAGCGAGACCAG
R-Col-I-S	CCCAGCGGTGGTTATGACTT
R-Col-I-A	TCGATCCAGTACTCTCCGCT

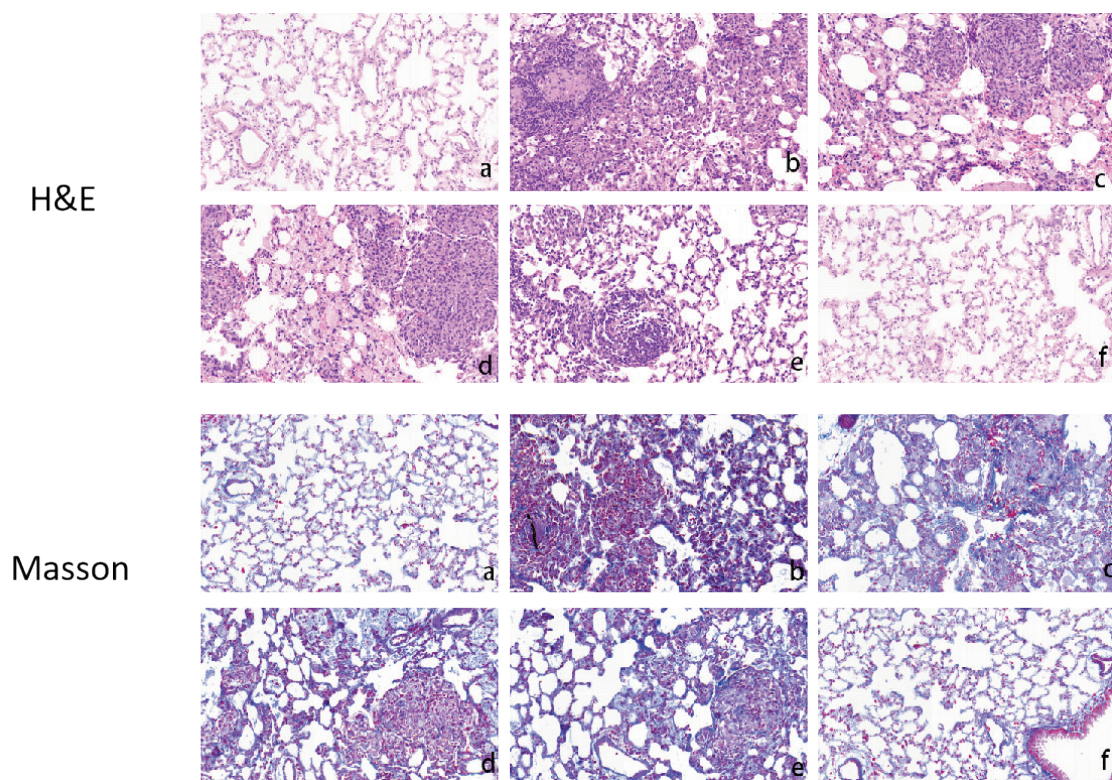
## Results

### Histopathological evaluation of lung tissue

HE staining showed normal lungs in the control group (Fig. 1a) and the Baicalin group (Fig. 1f) with a thin alveolar septum, well-organized alveolar space and no inflammation. Silicosis model group (Fig. 1b) showed serious inflammatory response, significantly increased alveolar septum thickness and initial formation of silicon

nodules, indicating silicosis model group occurred lung inflammation and silica lung fibrosis. In contrast, improvements in lung structure with less alveolar space collapse, reduction of widened and thickened alveolar septum and silica nodules were observed in the TAK-242 (Fig. 1c) and Baicalin groups (50mg/kg or 100mg/kg) (Fig. 1d,e), suggesting that the lung inflammation and lung fibrosis were relieved in TAK-242 and Baicalin groups (50mg/kg or 100mg/kg). With Masson staining, the model group presented obvious collagen fibers compared with control and Baicalin control groups (Fig. 1a,b,f), revealing that the lungs of model group have lung fibrosis.

Compared with the model group, the deposition area of blue collagen fibers in Baicalin groups (50mg/kg or 100mg/kg, Fig. 1d,e) and TAK-242 groups (Fig. 1c) was significantly smaller than that in the model group. These results showed that TAK-242 and Baicalin could ease lung fibrosis. Meanwhile, based on Szapiel's method, the degree of alveolitis and pulmonary fibrosis were quantified and calculated statistically by two professional pathologists, and the calculation results were shown in Table 2. The results displayed that the scores of alveolar inflammation and pulmonary fibrosis in the silicosis-model group were obviously higher than control



**Fig. 1.** Histopathological evaluation of lung tissue ( $\times 200$ ). **a)** Control group; **b)** Model group; **c)** TAK-242 group; **d)** Baicalin (50mg/kg) group; **e)** Baicalin (100mg/kg) group; **f)** Baicalin control group.

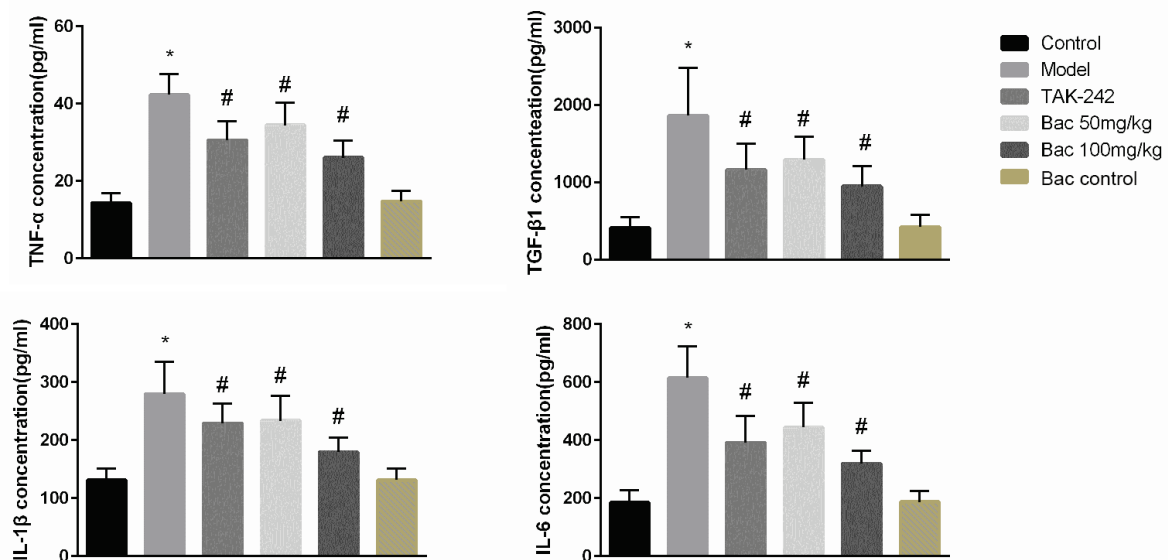
group ( $p<0.05$ ). After Baicalin group (50mg/kg or 100mg/kg) intervention, the scores of alveolar inflammation and pulmonary fibrosis were significantly lower than that of the silicosis model group ( $p<0.05$ ).

These results showed that Baicalin treated for 28 days in silica-induced rats could significantly alleviate alveolitis and pulmonary fibrosis (Table 2).

**Table 2.** Alveolar inflammation and pulmonary fibrosis score of lung tissues in different group

Group	Alveolar inflammation	Pulmonary fibrosis
Control	0	0
Model	2.30±0.46*	2.51±0.38*
TAK-242	1.14±0.44 <sup>#</sup>	1.71±0.35 <sup>#</sup>
Bac (50mg/kg)	1.30±0.23 <sup>#</sup>	1.90±0.17 <sup>#</sup>
Bac (100mg/kg)	0.86±0.24 <sup>#</sup>	1.30±0.46 <sup>#</sup>
Bac Control	0	0

\* $p<0.05$  vs. Control group. <sup>#</sup> $p<0.05$  vs. model group.



**Fig. 2.** The levels of TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$  and IL-6 in rat lungs after Baicalin treated for 28 days. Results showed Baicalin (50 mg/kg/day or 100 mg/kg/day) inhibited the increase of TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$  and IL-6 in rat lung tissues induced by silica. Data are presented as the means  $\pm$  standard deviation. \* $p<0.05$  vs. control group, <sup>#</sup> $p<0.05$  vs. model group.  $n=7$  in each group of animals.

#### Baicalin reduced the level of TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$ and IL-6 in silica-induced rats

As shown in Fig. 2, the levels of TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$  and IL-6 in model group were significantly higher than the control group ( $p<0.05$ ). Notably, the TNF- $\alpha$ , TGF- $\beta$ 1, IL-1 $\beta$  and IL-6 levels were significantly decreased in the Baicalin (50mg/kg or 100mg/kg) and TAK-242 groups compared to the model group ( $p<0.05$ ).

#### Effect of Baicalin on the expression of Col-I

Excessive Col-I deposition could laterally demonstrate the development of pulmonary fibrosis.

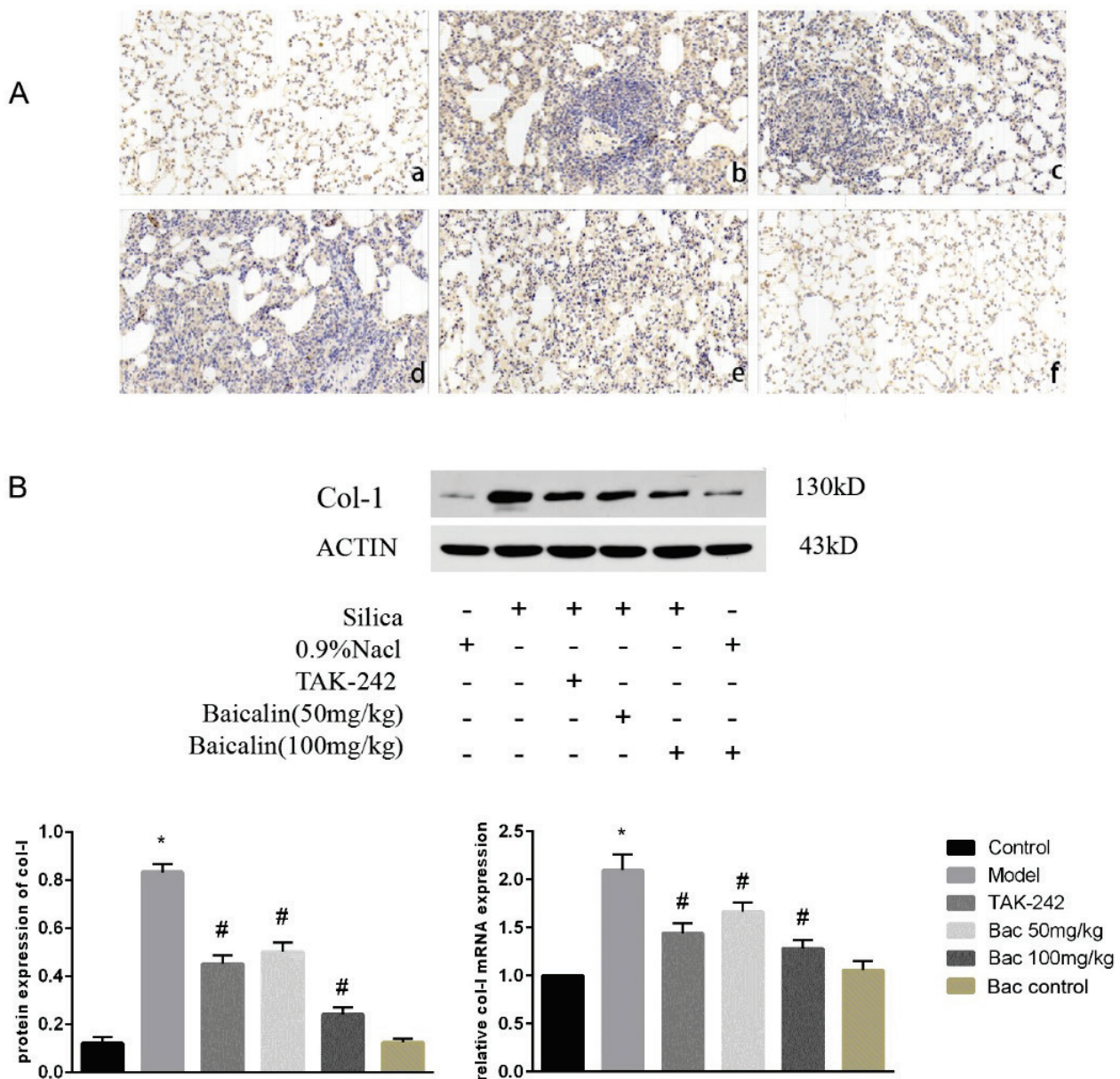
Therefore, we measured expression of Col-I using western blot to investigate the effect of Baicalin on the fibrosis. The results showed that Col-I expression significantly increased in the silicosis-model group. Meanwhile, Baicalin intervention reduced the Col-I protein level and relative Col-I mRNA expression as shown in Fig. 3. These findings were further confirmed by immunohistochemistry and qRT-PCR.

#### Effect of Baicalin on the EMT

EMT has been shown to promote fibroblast proliferation and collagen production, further promoting the evolution of pulmonary fibrosis [25]. We examined

the effect of Baicalin on EMT silica dust-induced through measuring the expression of EMT markers E-cad, vimentin and  $\alpha$ -SMA in the rat of each group. Western blot showed the E-cad expression was diminished, vimentin and  $\alpha$ -SMA were multiplied in the model group. Baicalin treated for 28 days significantly increased the

expression of E-cad, attenuated the levels of vimentin and  $\alpha$ -SMA (Fig. 4B). The immunohistochemical expression of  $\alpha$ -SMA was consistent with that of western blot (Fig. 4A). These indicated Baicalin intervention in silica-induced rats alleviated pulmonary fibrosis by inhibiting the process of EMT.



**Fig. 3.** Immunohistochemistry of Col-I (**A**) expression in rat lungs after Baicalin treated for 28 days. ( $\times 200$ ). a) Control group; b) Model group; c) TAK-242 group; d) Baicalin (50mg/kg) group; e) Baicalin (100mg/kg) group; f) Baicalin control group. Western blot and qRT-PCR (**B**) analyzed the protein expression of Col-I and the mRNA expression of Col-I. \* $p < 0.05$  vs. control group, # $p < 0.05$  vs. model group.  $n = 7$  in each group of animals.

#### Effect of Baicalin on the TLR4/ NF- $\kappa$ B signal pathway

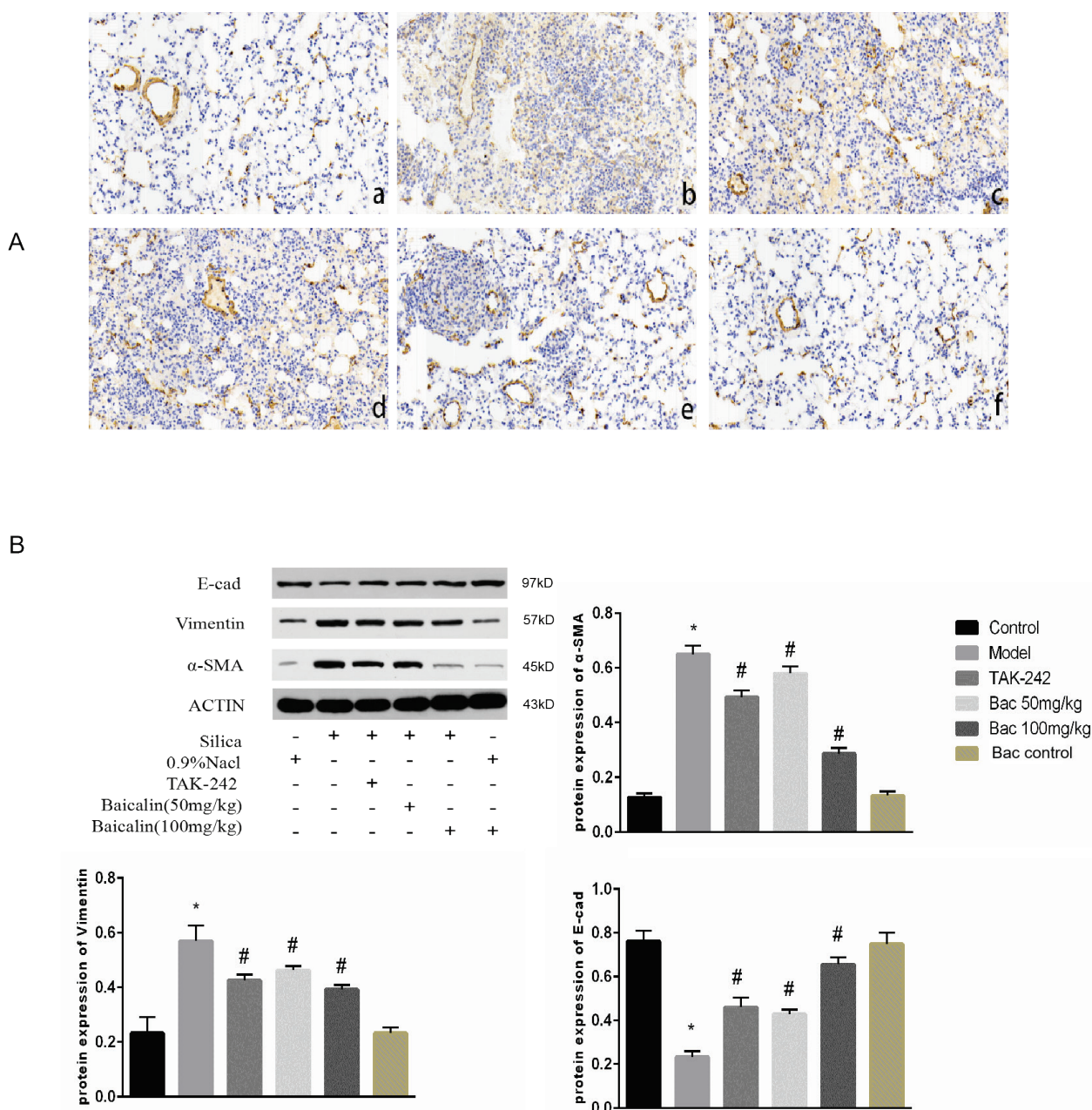
As a pattern recognition receptor, TLR4 can mediate the activation of NF- $\kappa$ B with the MYD88-dependent pathway. To further investigate the effects of

Baicalin on the TLR4/ NF- $\kappa$ B signal pathway in rats, using western blot to assess the level of TLR4 and MyD88 in lung tissues. As shown in Fig. 5B, inhalation of silica increased protein expression of TLR4 and

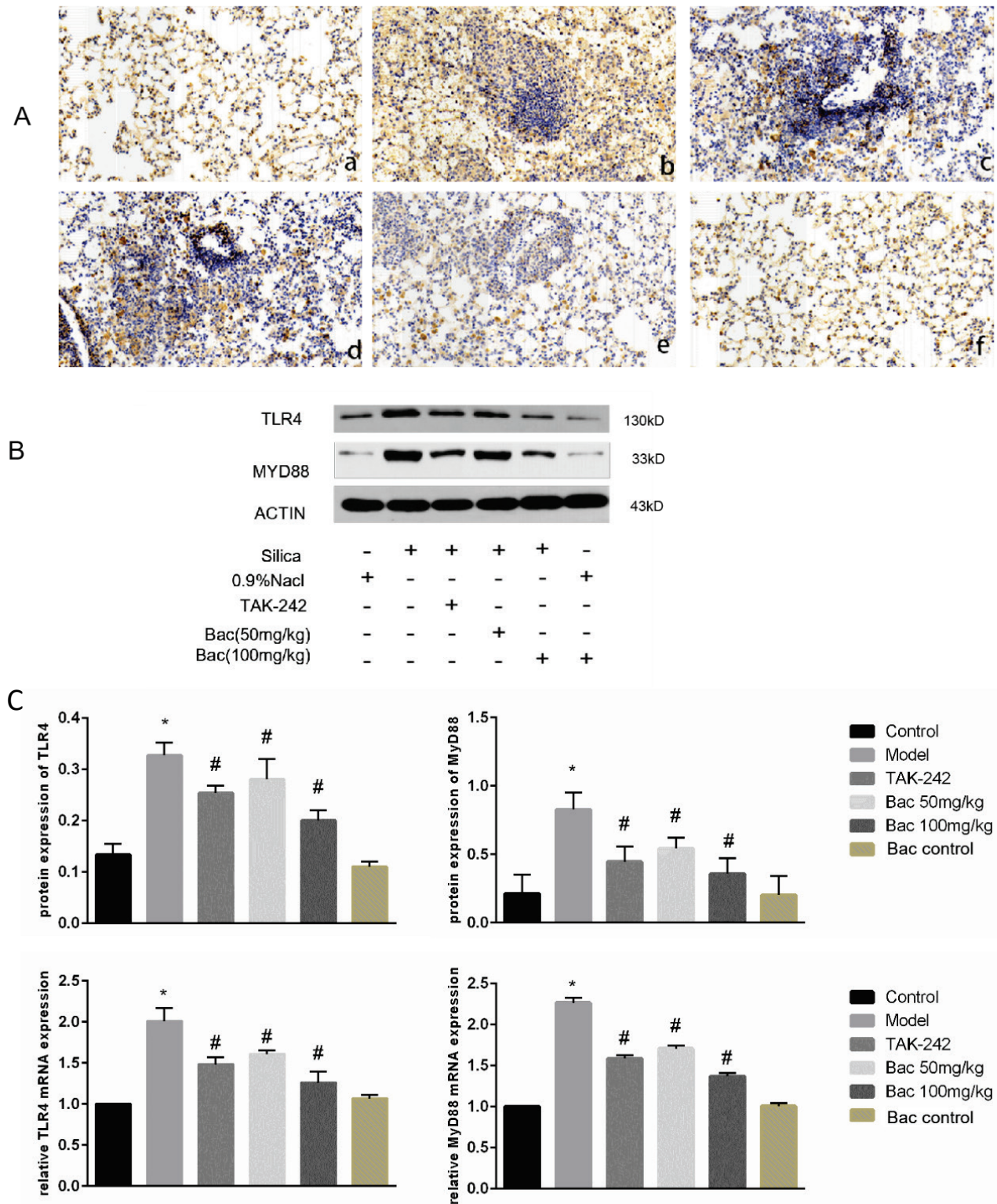
MyD88 in the lung tissues, while TAK-242 and Baicalin intervention for 28 days significantly decreased TLR4 and MyD88 protein levels. Besides, immunohistochemistry and qRT-PCR confirmed similar results (Fig. 5 A,C).

It has been well established that NF-KB is the downstream signaling of the TLR4 signaling pathway. The protein expression of NF-κBp65, p-NF-κBp65, IκBα, p-IκBα in lung tissue were evaluated by western blot to further study the mechanism of Baicalin anti-fibrosis in silicosis rats. As shown in Fig. 6A, silica inhalation could

increase the protein expression of p-NFκBp65 and p-IκBα in lungs, the relative mRNA levels of NF-κBp65 and IκBα were also up-regulated. Meanwhile, the protein NF-κBp65 and IκBα levels showed no significant change between the model and control groups. TAK-242 and Baicalin treated for 28 days significantly decreased p-NFκBp65 and p-IκBα protein expressions. Results displayed Baicalin could inhibit the increase of p-NF-κBp65 and p-IκBα levels in silica-induced rats. (Fig. 6B).

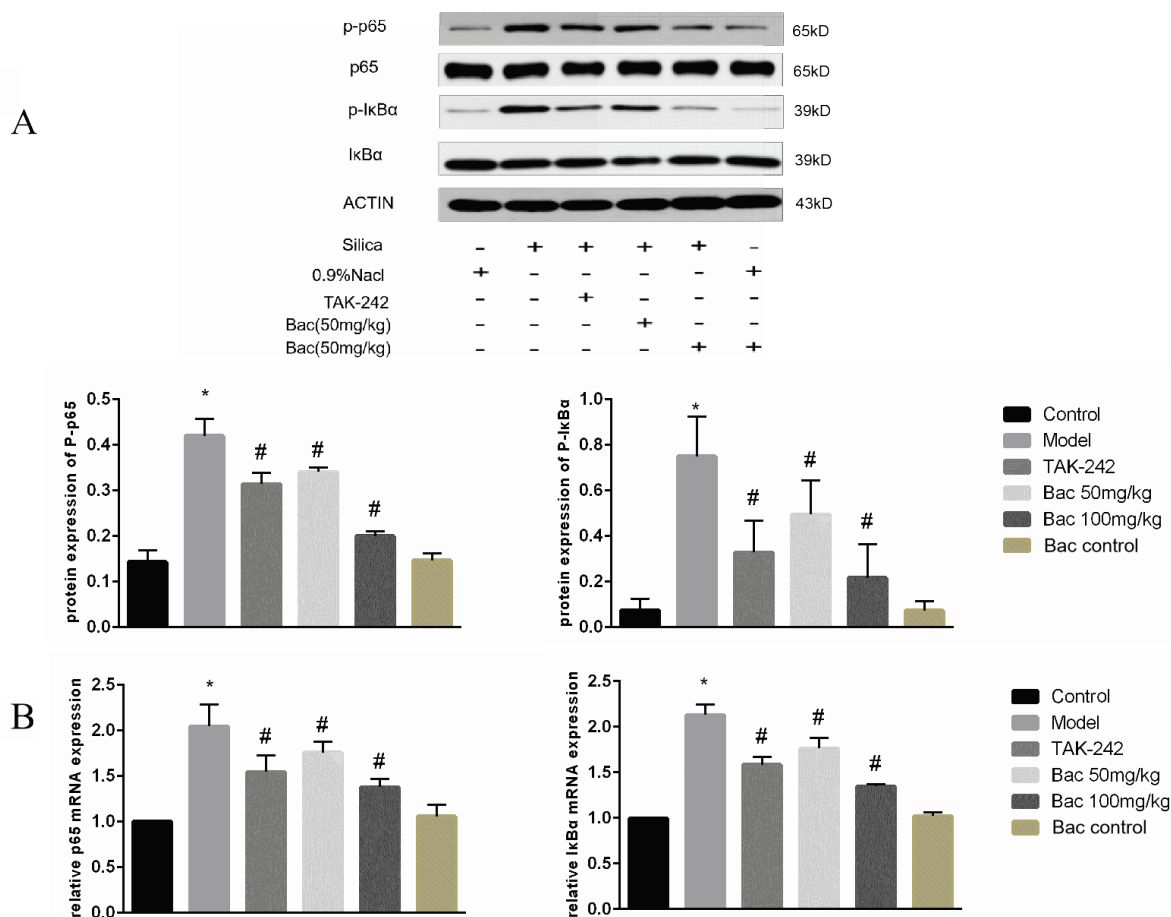


**Fig. 4.** Immunohistochemistry of α-SMA (A) expression in lungs tissue after Baicalin treated for 28 days. (×200). a) Control group; b) Model group; c) TAK-242 group; d) Baicalin (50mg/kg) group; e) Baicalin (100mg/kg) group; f) Baicalin control group. Western blot (B) displayed the protein expression of E-cad, Vimentin, and α-SMA. \*p<0.05 vs. control group, #p<0.05 vs. model group. n=7 in each group of animals.



**Fig. 5.** Immunohistochemistry of TLR4 (A) expression in lungs after Baicalin treated for 28 days. ( $\times 200$ ). a) Control group; b) Model group; c) TAK-242 group; d) Baicalin (50mg/kg) group; e) Baicalin (100mg/kg) group; f) Baicalin control group. Western blot (B) displayed the protein expression of TLR4 and MyD88. qRT-PCR (C) revealed the relative mRNA expression of TLR4 and MyD88. \* $p < 0.05$  vs. control group, # $p < 0.05$  vs. model group.  $n = 7$  in each group of animals.





**Fig. 6.** Western blot (A) displayed the protein expression of NF-κBp65, p-NF-κBp65, IκBα and p-IκBα. qRT-PCR (B) analyzed the relative mRNA expression of NF-κBp65 and IκBα. \* $p < 0.05$  vs. control group, # $p < 0.05$  vs. model group.  $n = 7$  in each group of animals.

## Discussion

The pathogenesis of silicosis has not been fully clarified, but numerous studies have shown that inflammation and EMT take part in the progression of silicosis [25-27]. Although a number of treatments have been proposed for silicosis, no curative treatment has been discovered. Therefore, it is crucial to further study the pathogenesis of silicosis and explore new drugs for the treatment of silicosis. In recent years, the identification of compounds from medicinal plants with therapeutic potential for ailment and diseases has been receiving increasing attention. Baicalin has been shown to have anti-inflammatory properties and to play a therapeutic role in renal fibrosis [28]. And Baicalin is reported that could ameliorated cigarette smoke-induced airway inflammation and relieved bleomycin-induced fibroblast proliferation and pulmonary fibrosis [29,30]. Meanwhile, Baicalin also play a protective role in inhibiting EMT in human non-small cell lung cancer through PDK1/AKT signaling pathway [31]. However,

there is few studies have explored the effects of Baicalin on silicosis inflammation and EMT process. In our study, we used the improved non-exposed endotracheal intubation method to build silica-induced silicosis rats, and found that silica exposure significantly increased the levels of pro-inflammatory factor IL-1, IL-6, TNF- $\alpha$ , TGF- $\beta$ 1 and fibrosis indicator Col-1. Furtherly, we found that the expression of TLR4 and NF- $\kappa$ B were significantly increased in silicosis rats. Interestingly, the administration of Baicalin remarkably reverse above variations in silicosis rats. These results shown that Baicalin may alleviate inflammation and fibrosis *via* TLR4/NF- $\kappa$ B pathway in silicosis rats, providing a new treatment option for silicosis.

It is widely accepted that silica exposure triggers lung inflammation, which could promote the development of silicosis [26]. The inflammatory factors of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and TGF- $\beta$ 1 are the primary indicators to promote silicosis fibrosis. In present study, we shown that silica exposure caused in lung inflammation with significantly high levels of IL-1 $\beta$ ,

TNF- $\alpha$ , IL-6 and TGF- $\beta$ 1, which was consistent with previous research results [25]. At the same time, H&E staining results also showed that silica caused obvious inflammatory cell infiltration and higher scores of alveolar inflammation. However, After Baicalin treatment, the levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, TGF- $\beta$ , the areas of inflammatory cell infiltration and scores of alveolar inflammation were significantly decreased, indicating Baicalin could attenuate lung inflammation.

Pulmonary fibrosis is the main characteristic of silicosis. Col-1 is a one of collagen, and the large amount of collagen deposition in the lung is a sign of pulmonary fibrosis [32]. Masson staining is a commonly method was used to observe collagen deposition in lung tissue. In current study, the results suggested that silica exposure remarkably increased the areas of collagen deposition, the scores of pulmonary fibrosis and the expression of Col-1, and those of changes could reverse after treatment of Baicalin. Characteristics of pulmonary fibrosis is fibroblast proliferation and accumulation and excessive ECM components. TGF- $\beta$ 1, a key mediator in fibrogenesis, is upregulated and activated in fibrotic diseases [33]. Moreover, TGF- $\beta$ 1 is a key mediator in inducing EMT, which played a vital role in accumulating fibroblast is considered to be one of the key mechanisms of pulmonary fibrosis [27]. Our data shown that the expression of E-cad was significantly reduced and the TGF- $\beta$ 1, vimentin and  $\alpha$ -SMA protein expression were increased in silica-induced rats and promote lung fibrosis, and after treatment of Baicalin remarkably attenuates lung fibrosis with reduction of TGF- $\beta$ 1 and inhibition of EMT process. The results indicated that Baicalin exerts anti-fibrosis effects on silicosis rats.

Lung inflammation caused by inhalation of silica is thought to be the initial factor in the progression of silicosis [26]. TLR4/NF- $\kappa$ B signaling pathways are classic signaling pathways related to inflammation [34-35]. Some evidences indicated that silica induced inflammation of U937 differentiated macrophages through the TLR4 signaling pathway, and activated the NF- $\kappa$ Bp65 cascade reaction with the release of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [36]. In addition, studies have demonstrated that Lipopolysaccharide (LPS)-induced acute lung injury could be attenuate by reducing the production of inflammatory mediators and inhibiting TLR4/NF- $\kappa$ B pathway [37]. As a TLR4 connector molecule, MyD88 includes two dependent and independent pathways. In dependent pathway, MyD88 mediate the activation of NF- $\kappa$ B and the production of

cytokine [38]. Normally, NF- $\kappa$ B is bound to inhibitory protein I $\kappa$ B $\alpha$  and has no activity. When I $\kappa$ B $\alpha$  is phosphorylated, NF- $\kappa$ B would escape from the nucleus and activate downstream gene transcription [39]. In this study, to further clarify the potential mechanism of Baicalin in alleviating silica-induced inflammatory and pulmonary fibrosis in rats, we set up the TLR4 inhibitor (TAK-242) group. Our data showed that the expression of TLR4, MyD88, p-NF- $\kappa$ B and p-I $\kappa$ B $\alpha$  were distinctly increased in lung tissue after silica exposure and those changes were weakened in TAK-242 group, suggesting that the pathogenesis of silicosis may be related to TLR4/NF- $\kappa$ B-mediated inflammation. Previous studies have showed that Baicalin protected against oxygen-glucose deprivation-induced inflammatory factors by attenuating TLR4 pathway [20]. Baicalin has been reported to prevent inflammation and TLR4/NF- $\kappa$ B p65 pathway activation induced by LPS [40]. And in our study, Baicalin have similar effects to TAK-242 in silicosis rats, accompanied with the significantly decreased expression of TLR4, MyD88, p-NF- $\kappa$ B and p-I $\kappa$ B $\alpha$  in lung tissues of rats. Based on the features of TAK-242, we reasonable to believe that Baicalin alleviated lung inflammation induced by silica may be related to inhibition of TLR4/NF- $\kappa$ B pathway.

EMT is increasingly recognized as one of the key mechanisms of pulmonary fibrosis. Previous study found that targeted involvement of TLR4 /NF- $\kappa$ B signaling pathway may efficiently inhibit EMT process induced by high Uric Acid-induced in renal tubular epithelial cells [41]. And tripterygium glycosides tablet could ameliorate renal tubulointerstitial fibrosis, and the mechanism may be associated with the amelioration of EMT by inhibiting the TLR4 /NF- $\kappa$ B pathway [42]. In addition, Baicalin was reported to reduce the EMT level of colorectal cancer cells [43]. In the current study, we found that Baicalin and TAK-242 significantly inhibited EMT process in lung tissues induced by silica, indicating that Baicalin may have alike properties to TAK-242 inhibiting the expression of TLR4, MyD88, p-NF- $\kappa$ B and p-I $\kappa$ B $\alpha$ . Thus, we considered that Baicalin attenuate EMT and ameliorate pulmonary fibrosis induced by silica *via* inhibiting TLR4 /NF- $\kappa$ B pathway.

## Conclusion

In summary, this study proved that TLR4/NF- $\kappa$ B pathway was activated in silicosis rats, and Baicalin intervention can significantly alleviate silica-induced

pulmonary inflammation and fibrosis in rats by inhibiting the TLR4/NF- $\kappa$ B pathway. These findings further explore the pathogenesis of silicosis and provide a new possible option for the treatment of silicosis.

### Conflict of Interest

There is no conflict of interest.

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