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EFFECT OF CHALCONE ON INFLAMMATORY FACTORS AND THE EXPRESSION OF P38 MAPK PROTEIN IN *STREPTOCOCCUS PNEUMONIAE* MICE

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Abstract

The effects of chalcone on inflammatory response and expression of mitogen-activated protein kinase p38 (p38 MAPK) in lung tissue of mice with pneumonia due to *Streptococcus pneumoniae* were investigated. Sixty male Kunming mice were divided into three groups (20 mice in each group), a control group, a pneumonia model group and a chalcone group. For the mice in model group and chalcone group, *Streptococcus pneumoniae* standard strain solution was used for modelling, while the control group was slowly dripped with 50 µL normal saline solution. After successful modelling, mice in the chalcone group were subcutaneously injected with 100 mg/kg chalcone, and mice in the model group and control group were subcutaneously injected with an equal volume of normal saline solution, once every 8h, for 3 days. Venous blood was collected to determine C-reactive protein (CRP) and procalcitonin (PCT) levels. Inflammatory markers were also detected in lung tissue. The results showed that in the pneumonia group there is a significant increase in CRP, PCT, IL-1β, TNF-α, IL-6 and IL-8 levels, along with increased expression of the p38 MAPK gene and protein compared to the control group. Chalcone treatment can significantly reduce the level of inflammatory factors in the lung tissue of mice with pneumonia and down-regulate the expression of p38 MAPK gene and protein. Therefore, this may be one of the mechanisms by which chalcone inhibits the inflammatory response of lung tissue in mice with *Streptococcus pneumoniae* pneumonia.

Rezumat

Au fost investigate efectele calconei asupra răspunsului inflamator și a expresiei genei și proteinei p38 MAPK în țesutul pulmonar la șoarecii cu pneumonie indusă cu *Streptococcus pneumoniae*. Șaizeci de șoareci Kunming masculi au fost împărțiți egal în trei grupuri astfel: un grup martor, un grup control și un grup test. Grupului control și grupului test le-a fost indusă pneumonie, folosind tulpină standard de *Streptococcus pneumoniae*, în timp ce grupul martor a fost tratat cu 50 µL de souție salină normală. După ce a fost indusă pneumonia, șoarecilor din grupul test le-a fost administră 100 mg/kg de calconă, iar șoarecii din celelalte două grupuri au primit un volum egal de soluție salină normală, o dată la fiecare 8 ore, timp de 3 zile. S-a recoltat sânge venos pentru a se determina nivelurile de proteină C-reactivă (PCR) și procalcitonină (PCT). Totodată, markerii inflamatori au fost detectați la nivelul țesutului pulmonar. Rezultatele arată că în grupul cu pneumonie se observă o creștere semnificativă a nivelurilor de PCR, PCT, IL-1β, TNF-α, IL-6 și IL-8, împreună cu creșterea expresiei genei și a proteinei p38 MAPK, în comparație cu grupul martor. Tratamentul cu calconă poate reduce în mod semnificativ nivelul factorilor inflamatori în țesutul pulmonar la șoarecii cu pneumonie și poate scădea expresia genei și a proteinei p38 MAPK. Prin urmare, acesta poate fi unul dintre mecanismele prin care calcona inhibă răspunsul inflamator la nivelul țesutului pulmonar la șoarecii cu pneumonie indusă cu *Streptococcus pneumoniae*.

Keywords: chalcone, *Streptococcus pneumoniae*, inflammatory factor, p38 MAPK, anti-inflammatory mechanism

Introduction

Streptococcus pneumoniae is a gram-positive diplococcus that often lives in the upper respiratory tract of the human body [1]. When the immunity of the human body is clearly decreased, it will develop into septicaemia, community-acquired pneumonia (CAP), meningitis and otitis media [2, 3]. The incidence rate and mortality rate of pneumonia due to *Streptococcus pneumoniae* are increasing year by year. At this time, *Streptococcus pneumoniae* remains the most common pathogen involved in the appearance of CAP [4]. Antibiotics are still the first choice for the treatment of pneumonia due to *Streptococcus pneumoniae*. But *Streptococcus pneumoniae* has become more resistant to antibiotics [5]. Chalcone, referred to as 1,3-diphenyl-2-propene-1-one, is present in many natural plants [6]. At the same time, chalcone and its derivatives have a wide range of pharmacological activities. Venturelli S found that [7] licochalcone and its derivatives could inhibit the proliferation of a series of tumour

cells, including renal cell carcinoma, cervical cancer, pancreatic cancer and nerve cell carcinoma. Kumar D [8] reported the antifungal effects of chalcone and its derivatives and speculated that they prevented fungal cell wall formation by inhibiting glucan or chitin synthase. Cole AL *et al.* [9] and Deng J *et al.* [10] revealed that chalcone could inhibit HIV transcription by inhibiting the HIV translation initiation factor. These results indicate that chalcone has broad-spectrum antibacterial activity [11]. Mitogen-activated protein kinase (MAPK) signal pathway is one of the important signal transduction systems in cells, which is involved in cell proliferation, differentiation, apoptosis and inflammation [12]. p38 MAPK is also involved in cell proliferation, differentiation, apoptosis and inflammation [13]. When p38 is activated, it can activate other pathways and expand inflammatory response [14].

In this study, the mice model of pneumonia due to *Streptococcus pneumoniae* was established by intra-nasal instillation of standard strain solution of *S. pneumoniae*, and chalcone was used for intervention. The levels of inflammatory factors (C-reactive protein (CRP), procalcitonin (PCT), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8), tumour necrosis factor - α (TNF-α)) and the expression level of p38 MAPK in mice were observed to explore the effect of chalcone on the expression of p38 MAPK, to clarify the anti-inflammatory mechanism of chalcone on pneumonia due to *Streptococcus pneumoniae*, and to provide theoretical evidence for clinical application of chalcone treatment.

Materials and Methods

Animals

Sixty clean-grade male Kunming mice weighing about 25 g were selected. The animals were kept under standard laboratory conditions (ambient temperature of 20 - 25°C, humidity 60 ± 10%, and normal light and dark alternated). The mice were reared adaptively for 1 week to establish the mice model of pneumonia due to *Streptococcus pneumoniae*. The study protocol followed the ARRIVE guidelines and obtained the Institutional Ethics Committee approval.

Bacteria culture

The standard strain of *Streptococcus pneumoniae* ATCC 49619 was purchased from Shanghai Beinuo Biotechnology Co., Ltd., China. It was cultured in a blood agar medium (Jiangxi yimi Biotechnology Co., Ltd., China; culture environment: 37.0°C; constant temperature, 50 mL/L CO_2). After 18 hours, the growth of *Streptococcus pneumoniae* colonies could be observed. Sterile instruments were used to take *Streptococcus pneumoniae* colonies from the surface of agar plates and suspend them in sterile normal saline. The McFarland turbidimetric method was used to prepare the bacterial suspension with a bacterial concentration of approximately 1.5 × 108 CFU mL (0.5 McFarland).

Establishment of mice model of pneumonia

Mice in each group were weighed. The hind leg muscles were anaesthetized with 10% ethyl carbamate (800 mg/kg bw) (Shanghai Bohu Biology Science and Technology Co., Ltd., China). Then, the nasal mucosa of mice in the model group and chalcone group were damaged, and 50 µL standard strain solution of *Streptococcus pneumoniae* (bacterial amount of 3 × 10^7 CFU) was dropped. The nasal mucosa of normal mice was damaged and 50 µL sterile 0.9% sodium chloride solution was dropped. When sodium chloride was dripped, it was necessary to observe the breathing condition of mice. If there was asphyxia, stretching the forelimb and squeezing the heart needed to be done immediately.

Chalcone administration

Chalcone (Shanghai Baoman Biology Science and Technology Co., Ltd., China) was dissolved in DMSO (dimethyl sulfoxide) (AR 99%, Shanghai Bohu Biology Science and Technology Co., Ltd., China) and injected subcutaneously at a dose of 100 mg/kg bw. The administration started at 2 h after infection and continued once every 8 h, for 3 days. After the first administration (0 h), 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h were taken as time nodes to detect, record and plot the mortality of mice.

Specimen fixation and sampling

12 h after the last administration, all mice were weighed and anesthetized by intraperitoneal injection of 5% chloral hydrate (Beijing Xinhua Lvyuan Technology Co., Ltd., China). The eyeballs of mice were removed and venous blood was collected and put into an ethylenediamine tetraacetic acid (EDTA) anticoagulant tube. The chest was opened to remove the whole lung. The left lung was fixed in a 4% paraformaldehyde solution for haematoxylin and eosin (HE) staining. The upper, middle and lower lobes of the right lung of mice were taken and immediately frozen at -80°C for further analysis.

Detection of serum infectious index

After the mice were killed, the whole blood was taken and placed in an EP tube. Then, it was centrifuged for 10 min at 3500 r/min. Serum CRP and PCT levels were detected by ELISA using mice kits purchased from Guizhou Chaoyan Biotechnology Co., Ltd., China, according to the manufacturer's instructions.

Haematoxylin-eosin staining (HE)

The left lung tissue of mice was fixed with 4% paraformaldehyde (Shanghai Xinfan Biology Science and Technology Co., Ltd., China) for 24 h and then removed. The trimmed tissue was rinsed with tap water for 30 min and placed in a dehydration box to remove moisture. Then, the tissue blocks were treated with xylene (Nanjing Milan Chemical Co., Ltd., China) for 30 min each time for 3 times, and then embedded in paraffin. The samples were placed in the refrigerator,

cooled and solidified into blocks, removed and trimmed neatly. The wax block was cut into 4 - 6 μm thick slices, pasted on the slide, and dried in a 45°C incubator. After the slices were treated with xylene, ethanol gradient dehydration (Wuhan Jixinyibang Biology Science and Technology Co., Ltd., China) was carried out. Then, the slices were stained with haematoxylin (Hebei Biology Science and Technology Co., Ltd., China), differentiated with 70% hydrochloric acid alcohol (Wuhan Jixinyibang Biology Science and Technology Co., Ltd., China), washed with running water, stained with eosin, dehydrated by ethanol gradient, treated with xylene, and sealed with neutral gum. Finally, they were observed and photographed under the light microscope (Shenzhen Chensheng Optical Instrument Co., Ltd., China).

Detection of inflammatory factors in lung tissue of mice by ELISA

The right upper lobe lung tissue of mice was taken out from -80°C refrigerator and weighed. The tissue was transformed into lung homogenate and centrifuged at 4°C, 12000 r/min × 15 min. After centrifugation, the supernatant was taken. IL-1β, IL-6, IL-8 and TNF-α in lung tissue were detected by the double antibody sandwich method. The specific operation method is strictly in accordance with the kit instructions (Shanghai Enzyme Linked Biotechnology Co., Ltd., China).

RT-PCR detection of p38 MARK gene expression

First, total RNA was extracted by dissolving the tissue with Trizol (TRIzol Reagent; Shanghai lianmai biology engineering co., ltd., China). Reverse transcription was carried out under the action of reverse transcriptase, and then PCR amplification (ABI Company, American) was performed using complementary deoxyribonucleotide (cDNA) as a template. After PCR, the relative expression of the target gene was calculated. The primer sequences were GAPDH: Forward 5’-CAAGGTCATCCATGACAACTTTG-3’, reverse 5’-GTCCACCACCCTGTTGCTGTAG-3’ (the length of the product was 496bp) and p38 MAPK: Forward 5’-CGTTGTTTCCTGGTACAGACC-3’, reverse 5’-CCATTTCTCTTGGTCAAGGG-3’ (the length of the product was 430bp).

Western blotting detection of p38 MARK protein expression

The tissue from the lower lobe of the right lung was removed from -80°C for standby. It was homogenized, and the homogenate was put into a centrifuge tube and vibrated repeatedly. After 30 min of the ice bath, it was blown repeatedly and centrifugated for 5 min at 4°C and 13000 r/min. Then, the supernatant was collected. The operation was repeated 5 times. The protein concentration was determined using bicinchoninic acid (BCA) assay (BCA kit, Shanghai Enzyme Linked Biotechnology Co., Ltd., China). The sample concentration was determined as 40μg. Each sample was added with 5 × SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Beijing Baiaolaibo Technology Co., Ltd., China) loading buffer. After boiling water for 5min, TEMED (N,N,N',N'-tetramethylethylenediamine; Shanghai Enzyme Linked Biotechnology Co., Ltd., China) was added. A proper amount of water was added for 45 min, poured out and aspirated dry. TEMED and the electrophoresis buffer were put to the electrophoresis tank. Then, the sample was added into the sample hole and electrophoresis was carried out under constant pressure. Methanol was utilized for activation, the membrane was transferred, and a 300 mA constant current was used. The sealing solution was added at room temperature for 1 h and the first antibody (rabbit anti-mouse phosphorylate P38 MAPK polyclonal antibody; Sigma, America) after dilution (1:1000). After it was put at 4°C overnight and washed with Tris-buffered saline with Tween (TBST, Shanghai Double Helix Biological Technology Co., Ltd., China) for 5 min/time × 3 times, the secondary antibody (Goat anti-rabbit secondary antibody, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) was added after dilution (1:1000). Then, it was incubated at room temperature for 30 min, and TBST was used to wash for 5 min/time × 4 times. The prepared Enhanced ChemiLuminescence (ECL, Beijing Baiaolaibo Technology Co., Ltd., China) mixture was placed on the protein side of the membrane and exhibited. According to the light intensity, the exposure conditions were adjusted. Development and fixation were carried out. After scanning, Alpha Ease FC software (Nanjing Changxiang Instrument Equipment Co., Ltd., China) was used for analysis and processing.

Statistical analysys

SPSS19.0 statistical software (IBM, USA) was used for statistical analysis. All experimental data were expressed as mean ± standard deviation. One-way ANOVA was used to compare the sample mean between groups, and the least-significant difference (LSD) method was used for pairwise comparison when the contrast was uniform. On the contrary, if the contrast was not uniform, the Games-Howell method was applied. A $p < 0.05$ was an indication that the difference was statistically significant.

Results and Discussion

Observation on the general condition of mice

When the mice in the control group were given normal saline, they coughed occasionally, but not violently, and a few of them had neck extensions. After nasal drip, there were no signs of shortness of breath, dyspnoea, restlessness, sneezing, runny nose. During the period of subcutaneous injection of normal saline, the mice had no obvious changes in diet and drinking water, and they moved normally in the cage. When the mice in the model group were given the standard strain solution of *Streptococcus pneumoniae*,

they coughed more and had more neck extension reflexes than the control group. During the subcutaneous injection of normal saline, the respiratory rate of mice in the model group was slightly accelerated. The mice were agitated and frequently scratched their nose. Some of them developed a cough, runny nose, sneezing, cyanosis of oral mucosa and dyspnoea. In addition, some mice coughed and sneezed with gatism. Compared with the control group and chalcone group, the intake and drinking water of mice decreased, and the body weight also decreased. The mice in the chalcone group were treated with a standard strain solution of *Streptococcus pneumoniae* by nasal drip and chalcone intervention, resulting in restlessness, nasal scratching and shortness of breath. Some mice showed nasal runny nose, sneezing and slight cyanosis of the oral mucosa. However, compared with the model group, the above-mentioned manifestations were attenuated, and gatism was found in mice occasionally.

Effect of chalcone on the mortality of Streptococcus pneumoniae in mice

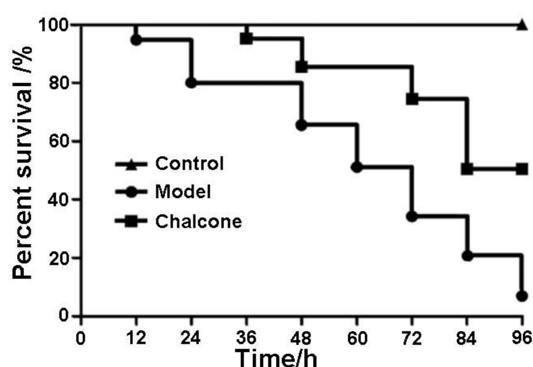

Figure 1.
Protective rate of chalcone against *Streptococcus pneumoniae* infection in mice model

Figure 1 showed that no mice died in the control group. In contrast, almost all mice in the model group died within 96 h after infection. The survival time and survival rate of the chalcone treatment group were significantly higher than that of the model group.

Pathological findings of lung tissue in each mice group

In the control group, it could be seen with free eyes that the lung tissue was pink, without swelling and necrosis. Under the light microscope, it was noted that the alveolar structure was intact and there were no secretions in the alveolar cavity, no thickening of the alveolar septum, obvious congestion, expansion and infiltration of inflammatory cells in the capillaries of the alveolar septum.

In the model group it was observed by free eyes that the colour of the lung tissue was dark red compared to the control group, the lung lobe was obviously swollen and there was no necrosis. Under the light microscope, it could be seen that the alveolar cavity was filled with exudative cellulose and the adjacent alveolar cellulose filaments were often connected by interalveolar pores. The alveolar structure was destroyed, the alveolar septum was thickened, and capillary dilatation was evident. A large number of inflammatory cells were infiltrating the pulmonary interstitium.

In the chalcone group, it was observed with the free eye that the colour of the lung tissue was between the model and control groups, and the lung lobes were slightly swollen without necrosis. Under the light microscope, it could be seen that there was less cellulose loading in the alveolar cavity than in the model group. The connection of adjacent alveolar cellulose filaments through the interalveolar pores was less than in the model group. Some alveolar structures were destroyed and fused, and some alveolar septa were thickened. There was a small amount of inflammatory cell infiltration into the lung interstitium (Figure 2).

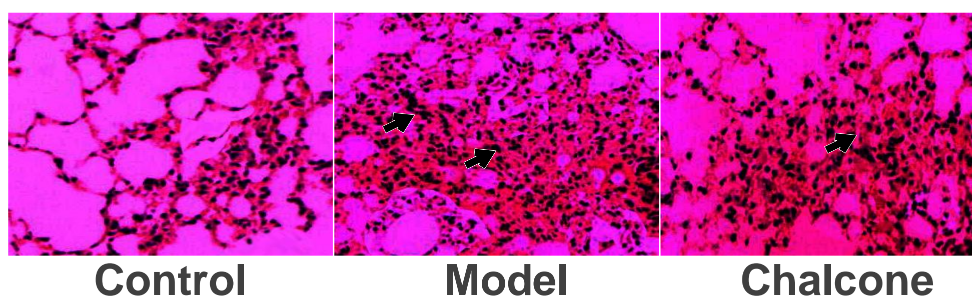

Figure 2.
Pathological section of lung tissue in mice (×200). The black arrow marked area in the figure indicates the inflammatory infiltration site

Expression of inflammatory factors in each group of mice

Table I showed that CRP and PCT levels in the model group were higher than those in the control group ($p < 0.05$). In the chalcone group, the level of CRP and PCT were significantly increased compared with the control group ($p < 0.05$) but significantly decreased compared with the model group ($p < 0.05$).

The level of IL-1β, IL-6, IL-8 and TNF-α in lung tissue in the model group were significantly increased compared with the control group ($p < 0.05$). In the chalcone group, the level of IL-1β, IL-6, IL-8 and TNF-α were significantly increased compared with the n control group ($p < 0.05$), but significantly decreased compared with the model group ($p < 0.05$).

Table I

Comparison of inflammatory factors in each group

Indexes	Control group	Model group	Chalcone group
CRP(μg/mL)	6.83 ± 1.73	35.72 ± 7.48*	18.26 ± 4.03*#
PCT(ng/mL)	0.42 ± 0.11	8.53 ± 1.87*	4.97 ± 1.43*#
IL-1β(pg/mL)	106.33 ± 9.52	547.46 ± 68.56*	308.14 ± 46.52*#
IL-6(pg/mL)	53.17 ± 12.37	346.41 ± 28.58*	201.34 ± 16.63*#
IL-8(pg/mL)	19.67 ± 5.39	184.03 ± 30.22*	98.46 ± 18.36*#
TNF-α(pg/mL)	68.35 ± 14.10	397.86 ± 29.67*	159.63 ± 17.78*#

Note: *$p < 0.05$ compared with the control group; #$p < 0.05$ compared with the model group.

Comparison of p38 MAPK mRNA expression in lung tissue of mice in each group

Figure 3A showed that the expression of p38 MAPK mRNA in lung tissue in the model group was higher than that of the control group ($p < 0.05$). In the chalcone group, it is observed that the expression of p38 MAPK mRNA in lung tissue is significantly increased compared with the control group ($p < 0.05$), but significantly decreased compared with the model group ($p < 0.05$).

Comparison of p38 MAPK protein expression in lung tissue of mice in each group

Figure 3B and Figure 3C showed that the expression of p38 MAPK protein in the model group was significantly increased compared with the control group ($p < 0.05$). In the chalcone group, the expression of p38 MAPK protein was significantly increased compared with the control group ($p < 0.05$), but significantly decreased compared with the model group ($p < 0.05$).

CRP and PCT have been recognized in the diagnosis of infectious diseases [15, 16, 17]. Serum PCT, as a new bacterial infection marker, is often widely used in the diagnosis of infectious diseases. CRP is an acute-phase protein, which is not affected by anti-inflammatory drugs, immunosuppressants or adrenocortical hormones and can contribute to timely diagnosis and reasonable treatment of infectious diseases [18]. The results show that the serum levels of CRP and PCT in the model group and chalcone group are higher than those in the control group, suggesting that *Streptococcus pneumoniae* infection can lead to abnormal changes of CRP and PCT. After treatment with chalcone, the levels of CRP and PCT in mice are significantly lower than those in model group, suggesting that chalcone may have anti-infection and anti-inflammatory effects.

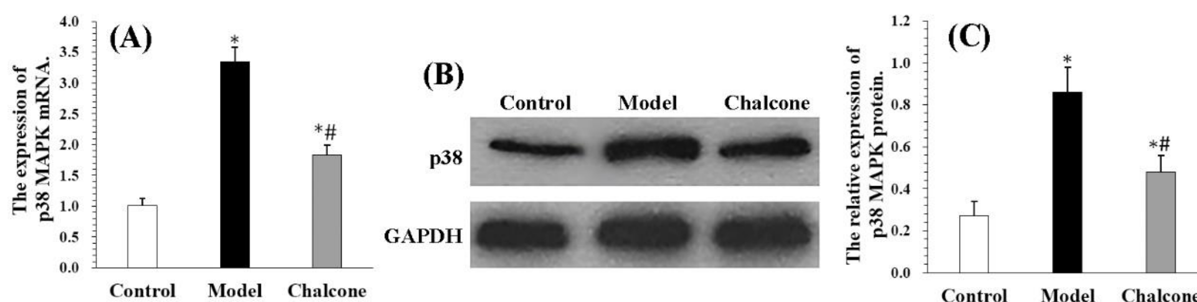

Figure 3.

Expression of p38 MAPK gene and protein in lung tissue of each group. A: p38 MAPK mRNA expression in lung tissue of each group; B: Western blotting detection results; C: p38 MAPK protein expression level in lung tissue of each group. *$p < 0.05$ compared with the control group; #$p < 0.05$ compared with the model group

Pneumonia leads to severe inflammatory response in the body of patients, causing activated monocyte macrophages to release inflammatory mediators [19]. Inflammatory response is one of the main manifestations of infectious diseases, which is an important index of infection and inflammatory response. IL-1β, TNF-α, IL-6 and IL-8 are widely used in the diagnosis of inflammatory conditions, mainly obesity relates diseases in mammals [20] and treatment of infectious diseases. Moreover, IL-1β, IL-6 and TNF-α can also promote the expression of inflammatory factors and inflammatory response by activating p38 MAPK signalling pathway [21]. Therefore, effective regulation of the positive feedback chain of inflammatory response caused by cytokines can reduce the damage of organs and tissues, and

alleviate the patient's condition [22]. Anderson *et al.* [23] and Cockeran *et al.* [24] found that *Streptococcus pneumoniae* could induce the synthesis and release of IL-8 in neutrophils. Early diagnosis of pathogen infection and effective anti-infection treatment is the key to improve the clinical efficacy of pneumonia patients. The results show that *Streptococcus pneumoniae* infection can lead to abnormal increase of IL-1β, TNF-α, IL-6 and IL-8 levels, but the levels of IL-1β, TNF-α, IL-6 and IL-8 in mice treated with chalcone are lower than those in model group, suggesting that chalcone can reduce the inflammatory response of *Streptococcus pneumoniae* to the body. In addition, the pathological changes of lung tissue in chalcone group are significantly lighter than those in model group, which is basically consistent with the results of inflammatory factors, suggesting that chalcone has preventive and therapeutic effect on pneumonia due to *Streptococcus pneumoniae*. P38 MAPK signalling pathway can be induced and activated by a variety of stress stimuli, inflammatory factors (IL-1β, IL-6, TNF-α and FGF), lipopolysaccharide (LPS), and can be phosphorylated and activated by activated MAPK kinase MKK3/MKK6 [25, 26]. Activated p38 MAPK can regulate the inflammatory response in lung tissue by increasing neutrophil recruitment and chemotaxis in vivo and regulating macrophage immune response [27]. Previous studies revealed that [28] blocking the p38 MAPK signal pathway could effectively reduce the inflammatory response of lung tissue, and then effectively protect alveolar epithelial cells and capillary endothelial cells. The research results show that the expression of p38 MAPK protein and mRNA in lung tissue of pneumonia due to *Streptococcus pneumoniae* is significantly higher than that of the control group, which confirms that the inflammatory response of pneumonia due to *Streptococcus pneumoniae* is positively correlated with p38 MAPK. After chalcone treatment, the expression of p38 MAPK in the lung tissue of mice is significantly decreased both in protein and gene levels, indicating that chalcone inhibits the expression of p38 MAPK in lung tissue.

Conclusions

Pneumonia caused by *Streptococcus pneumoniae* can increase serum CRP and PCT levels, increase IL-1β, TNF-α, IL-6 and IL-8 levels in lung tissue and increase expression of the p38 MAPK gene and protein in lung tissue. Chalcone treatment reduces the level of inflammatory factors in lung tissue of mice with pneumonia caused by *Streptococcus pneumoniae* and down-regulates the expression of the p38 MAPK gene and protein, thereby reducing the inflammatory response in lung tissue. Inhibition of p38 MAPK expression may be one of the mechanisms by which chalcone inhibits the inflammatory response in lung tissue of mice with *Streptococcus pneumoniae* pneumonia.

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Conflict of interest

The authors declare no conflict of interest.

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