Qufeng Xuanbi Formula ameliorates airway remodeling in asthmatic mice by suppressing airway smooth muscle cells proliferation through MEK/ERK signaling pathway

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Research

**Keywords:** asthma, Qufeng Xuanbi Formula, MEK/ERK signaling pathway, airway remodeling, airway smooth muscle cells
Qufeng Xuanbi Formula ameliorates airway remodeling in asthmatic mice by suppressing airway smooth muscle cells proliferation through MEK/ERK signaling pathway

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Abstract

Background: Asthma is a common chronic respiratory disease. Qufeng Xuanbi Formula (QFXBF), a Chinese herbal decoction, has shown efficiency for the management of asthma. The purpose of current study is to investigate the potential therapeutic effects of QFXBF for the treatment of asthma both in vitro and in vivo.

Methods: PDGF-induced ASMCs proliferation model and MTT assay have been applied for exploring the effects of QFXBF on the proliferation of ASMCs. Moreover, 40 female BALB/c mice were randomly divided into five groups: control group, OVA group, High QFXBF group, Low QFXBF group, and dexamethasone (DEX) group (n = 8 per group). The mouse allergic asthma model has been established by intranasally administered ovalbumin (OVA) sensitization method. Morphological changes of the lung tissue have been examined by hematoxylin and eosin (H&E) staining and Masson’s staining. Finally, the protein expressions of α-SMA, PCNA, p-MEK1/2, MEK1/2, p-ERK1/2, and ERK1/2 in ASMCs and lung tissue were determined by western blotting and immunofluorescent staining assays.

Results: PDGF induced significant increase in viability of ASMCs. Compared with mice in control group, the airway walls and airway smooth muscle of mice in OVA group mice thickened, and the inflammatory cells around the bronchus increased significantly. Moreover, administration of QFXBF markedly inhibited the proliferation of ASMCs and alleviated the
pathologic changes induced by OVA. Furthermore, the protein expressions of p-ERK1/2, p-MEK1/2, PCNA, and α-SMA were significantly increased in OVA-treated mice and PDGF-treated ASMCs. Finally, treatment of QFXBF also significantly decreased the protein expression of p-ERK1/2, p-MEK1/2, α-SMA and PCNA.

Conclusion: QFXBF can inhibit the proliferation of ASMCs via suppressing the MEK/ERK signaling in PDGF-induced ASMCs and OVA-induced mice.

Key Words: asthma; Qufeng Xuanbi Formula; MEK/ERK signaling pathway; airway remodeling; airway smooth muscle cells

Introduction

Asthma is a chronic respiratory disease that affects nearly 400 million individuals worldwide. Increasing evidence suggested that asthma has become a global healthcare issue that significantly affected the quality of life of patients, and also induced a massive increase in healthcare burden[1]. Chronic airway inflammation and airway remodeling were known as key pathological changes in the pathogenesis of asthma[2]. Airway remodeling is the main factor that regulates the progression of asthma, which leads to incompletely reversible obstruction of airflow[3]. At present, glucocorticoid drugs are still the first-line medication for the management of asthma; however, the safety issue remains to be solved, and whether glucocorticoid medications could fundamentally reduce the process of airway remodeling remains further investigation[4-5]. Therefore, it is vital to explore the pathogenesis of airway remodeling in asthma and develop potential anti-asthma therapies with improved therapeutic efficacy.

Airway remodeling caused by abnormal proliferation of airway smooth muscle cells (ASMCs) is a typical pathological feature of asthma[6-7]. Previous results suggested that multiple factors can regulate the proliferation of AMSCs, for example, it has been reported that mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway plays key role in the development of asthma[8]. ERK is widely distributed in differentiated cells[9], and ERK is known to participate in multiple cellular activities, including the differentiation, secretion, and proliferation of smooth muscle cells[10] and tumor cells[11]. Studies have shown that phosphorylated ERK can regulate the expression of various growth
factors as well as inflammatory mediators, which further inhibit the over-proliferation of ASMCs and consequentially leading to the airway remodeling of asthma \(^{[12]}\). On the other hand, inhibition of the MEK/ERK signaling pathway can suppress the over-proliferation and migration of endothelial cells \textit{in vitro} \(^{[13-14]}\). Therefore, exploring the potential roles of the MEK/ERK signaling pathway in the pathogenesis of asthma may provide a new method for the treatment of asthma.

Qufeng Xuanbi Formula (QFXBF) has been an approved for hospital prescription in Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China), and it has been used for the treatment of asthma during the past 30 years with its high efficiency for the management of refractory asthma. Result of previous studies have proved that QFXBF can reduce eosinophils (EOS) infiltration and decrease airway inflammation, and consequentially inhibit occurrence and development of asthma via regulating the imbalance of Th1/Th2 \(^{[15]}\). However, the pharmacological effects of QFXBF on the MEK/ERK signaling pathway during the process of airway remodeling remains to be further investigated. Therefore, we treated ASMCs with PDGF to create the proliferation cell model \textit{in vitro}, and established the asthmatic mice model by OVA stimulation. The objective of this study was to investigate the effects of QFXBF on alleviating the airway remodeling of asthma through modulating the MEK/ERK signaling pathway.

**Material and methods**

**Chemicals and Reagents**

Platelet-derived growth factor (PDGF) were purchased from Proteintech (Wuhan, China); U0126 was provided by Selleck Chemicals (Shanghai, China); Fetal bovine serum (FBS), RPMI-1640 and penicillin-streptomycin solution were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA); ovalbumin (OVA) was obtained from Sigma–Aldrich (Saint Louis, MO, United States); Dexamethasone (DEX) was purchased from Xianjv Pharmaceuticals (Zhejiang, China). phospho-ERK1/2(p-ERK1/2); ERK1/2 antibodies and HRP conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, United
States); phospho-MEK1/2 (p-MEK1/2), MEK1/2 and β-actin antibodies were obtained from Affinity (Changzhou, China); PCNA, α-SMA and GAPDH antibodies were purchased from Proteintech (Wuhan, China); RIPA lysis buffer and BCA protein assay kit were obtained from Beyotime (Shanghai, China).

**Preparation of QFXBF**

QFXBF is composed of *Belamcandae Rhizoma* 10g, *Ephedrae Herba* 5g, *Armeniacae Semen Amarum* 10g, *Glycyrrhizae Radix et Rhizoma* 5g, *Pheretima* 10g, *Trichosanthis Fructus* 10g, *Allium macrostemon Bunge* 10g, *Bombyx Batryticatus* 10g, *Kadsura Pepper Stem* 15g, *Cnidii Fructus* 15g. All herbs were obtained from the Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China). QFXBF decoction was prepared according to the conventional method. All of the herbs were impregnated in 1000 ml water for 1 h, boiled for 45 minutes and then the solution was collected; then 500 ml of water have been added to the residual liquid and boiled for further 45 min; the two extracts were then filtered, concentrated to 1g/ml, and stored at 4°C. Chromatographic analysis of QFXBF sample showed that the active ingredients in this prescription included tectorigenin, glycyrrhizic acid, amygdalin, and osthol[15].

**Cell Culture and Treatment**

Mice ASMCs cell was purchased from the Chinese Academy of Science (Shanghai, China). ASMCs were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin, cultured in an incubator at 37 °C with 5% CO2. ASMCs were seeded onto 2×10^5/well in a 6-well plate. After the cells were adhered, 10 ng/ml PDGF was added to the cell culture medium. Treatment of QFXBF (2 mg/ml, 4 mg/ml, 8 mg/ml) or U0126 (10μmol/L) begins after the induction of PDGF, and lasted for an additional 48 h before harvesting.

**Cell Proliferation Assay**

Cell proliferation was determined by the MTT Cell Viability Assay Kit (Beyotime Biotechnology, Shanghai, China). ASMCs were seeded onto 96-well plates with 8000 cells/well, and after treatment, 15 μL MTT (5 mg/ml) was added to each well and incubated for 4 h. The supernatant was then removed and 150 μL dimethyl sulfoxide(DMSO) was added to each well. The viability of the cell was determined by measuring the absorbance of each well at 490 nm
using an ELX800 automatic microplate reader (Bio-Tek, USA).

**Animals and Treatment**

The animal protocol was approved by the Animal Care and Use Committee of Affiliated Hospital of Nanjing University of Chinese Medicine (2021DW-07-02, Nanjing, China). Forty female BALB/c mice (6-8 weeks old, weighing 18-22 g) were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China) and housed in Animal Laboratory of Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, China). After one week of adaptive feeding, the mice were randomly divided into five groups: control group, OVA group, High QFXBF group, Low QFXBF group and DEX group. On day 0 and day 14, mice of the OVA group, High QFXBF group, Low QFXBF group and DEX group were sensitized via intraperitoneal injection of 100 µg of OVA combined with aluminum hydroxide. On day 14, 25, 26 and 27, every mouse of the above groups was intranasally administered with 50 µL of OVA. For the control group, normal saline was used following the same operation. Between day 14 and 27, mice of High QFXBF group (25g/kg) and Low QFXBF group were intragastrically administered with QFXBF (12.5g/kg) once/day, while mice of the DEX group were intragastrically administered 2mg/kg DEX once/day, and mice of the saline group and OVA group received normal saline (0.5 mL). On day 28, mice were sacrificed, and the pathophysiological and immunological features of asthma were determined.

**Histopathology**

Lung tissues were fixed with 10% formalin liquid, and the fixed sections were embedded into paraffin, sectioned and stained with H&E and Masson. Tissue sections were visualized using light microscopy with the magnification of 400x. The bronchial basement membrane perimeter (Pbm), bronchial wall area (WAt), bronchial smooth muscle area (WAm) and the Masson stained area were determined using Image-Pro Plus software. The bronchial wall thickness (WAt/Pbm), bronchial smooth muscle thickness (WAm/Pbm) and Masson stained area/Pbm were calculated for indicating the degree of airway remodeling as previously described [16].

**Western blot assay**

Total proteins were extracted from lung tissues and ASMCs, and the concentration of the proteins were quantified using the bicinchoninic acid (BCA) protein assay kit. Then, equal
amounts of protein samples were separated by 10% SDS-PAGE (80 V, 30 min and then 120 V, 60 min). Next, the separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (250mA, 90 min), and then blocked with 5% bovine serum albumin (BSA) for 60 min at room temperature. The membrane were then incubated with primary antibodies (anti-PCNA (1:2,000 dilution), anti-α-SMA (1:1,000 dilution), anti-p-MEK1/2 (1:1,000 dilution), anti-MEK1/2 (1:500 dilution), anti-p-ERK1/2 (1:1,000 dilution), anti-ERK1/2 (1:1,000 dilution)) overnight at 4 °C. On day 2, the membranes were incubated with the fluorescence-conjugated secondary antibodies (1: 3,000 dilution). ECL luminescent liquid was used for visualization. The results were analyzed and quantified by Image Lab software (BIO-RED, USA).

**Immunofluorescence assay**

After fixing in Immunol Staining Fix Solution, the cells were permeabilized with 0.3% Triton X-100 in PBS for 3 times (5 mins each time) and blocked with 5% BSA for 30 mins at room temperature. Then the cells were incubated with the primary antibodies (anti-PCNA (1:50 dilution) and anti-α-SMA (1:100 dilution)) overnight at 4°C. On day 2, cells were washed with PBS for 3 times, and then incubated with the secondary antibodies (1:100 dilution) for 60 mins at room temperature; Next, the cells were washed with PBS for 3 mins, and the nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI). Finally, the cells with immunofluorescence staining were visualized by using a fluorescence microscope (NIKON, Japan).

**Statistical Analysis**

Data are expressed as the mean±standard deviation. Statistical analyses were performed using SPSS 23.0 software. The difference among multiple groups was analyzed by one-way ANOVA, and p< 0.05 was considered as the difference was statistically significant.

**Results**

**QFXBF inhibited the hyperproliferation of ASMCs induced by PDGF**

To verify the inhibitory effects of QFXBF on the hyperproliferation of ASMCs, PDGF was used to stimulate ASMCs in order to build up the over-proliferation cell model(Figure 1A). The cytotoxic effects of QFXBF on ASMCs was examined by MTT assay. ASMCs were treated by
QFXBF with different concentrations (2–32mg/mL) for 48 h, and the maximum non-toxic concentration of QFXBF was 8mg/ml(Figure 1B). Moreover, as shown in Figure 1C, compared with the control group, treatment of PDGF significantly promoted the proliferation of ASMCs, while the administration of QFXBF markedly reduced the proliferation of ASMCs in comparison with the PDGF group in a dose-dependent manner.

**Figure 1** QFXBF inhibited the hyperproliferation of ASMCs induced by PDGF. (A) The protocol of current work. (B) MTT assay has been applied to determine the cytotoxic effect of QFXBF on the viability of ASMCs. (C) Cell viability was detected by MTT assay. All data were presented as means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

**QFXBF inhibited the expressions of α-SMA and PCNA in ASMCs**

Overexpression of PCNA and α-SMA is a significant hallmark of ASMCs proliferation, so next, the protein expressions of PCNA and α-SMA were detected by western blot assay. As shown in Figure 2A-B, PDGF induced significant up-regulation of PCNA and α-SMA in ASMCs compared with the control group, while administration of QFXBF reduced the expressions of PCNA and α-SMA. In addition, immunofluorescence staining has also been performed to examine the expressions of α-SMA and PCNA (Figure 2C-D), and the results were similar the
western blot.

**Figure 2** QFXBF inhibited the expression of α-SMA and PCNA in ASMCs. (A) Expressions of PCNA and α-SMA protein in ASMCs by western blot. (B) Quantification of WB results in (A). (C) Expression of PCNA(C) and α-SMA(D) in ASMCs by immunofluorescence staining. All data are presented as means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

**PDGF induced hyperproliferation of ASMCs via regulating MEK/ERK signaling pathway**

The experimental protocol is illustrated in **Figure 3A**. As shown in **Figure 3B-C**, the expressions of p-MEK1/2 and p-ERK1/2 were significantly increased in the PDGF-induced hyperproliferation model in comparison with the control; on the other hand, U0126, an inhibitor of the MEK/ERK signaling pathway, markedly reduced the phosphorylation of MEK and ERK induced by PDGF, and also inhibited the proliferation of ASMCs through inhibiting the expressions of α-SMA and PCNA (**Figure 3D-E**).
QFXBF inhibits PDGF-induced hyperproliferation of ASMCs via regulating MEK/ERK signaling pathway

Furthermore, we explored the effects of QFXBF on activation of the MEK/ERK signaling in PDGF-induced hyperproliferation of ASMCs by western blot methods. As shown in Figure 4A-B, the expressions of p-MEK1/2 and p-ERK1/2 in ASMCs were significantly upregulated in
PDGF-induced hyperproliferation model in comparison with the control group. On the other hand, QFXBF treatment significantly reduced the phosphorylation of p-MEK1/2 and p-ERK1/2 in a dose-dependent manner.

**Figure 4** QFXBF inhibits PDGF-induced hyperproliferation of ASMCs via regulating the MEK/ERK signaling pathway. (A) Expressions of p-MEK1/2, MEK1/2, p-ERK1/2, and ERK1/2 in ASMCs by western blot method. (B) Quantification of results in (A). All data are presented as means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

**QFXBF alleviated the inflammation and airway remodeling in OVA-induced asthmatic mice models**

To verify the protective effects of QFXBF in airway remodeling process of asthma, OVA-induced asthmatic mice model was established (*Figure 5A*). As shown in *Figure 5B*, the airway mucosa epithelium and alveolar structure of the mice were relatively complete in control group, and there were no significant inflammatory condition in the lung tissues; meanwhile, infiltration of peribronchial and perivascular inflammatory cells were observed in OVA group; on the other hand, treatment of High QFXBF or DEX significantly alleviated inflammatory condition and infiltration of the immune cells, while the curative effects were not obvious in Low QFXBF group. Furthermore, QFXBF effectively alleviated the remodeling of the airway structure. Finally, collagen deposition was examined by Masson trichrome staining(*Figure 5B*), and over-expression of collagen was observed in OVA group, while QFXBF significantly decreased the deposition collagen. Compared with the control group, the thicken of bronchiolar wall as well as airway smooth muscle was shown in the lung tissue of
the OVA mice, while treatment of High QFXBF and DEX alleviated the above condition (Figure 5C-D).

**Figure 5** QFXBF inhibited the inflammatory condition and airway remodeling process of OVA-induced asthmatic mice. (A) Animal experimental protocol of this study. (B) Representative images of the mice lung tissue by HE and Masson staining. Scale bar, 50 μm. (C) Quantitative analyses of WAt/Pbm. (D) Quantitative analyses of WAm/Pbm. All data are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

**QFXBF inhibits the expressions of α-SMA and PCNA in lung tissue**

In addition, we further detected the expressions of α-SMA and PCNA by western blot methods. Compared with the control group, the protein expressions of PCNA and α-SMA were significantly increased ($P < 0.05$) in OVA group. Meanwhile, administration of High QFXBF and DEX significantly inhibited the protein expressions of PCNA and α-SMA (Figure 6A-B).
Figure 6 QFXBF inhibited the expression of α-SMA and PCNA in lung tissue. (A) Protein expressions of PCNA and α-SMA in lung tissue by western blot assay. (B) Quantification of results in (A). All data are presented as means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

Effects of QFXBF on airway remodeling were regulated by MEK/ERK signaling pathway

In order to determine whether QFXBF could affect MEK/ERK signaling during the process of OVA-induced airway remodeling, the protein expressions of p-MEK1/2, MEK1/2, p-ERK1/2, and ERK1/2 in mice with different treatments were examined. As shown in Figure 7A-B, the expressions of p-MEK1/2 and p-ERK1/2 in the lung tissues of the mice were markedly increased in the model group compared with the control group, while treatment of High QFXBF and DEX significantly decreased the protein expressions of p-MEK1/2 and p-ERK1/2.

Figure 7 Effects of QFXBF on airway remodeling were regulated by MEK/ERK signaling pathway. (A) Protein expressions of p-MEK1/2, MEK1/2, p-ERK1/2, and ERK1/2 in lung tissue
of the mice by western blot. (B) Quantification of results in (A). All data are presented as means ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

Discussion

Airway remodeling is an important pathological feature of asthma \(^{[17]}\), and it is also an important cause of repeated attacks of asthma, causing the hyperresponsiveness of airway and the chronic decline of the lung function. Airway remodeling is described as the pathological reorganization of cells and molecular components of the airway wall, including airway epithelial detachment, goblet cell proliferation, increased mucus gland secretion, subepithelial fibrosis, hyperproliferation and hypertrophy of ASMCs \(^{[18-20]}\). Results of previous studies have shown that the proliferation of ASMCs is a main characteristic of airway remodeling in asthma \(^{[21-23]}\), which induces the remodeling process by increasing the thickness of bronchial wall and airway smooth muscle \(^{[24-25]}\). Therefore, drugs that inhibited the proliferation of ASMCs may improve the process of airway remodeling in the pathogenesis of asthma. Traditional Chinese medicine (TCM) has a long history for the treatment of asthma with high therapeutic efficacy \(^{[26]}\). As a TCM for the treatment of asthma in China for many years, QFXBF has shown remarkable curative effects for the treatment and prevention of asthma. Results of previous studies indicated that QFXBF could alleviate the inflammatory condition in mice asthma model \(^{[15]}\). However, little was known regarding the effects of QFXBF on the process of airway remodeling. Our study demonstrated that treatment of QFXBF inhibited the proliferation of airway smooth muscle cells \textit{in vitro} and airway remodeling \textit{in vivo}. Our data also confirmed the protective effects of QFXBF was affected by the MEK/ERK signaling pathways \textit{in vitro} and \textit{in vivo}.

Furthermore, we also explored the effects of QFXBF on the process of airway remodeling \textit{in vitro} and \textit{in vivo}. PDGF is known as the major stimulus that induces the proliferation of ASMCs\(^{[27]}\) and participate in the process of airway remodeling \(^{[28-30]}\). Therefore, PDGF is often used to induce excessive proliferation of the ASMCs. In this study, we used PDGF to induce the excessive proliferation of ASMCs. Our results suggested that QFXBF could inhibit the
proliferation and viability of ASMCs in a dose-dependent manner. In asthma animal models, the thickness of the bronchial walls and increased airway smooth muscles in OVA-induced mice were alleviated in the High QFXBF group. PCNA is an indicator of cell proliferation, and it is also an accessory protein of a polymerase that is necessary for the replication of DNA \cite{31}. α-SMA is a primary marker of airway smooth muscle, and it is considered as a crucial index for the airway remodeling \cite{32}, and it can significantly affect the contractility of the airway smooth muscle \cite{33}. In our study, expressions of PCNA and α-SMA were used to examine the effects of QFXBF on airway remodeling. Our results showed that expressions of PCNA and α-SMA were increased in the lung tissues of mice asthma models and the hyperproliferative ASMCs, while QFXBF could alleviate the overexpression of α-SMA and PCNA. These results indicate that QFXBF could exert therapeutic effects on airway remodeling.

To further explore the underlying mechanisms of QFXBF on airway remodeling, we investigated the changes of MEK/ERK signaling pathway with different treatment. MEK/ERK signaling pathway plays a vital role in the pathogenesis and development of asthma, and it was known to aggravate the inflammatory condition as well as the airway remodeling process. As an important part of the MAPK signaling pathway, ERK-induced signaling can be transmitted from extracellular region to the nucleus, which could further affect the proliferation of ASMCs and participate in the airway remodeling process in the development of asthma \cite{34}. It has been reported that Shenmai injection can improve the airway remodeling by regulating ERK signaling pathway and affect the proliferation of ASMCs \cite{35}. Moreover, vasoactive intestinal peptide has been reported to inhibit the proliferation of airway smooth muscle cells in a mouse model of asthma via regulating the ERK1/2 signaling \cite{36}. Our results were consistent with the results of the previous works. In our study, the phosphorylation of MEK and ERK in the OVA-induced mice and PDGF-induced ASMCs were markedly increased, indicating that the activation of MEK/ERK signaling pathway is closely related to the development of asthma. Moreover, we used the MEK/ERK signaling pathway inhibitor U0126 to confirm that PDGF induced the hyperproliferation of ASMCs through regulating the MEK/ERK signaling. Like U0126, QFXBF could inhibit PDGF-induced hyperproliferation of ASMCs and the
phosphorylation of ERK and MEK, which further confirmed the protective roles of QFXBF in asthma.

**Conclusion**

In this study, we found the protective roles of QFXBF in asthma. QFXBF can inhibit the proliferation of ASMCs by suppressing the activation of MEK/ERK signaling in OVA-stimulated mice and PDGF-treated ASMCs, eventually suppressing the process of airway remodeling. Our findings provided new evidence for the use of QFXBF as a potential anti-remodeling candidate for the treatment of asthma.

**Abbreviations**

QFXBF: Qufeng Xuanbi Formula; DEX: dexamethasone; OVA: ovalbumin; H&E: hematoxylin and eosin; ASMCs: airway smooth muscle cells; MEK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; PDGF: Platelet-derived growth factor; Pbm: bronchial basement membrane perimeter; WAt: bronchial wall area; WAm: bronchial smooth muscle area; TCM: Traditional Chinese medicine.

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**Authors’ contributions**

Study concept and design: SS and LL. Animal handling and tissue collection: BW and LT. Cell Culture: BW. Molecular biology experiments and data analysis: BW, LT, XS and XZ. Histopathology analysis: CL. Drafting of the manuscript: BW, YY and LT. Critical revision of the manuscript for important intellectual content: LL and SS. Obtained funding: SS. All authors have read and approved the manuscript.
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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All experiments were evaluated and approved by the Animal Care and Use Committee of Affiliated Hospital of Nanjing University of Chinese Medicine (2021DW-07-02, Nanjing, China).

Consent for publication

Not applicable.

Competing interests

All authors declare no conflicts of interest.

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