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Original Article

Traditional Chinese medicine Ze-Qi-Tang formula reduces inflammation in mice with asthma by inhibiting PI3K/AKT/NF-κB signaling pathway

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Abstract

Asthma is a chronic airway inflammatory disease. The excessive proliferation of airway smooth muscle cells (ASMCs) is associated with airway remodeling. Ze-Qi-Tang (ZQT) is a popular traditional Chinese medicine preparation and has been confirmed to have therapeutic effects on lung diseases. This study is aimed to probe the biological function of ZQT in asthma. RT-qPCR and ELISA were utilized for testing the mRNA levels and concentrations of pro-inflammatory factors. Colony formation and transwell assay were applied to test cell viability and migration. The mouse model with asthma was established by ovalbumin (OVA) induction. Western blot was utilized for detecting the activation of PI3K/AKT/NF-κB pathway. We found that the concentrations of proinflammatory factors in cells induced by PDGF-BB could been suppressed by ZQT. ZQT-H treatment notably repressed cell viability and proliferation. Furthermore, we proved the suppressive effect of ZQT on airway inflammation in asthma mice. Additionally, we discovered that ZQT could suppress the PI3K/AKT/NF-κB pathway in PDGF-BB-induced ASMCs. To sum up, ZQT reduced airway inflammation and remodeling in mice with asthma via inactivating PI3K/AKT/NF-κB pathway.

Keywords: Asthma, Airway inflammation, Airway smooth muscle cells, PI3K/AKT/NF-κB pathway, Ze-Qi-Tang.

1. Introduction

Asthma is a chronic inflammatory respiratory system disease, with main clinical manifestations such as cough, chest tightness, wheezing, shortness of breath, and chest discomfort [1]. Airway inflammation, airway remodeling and hyperresponsiveness are the main pathological features of asthma [2]. Airway remodeling is an airway repair response to persistent injury caused by inflammation [3]. Airway smooth muscle cells (ASMCs) are multifunctional cells, whose excessive proliferation and migration directly lead to airway remodeling [4]. It has been reported that elevated platelet-derived growth factor (PDGF)-BB levels in the lungs of asthmatic patients can lead to proliferation and migration of ASMCs and facilitate airway remodeling [5]. Currently, the common anti-inflammatory therapy is the inhalation of glucocorticoids [6]. However, some patients will have serious adverse reactions after long-term use of glucocorticoids. Therefore, in-depth exploration of the pathogenesis of asthma is helpful to develop new therapeutic strategies.

Ze-Qi-Tang (ZQT) is a popular traditional Chinese medicine preparation composed of nine different herbs, first recorded in the Classic Jin Gui Yao Lue [7]. In ZQT formula, Zeqi is considered the main ingredient [8]. Sovereign drugs always play the most vital function in the entire formula. Modern pharmacological research has found that Zeqi contains mushrooms, natural flavonoids, and polyphenols. Studies have confirmed ZQT can be effectively used to treat lung diseases such as cough, asthma, chest tightness, shortness of breath, pleural effusion, and lung cancer. For example, ZQT formula can induce cell apoptosis to repress the development of lung cancer in mice [7]. ZQT is reported to alleviate chronic cough in lung cancer patients. Additionally, the therapeutic effect of ZQT on acute nephritis has also been confirmed. However, the specific roles ZQT in asthma are not yet clear.

This study is aimed to probe the specific roles and regulatory mechanisms of ZQT in asthma. Our findings may provide new evidence for ZQT as a treatment for asthma.

2. Materials and methods

2.1. Preparation of ZQT

The crude drugs were purchased from Kangqiao Chinese Medicine Pieces Company (Shanghai, China). For preparing the lyophilized ZQT powder, herbs are mixed in a ratio of 30: 15: 10: 10: 10: 6: 6: 6: 6 (Table 1). The mixture was subjected to extraction with boiling water and further steamed into 0.144 g/mL extracts prior to being lyophilized into powder.

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2.2. Cell culture

Human ASMC was purchased from Bnbio (Beijing, China) and incubated in DMEM (Invitrogen, USA) added with 10% FBS with 5% CO_2 at 37°C. For PDGF-BB induction, cells were treated with 10 ng/ml PDGF-BB (Sigma-Aldrich, USA) for 24 h. For treatment, cells were treated with 0.5mg/ml or 1mg/ml ZQT for 24 h.

2.3. RT-qPCR

Total RNA from cells was isolated through the TRIzol method. RNA was reverse-transcribed to cDNA utilizing the PrimeScript Reverse Reagent Transcriptase Kit (Takara, Japan). After that, the cDNA was amplified utilizing SYBR mix (Takara). The relative fold changes were estimated by the $2^{-\Delta\Delta Ct}$ method.

2.4. Colony formation assay

Cells were put in the 6-well plates and incubated for 1e4 days. After that, cells were subjected to fixation with 4% PFA and dyed with 0.1% crystal violet for 1 h. The quantity of colonies was calculated manually.

2.5. CCK-8 assay

A Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was utilized for testing cell viability. Cells were put in 96-well plates and cultured for 24 h. Next, cells were supplemented with 10 μL CCK-8 solution for 1 h. The absorbance was estimated via a microplate reader (Molecular Devices, USA).

2.6. Transwell assay

Cell migration was determined using a Transwell chamber (Corning, USA) with an 8-μm pore filter Cells were suspended in serum-free basic medium and placed in the upper chamber. The lower chamber was full of culture medium supplemented with 20 % FBS for 24 h. After that, the migrated cells were fixed by 4% PFA and then dyed by crystal violet. A light microscope (Olympus, Tokyo, Japan) was utilized for analysis.

2.7. Western blot

Cells were lysed in the RIPA lysis buffer (Thermo Fisher Scientific, USA). 20 ug proteins were subjected to separation utilizing 12% SDS-PAGE, followed by transferring to PVDF membranes. After being blockaded with 5% non-fat dry milk, the membranes were cultured with the primary antibodies (Abcam, USA) for one night at a temperature of 4 °C. The membranes were further incubated with secondary antibodies (Abcam) for 2 h. In the end, proteins were visualized using ECL luminescent liquid (Thermo Fisher Scientific).

2.8. Animal experiments

BALB/c mice (female; 8 weeks; 20-23 g) were purchased from Gempharmatech Co., Ltd (Nanjing, China) and maintained in an SPF facility for seven days of adaptive feeding. All the mice experiments were approved by the Experimental Animal Ethics Committee of the First Clinical Medical College of Heilongjiang University of Chinese Medicine. For establishing a mouse model with asthma, mice were intraperitoneally injected with 20 μg OVA (Sigma-Aldrich) on day 0 and day 14 with a total volume of 200 μl. After sensitization, 1% OVA aerosol particles were utilized to attack mice on days 21, 22, and 23. Control mice were challenged with PBS for half an hour. For treatment, mice were subjected to intraperitoneal injection of ZQT (0.2g/kg and 0.4g/kg) from day 21 to day 23. After the final OVA challenge, mice were euthanized.

2.9. ELISA

The concentrations of TNF-α, IL-4, IL-6, and IL-13 in cell supernatant were tested through their corresponding ELISA kits (R&D Systems, USA) in accordance with user guides. Total IgE and OVA-specific IgE in the serum of mice were determined by the IgE ELISA Kit and the OVA sIgE ELISA Kit (Mlbio, Shanghai, China).

2.10. Statistical analysis

Statistical analyses were implemented through GraphPad Prism 8 software. Data were presented as mean ± SD from three individual repeats. Group difference was analyzed by one-way ANOVA. $p<0.05$ was considered significant.

3. Results

3.1. ZQT suppresses inflammatory response in asthma

It has been reported that PDGF-BB is abundant in patients with asthma, and it can trigger tracheal remodeling induced by ASMC proliferation [9]. In order to study the role of ZQT in asthma, we first constructed an *in vitro* cell model of asthma and treated model cells with low or high concentrations of ZQT. Through RT-qPCR and ELISA, we proved that PDGF-BB induction notably elevated the mRNA expression levels and concentrations of inflammatory factors (TNF-α, IL-4, IL-6, and IL-13) in ASMC, while ZQT administration sensibly repressed their expressions and concentrations (Fig. 1A-B). The inhibitory effect of high concentration of ZQT on inflammatory factors was

Table 1. The main composition of Ze-Qi-Tang formula.

Main composition	Latin scientific name	Parts used	Amount (g)
Euphorbia helioscopia (Ze Qi)	*Euphorbia helioscopiaL.*	Herba	30
Processed Pinellia ternate (Zhi Ban Xia)	*Pinellia ternata (Thunb.) Breit.*	Tuber	15
Cynanchum glaucescens (Bai Qian)	*Cynanchum glaucescens (Decne.) Hand-Mazz.*	Rhizome	10
Fresh ginger (Sheng Jiang)	*Zingiber officinale Roscoe*	Rhizome	10
Salvia chinensis (Zi Shen)	*Rubia yunnanensis Diels*	Herba	10
Cassia twig (Gui Zhi)	*Cinnamomum cassia Presl*	Twig	6
Ginseng (Ren Sheng)	*Panax ginseng C. A. Mey.*	Radix	6
Liquorice (Gan Cao)	*Glycyrrhiza uralensis Fisch.*	Rhizoma	6
Scutellaria baicalensis (Huang Qin)	*Scutellaria baicalensis Georgi*	Radix	6

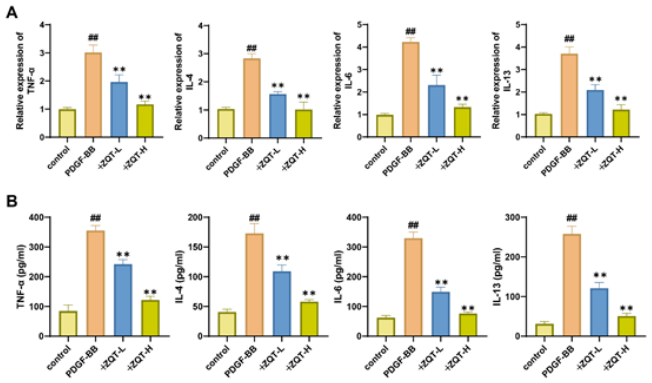

Fig. 1. ZQT suppresses inflammatory response in asthma. (A) RT-qPCR outcomes of TNF-α, IL-4, IL-6, and IL-13 expressions in ASMC of the control group, PDGF-BB group, ZQT-L group, and ZQT-H group. (B) ELISA outcomes of TNF-α, IL-4, IL-6, and IL-13 concentrations in different cells. **P<0.01; ##P<0.01.

sensibly higher than that of low concentration of ZQT. Therefore, we believe that ZQT can suppress inflammatory responses in asthma.

3.2. ZQT restrains ASMC proliferation

Studies have shown that the proliferation and migration of ASMC are associated with the development of severe asthma [10, 11]. Therefore, we examined the effects of ZQT on ASMC proliferation and migration. By CCK-8 assay, we found that ZQT treatment sensibly inhibited PDGF-BB-enhanced cell viability (Fig. 2A). Clone formation assay further showed that the promotion of cell proliferative ability by PDGF-BB was counteracted by ZQT treatment (Fig. 2B-C). In addition, the suppressive function of ZQT-H on cell viability and proliferation was sensibly higher than that of ZQT-L.

3.3. ZQT suppresses ASMC migration

Next, we examined the effect of ZQT on cell migration by transwell assay. The results manifested that PDGF-BB induction sensibly enhanced the quantity of migratory cells, while ZQT administration notably reduced this number (Fig. 3A-B). In addition, western blot results proved that PDGF-BB induction increased the levels of migration-related proteins (MMP-9 and MMP-3), while their levels markedly declined after ZQT treatment (Fig. 3C-D). In addition, the suppressive effect of ZQT-H on cell migration was notably higher than that of ZQT-L (Fig. 3A-D). Therefore, we concluded that ZQT alleviated asthma process by suppressing the proliferation and migration of ASMC.

3.4. ZQT alleviates inflammatory response in asthma mice

To further validate the role of ZQT in asthma, we constructed a mouse model of asthma by OVA induction. ELISA results manifested that the elevated concentrations of TNF-α, IL-4, IL-6, and IL-13) in model mice were sensibly declined by ZQT-L or ZQT-H administration (Fig. 4A). Additionally, we discovered that ZQT-L and ZQT-H administration sensibly suppressed the elevated IgE and OVA-IgE concentrations in the model mice (Fig. 4B). Thus, we confirmed the suppressive effect of ZQT on airway inflammation in asthma mice.

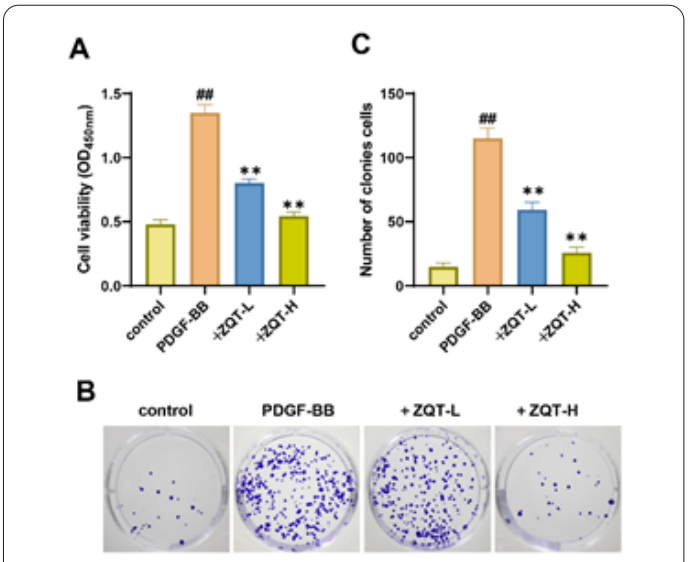

Fig. 2. ZQT restrains ASMC proliferation. (A) CCK-8 assay was utilized to detect cell viability of ASMC in the control group, PDGF-BB group, ZQT-L group, and ZQT-H group. (B-C) Colony formation assay was applied to test cell proliferative capability. **P<0.01; ##P<0.01.

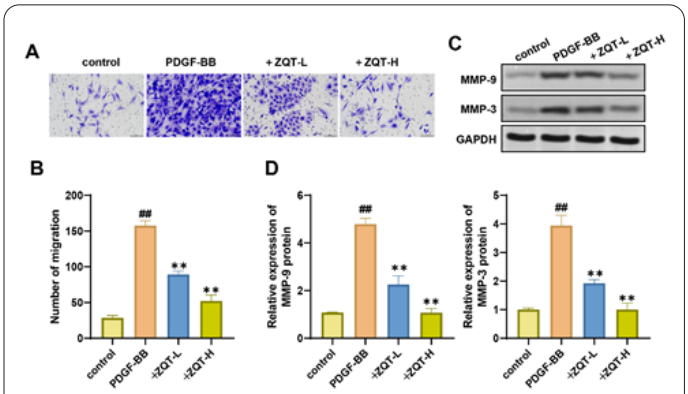

Fig. 3. ZQT suppresses ASMC migration. (A-B) Transwell assay was employed for detecting cell migratory capability in the control group, PDGF-BB group, ZQT-L group, and ZQT-H group. (C-D) Western blot outcomes of MMP-9 and MMP-3 protein levels in different cells. **P<0.01; ##P<0.01.

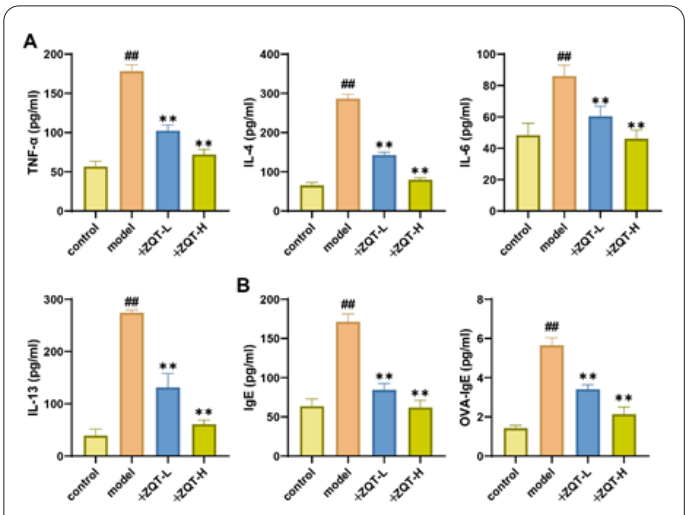

Fig. 3. ZQT alleviates inflammatory response in asthma mice. (A-B) ELISA was implemented for detecting the concentrations of TNF-α, IL-4, IL-6, IL-13, IgE, and OVA-IgE in lung tissues of mice in the control group, model group, ZQT-L group, and ZQT-H group. **P<0.01; ##P<0.01.

3.5. ZQT relieves asthma by inactivating the PI3K/AKT/NF-κB pathway

Studies have shown that the PI3K/AKT/NF-κB signaling pathway is associated with the development of asthma [12]. We further investigated whether ZQT suppresses asthma development by modulating the PI3K/AKT/NF-κB pathway. Western blot results illustrated that OVA induction sensibly elevated the protein levels of p-p65, p-AKT, and p-PI3K; however, these levels were reduced by ZQT-L and ZQT-H treatment (Fig. 5A-B). Protein levels of total p65, AKT, and PI3K did not change sensibly in the control, model, and treatment groups (Fig. 5A-B). Therefore, we concluded that ZQT alleviated asthma via suppressing the PI3K/AKT/NF-κB signaling pathway.

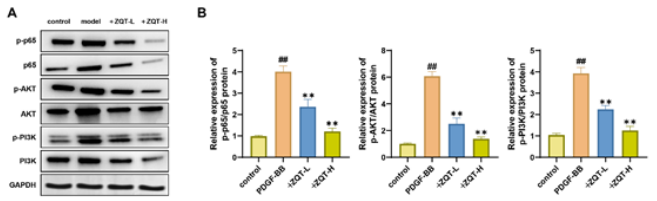

Fig. 5. ZQT relieves asthma by inactivating the PI3K/AKT/NF-κB pathway. (A-B) Western blot outcomes of p-p65, p65, p-AKT, AKT, p-PI3K, and PI3K protein levels in cells of the control group, PDGF-BB group, ZQT-L group, and ZQT-H group. **P<0.01; ##P<0.01.

4. Discussion

Asthma is a chronic airway inflammatory disease with a high incidence rate worldwide [13]. Airway inflammation is a vital pathological process in asthma, caused by infiltration of various cells and the release of various inflammatory cytokines [14]. ZQT is a traditional Chinese medicine prescription that has been frequently utilized to relieve cough, chest tightness, asthma, and other lung diseases There are nine commonly used Chinese medicinal herbs in ZQT, and these herbs combined can produce synergistic therapeutic effects. In our study, we found PDGF-BB induction sensibly elevated the concentrations and mRNA expressions of TNF-α, IL-4, IL-6, and IL-13; however, these increases were reduced by ZQT treatment. In addition, we further confirmed the suppressive effect of ZQT on inflammatory factors in OVA mice.

ASMC exerts vital functions in the occurrence of chronic respiratory diseases such as asthma, and its excessive proliferation and migration are the main pathological changes in asthma [15]. The stimulation of inflammatory cytokines, growth factors, and extracellular matrix proteins can induce ASMC proliferation and migration [16]. Evidence shows that under PDGF-BB stimulation, the contractile phenotype of ASMC can be transformed into a synthetic phenotype, resulting in an elevation in the production of cytokines, growth factors, and matrix proteins, thereby promoting the proliferation of the airway smooth muscle layer [17, 18]. Our research indicated that ZQT treatment sensibly counteracted the promoting effect of PDGF-BB stimulation on ASMC proliferation and migration. We confirmed that the expressions of MMP-3/9 increased by PDGF-BB stimulation were notably reduced after ZQT treatment. Therefore, we believed that ZQT suppressed ASMC proliferation and migration in asthma.

NF-κB is a key transcription factor that exerts vital functions in inflammatory pathways [19]. The activated NF-κB translocates into the nucleus and modulates the transcription of pro-inflammatory factors, thereby controlling cell proliferation, differentiation and inflammation [20]. PI3K/Akt signal transduction is the main upstream element of NF-κB signaling [21]. Accumulating researches have confirmed that PI3K/Akt pathway takes part in the occurrence and regulation of asthma, and is associated with allergic airway inflammation and remodeling [22, 23]. For example, FGF10 alleviates allergic airway inflammation in asthma via suppressing PI3K/AKT/NF-κB pathway [12]. A recent study has proven that Baiheqingjin formula inhibits inflammatory reactions in asthma through inactivating PI3K/AKT/NF-κb pathway [24]. Imperatorin relieves airway remodeling by targeting PI3K/AKT/NF-κB pathway [25]. Here, we proved the phosphorylation expressions of p65, AKT, and PI3K were sensibly elevated in OVA mice, while treatment with ZQT sensibly suppressed their levels. This indicated that ZQT can inactivate the PI3K/AKT/NF pathway in asthma.

5. Conclusion

Taken together, this study proved that ZQT reduced airway inflammation and remodeling in mice with asthma via inactivating PI3K/AKT/NF-κB pathway. These findings provide evidence for the clinical efficacy of ZQT in treating asthma.

Conflict of interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Experimental Animal Ethics Committee of First Clinical Medical College of Heilongjiang University of Chinese Medicine.

Informed consent

Not applicable.

Availability of data and material

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

Authors' contributions

LH contributed to the study conception and design. Experimental operation, data collection and analysis were performed by LY and GY. The first draft of the manuscript was written by LH and all authors commented on previous versions of the manuscript.

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Not applicable.

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