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清肺口服液减轻 RSV 感染加重哮喘小鼠气道炎症作用的研究(英文)

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【摘　要】 清肺口服液是临床治疗儿童呼吸道合胞病毒(RSV)性肺炎的有效经验方。前期研究发现,清肺口服液可以减轻 RSV 感染加重哮喘小鼠的气道炎症,本研究拟探讨清肺口服液对 RSV 感染的哮喘小鼠肺组织自噬及凋亡蛋白表达的影响。将 50 只雄性 BALB/c 小鼠随机分为对照组、哮喘组、RSV 感染+哮喘组、清肺口服液组、利巴韦林组。采用卵清蛋白(OVA)致敏及雾化激发哮喘模型;采用 RSV 隔日滴鼻、连续 3 次诱导感染,构建 RSV 感染哮喘模型。各药物组给予相应干预。末次激发 24 h 后,采集血清,分离肺组织。HE 染色后观察气道炎症反应;ELISA 法检测血清炎症因子白介素-6(IL-6)、白介素-8(IL-8)水平;Western blot 检测肺组织 Atg5、Bax、Bcl-2 蛋白表达情况。结果显示,清肺口服液可以明显减轻 RSV 感染哮喘小鼠肺组织炎症细胞浸润及充血水肿,显著降低血清炎症因子 IL-6、IL-8 的水平($P<0.01$),显著下调 Atg5、Bax 蛋白表达水平($P<0.01$),并上调 Bcl-2 蛋白表达水平($P<0.01$)。以上结果提示清肺口服液能够减轻 RSV 感染哮喘小鼠的气道炎症,其机制可能与抑制自噬和调节凋亡相关。

【关键词】 清肺口服液;呼吸道合胞病毒(RSV);哮喘;气道炎症;自噬;凋亡;小鼠

Qingfei Oral Liquid alleviates airway inflammation in RSV-infected asthmatic mice

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ABSTRACT Qingfei Oral Liquid is an effective empirical prescription in the treatment of children with respiratory syncytial virus (RSV) pneumonia. Previous studies have found that Qingfei Oral Liquid can alleviate airway inflammation in RSV-infected asthmatic mice. This study aims to investigate the effects of Qingfei Oral Liquid on autophagy and apoptosis protein expressions in lung tissue of asthmatic mice with RSV infection. Fifty male BALB/c mice were randomly divided into the control group, asthma group, RSV infection plus asthma group, Qingfei Oral Liquid group and Ribavirin group. The asthma model was induced by ovalbumin (OVA) sensitization and atomization, and the RSV-infected asthmatic model was established by nasal instillation of RSV every other day, for three times. Drug treatment groups were treated with the corresponding intervention. Twenty-four hours after the last stimulation, serum was collected and lung tissue was isolated. The airway inflammation was observed after HE staining, the serum levels of inflammatory factors interleukin (IL)-6 and IL-8 were detected by ELISA, and the protein expressions of Atg5, Bax and Bcl-2 in lung tissue were detected by Western blot. The results showed that Qingfei Oral Liquid could obviously alleviate the inflammatory cell infiltration, hyperemia and edema in lung tissue of asthmatic mice infected with RSV, significantly reduce the serum levels of inflammatory cytokines IL-6 and IL-8 ($P<0.01$), significantly down-regulate the protein expression levels of Atg5 and Bax

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($P<0.01$), and up-regulate the expression level of Bcl-2 protein ($P<0.01$). These findings suggest that Qingfei Oral Liquid can reduce airway inflammation in RSV-infected asthmatic mice, and its mechanism may be related to inhibiting autophagy and regulating apoptosis.
KEYWORDS Qingfei Oral Liquid; RSV; asthma; airway inflammation; autophagy; apoptosis; mouse

Introduction

Asthma is a heterogeneous and chronic airway inflammation disease. It is defined by the respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that change with time and in intensity, together with variable expiratory airway limitation[1]. According to the large sample survey conducted by domestic scholars, the prevalence of children's asthma in most cities in China has increased significantly[2]. Respiratory syncytial virus (RSV) is a negative single chain stranded RNA virus of *Paramyxoviridae*, which is the most common respiratory pathogen[3]. RSV infection can promote airway obstruction and repeated wheezing, causing airway damage, which is a huge disease burden for infants and young children worldwide[4]. RSV infection is one of the main pathogens of respiratory tract infections in infants and young children[5]. Epidemiological data showed that infants and young children were at risk of asthma for several years or decades after repeated RSV infections[6]. RSV-associated asthma in some cases could lead to aggravation, higher risk, intensive care and higher mortality[7]. After RSV infection, inflammatory factors are secreted by respiratory epithelial cells, airway inflammation is mediated by inflammatory mediators, and airway hyperresponsiveness is an important factor affecting disease outcome[8]. At present, there is no safe and effective treatment for RSV infection.

Recent studies have found that autophagy and apoptosis are closely related to RSV infection and asthma aggravating airway inflammation[9-10]. Research showed that an increase of Bax/Bcl-2 ratio was observed at post-sepsis, suggesting the apoptosis in lung tissue[11]. Study also revealed that autophagy-related gene 5 (Atg5) promoter region was associated with childhood asthma[12]. Interleukin-6 (IL-6) is an immune factor secreted by Th2 cells, which plays an important role in immune response and inflammatory reaction. The serum level of IL-6 in patients with acute attack of asthma was significantly increased[13]. IL-8, also known as neutrophils factor, is an important chemokine involved in recruitment and activation, and can delay neutrophil apoptosis and activate the status of inflammation. The level of IL-8 was significantly increased[14-15], such as patients with asthma and chronic obstructive pulmonary disease (COPD).

Traditional Chinese medicine shows unique advantages in fighting RSV infection[16-18]. Our previous study found that Qingfei Oral Liquid ("QF" for abbreviation) could inhibit the replication of RSV and alleviate the airway inflammation and mucus hypersecretion damage caused by RSV-infected asthma. QF is made based on the clinical experience of Professor Wang Shouchuan, a famous Chinese medical physician. It is an experimental prescription developed from the classic ancient prescriptions Maxin Shigan Decoction (consisted of Ephedrae Herba, Armeniacae Semen Amarum, Gypsum Fibrosum and Glycyrrhizae Radix et Rhizoma, with effects of clearing lung heat and relieving asthma) and Tili Dazao Xiefei Decoction (consisted of Descurainiae Semen Lepidii Semen and Jujubae Fructus, with efficacy of dispelling phlegm and relieving asthma), which has been used clinically for nearly 20 years. Moreover, two phases of randomized, large-sample, multi-center clinical studies have been carried out successively, and the results showed that its treatment on childhood viral pneumonia (syndrome of phlegm-heat blocking lung) was effective[19].

Due to the therapeutic effects of QF in treating asthma in clinic and experimental research, in this study the aggravated asthma model induced by ovalbumin (OVA) plus RSV was duplicated in mice to explore the effect and mechanism of QF in treating asthma exacerbation. The effects of QF were observed

through airway inflammation, pulmonary histopathology, as well as serum levels of cytokines in mice. Also, we further elaborated Atg5, Bax and Bcl-2 protein expressions in different groups, so as to explore the possible mechanism of QF on alleviating airway inflammation in RSV-infected asthma.

Materials and Methods

Drug and Virus

QF is a compound preparation of traditional Chinese medicine, with license from Jiangsu Province Food and Drug Administration (No. Z04000512), which is composed of Ephedrae Herba (麻黄, honey-fried, 3 g), Gypsum Fibrosum (石膏, 20 g), Armeniacae Semen Amarum (苦杏仁, 10 g), Scutellariae Radix (黄芩, 6 g), Descurainiae Semen Lepidii Semen (葶苈子, 10 g), Mori Cortex (桑白皮, 10 g), Polygoni Cuspidati Rhizoma et Radix (虎杖, 12 g), Peucedani Radix (前胡, 10 g), Bistortae Rhizoma (拳参, 12 g) and Salviae Miltiorrhizae Radix et Rhizoma (丹参, 10 g), and purchased from Jiangsu Province Hospital of Traditional Chinese Medicine. All reagents or solvents used in this study were commercially available and of reagent grade.

According to our previous study using Hep-2 cell inoculated RSV A2 strain (ATCC, USA), the RSV were harvested after 3-5 days later and the cytopathic effect appeared. Then the titer of RSV was determined by 50% tissue culture infective dose ($TCID_{50}$). This virus titer value was 1×10^6.

Animals, Grouping and Treatment

Fifty male BALB/c mice (6-8 weeks old) weighing (20 ± 2) g were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (Shanghai, China). Mice were placed in a controlled environment of (23 ± 1) ℃ and (50 ± 5)% relative humidity with free access to food and water, under a light/dark cycle of 12/12 hours. This study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (No. 2016001).

All the mice were randomly divided into five groups: the control, OVA, OVA + RSV, QF and Ribavirin groups ($n=10$). The OVA (Sigma Aldrich, USA) sensitization and challenge procedures were performed as indicated previously with slight modifications[14]. Briefly, the control group was intraperitoneally injected with PBS containing aluminum hydroxide gel, but without intranasal challenge. The model mice were sensitized by intraperitoneal injection of OVA (OVA 20 μg plus aluminum 2 mg) on day 0, day 14 and day 21, and aerosol nebulization with 1% OVA in PBS from day 22 to day 30 (using a 402 AI ultrasonic atomizer, Shanghai Yuyue Medical Equipment Co., Ltd.). Based on the treatment for the OVA group, the OVA + RSV group was additionally treated with RSV (1.0×10^6 PFU/ml in 50 μl) by nasal administration in the morning of day 28, day 30 and day 32. Based on the treatment for the OVA + RSV group, QF group and Ribavirin group were treated with the corresponding drugs (QF 1.17 $g\cdot kg^{-1}\cdot d^{-1}$, Ribavirin 0.1 $g\cdot kg^{-1}\cdot d^{-1}$) in the afternoon of day 25, day 27, day 29 and day 31, respectively. In the end challenge, all mice were anaesthetized with 2.5% pentobarbital sodium to collect blood and lung tissue for further research.

Histopathological Evaluation

Lung specimens of mice were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin wax, then sliced into 4 μm sections (RM2235, Leica, Germany) and stained with hematoxylin and eosin (HE), and observed under the light microscope (BX42, Leica, Germany) at high magnification (×200) to evaluate the morphological changes.

ELISA

ELISA (USCN Business Co., Ltd., China) according to the protocol instructions of manufacturer can be used to determine the levels of inflammation factors. In the study, the serum levels of IL-6 and IL-8 were measured by ELISA.

Western Blot

The protein from mice lung tissue were harvested in RIPA lysis buffer (containing 10% PMSF, Beyotime, China) on ice. The concentration of the

lysates was determined by BCA protein assay kit (P0009, Beyotime, China). The lysates were mixed with sample loading buffer (P0015L, Beyotime, China) to heat 10 minutes by metal bath at 100 ℃, then subjected to 6%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, at 80 V for 30 minutes). The protein was transferred onto a polyvinylidene difluoride (PVDF, Bio-Rad, USA) membrane via 120 V for one hour. The membrane was incubated with 5% BSA blocking buffer at room temperature for 2 hours, and then incubated in buffer containing rabbit polyclonal antibodies against Bax, Bcl-2 and Atg5 (1∶1 000) overnight. The next day, the membrane was washed with TBST and incubated with 5% BSA added horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1∶5 000) at room temperature for 2 hours. After washing, the immunoreactive membranes were visualized by enhanced chemiluminescence (ECL) system (Thermo Scientific, USA), with β-actin as the control reference to analysis the expressions of proteins in different groups.

Statistical Analysis

Statistical analysis was carried out using SPSS 18.0 (IBM software, USA). The obtained data were presented as the mean ± standard deviation (SD). Datasets with three or more groups were analyzed by one-way ANOVA, and the least significant difference (LSD) post hoc test was used for multiple comparisons. $P<0.05$ was considered to be statistically significant.

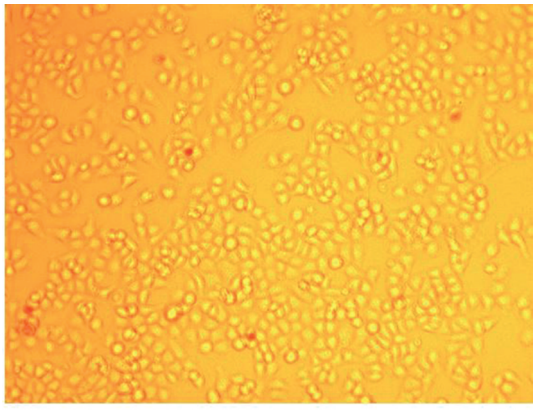
Results

RSV infected Hep-2 cells

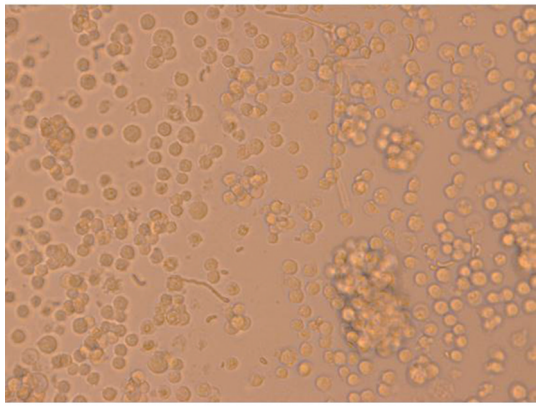
As shown in Figure 1, Hep-2 cells adhered to the culture plate wall to form a single layer of cells, which was the shape of paving stones. After RSV infected the cells, the cells in the lesions fused with each other to form a syncytium with multinucleated giant cell.

Effects of QF on lung histomorphology

After HE staining, the pulmonary tissue showed typical interstitial pneumonia in the OVA group, the alveolus cavity was obviously narrowed, and the inflammatory cell infiltration was significant. Further, we found the significant inflammatory cells infiltration into peribronchiolar and perivascular connective tissues in OVA+RSV group, and the capillary was dilated and congested. QF treatment remarkably attenuated the airway inflammation in the lung tissue, especially significantly reduced the inflammatory cell infiltration around the airway and the interstitial tissue, compared with other groups (Figure 2).



Control



RSV

Figure 1. RSV infected Hep-2 cells(×200).

Effects of QF on serum IL-6 and IL-8 levels

To determine whether QF could reduce the inflammatory reaction, the levels of IL-6 and IL-8 were measured by ELISA in the serum of mice. As shown in Figure 3, we found that the serum levels of IL-6 and IL-8 in the OVA group and OVA + RSV group were higher than those in the control group ($P<0.01$). Compared with the OVA + RSV group, QF could significantly decrease the serum levels of IL-6 and IL-8 ($P<0.01$), and their levels were also decreased in the Ribavirin group ($P<0.01$).

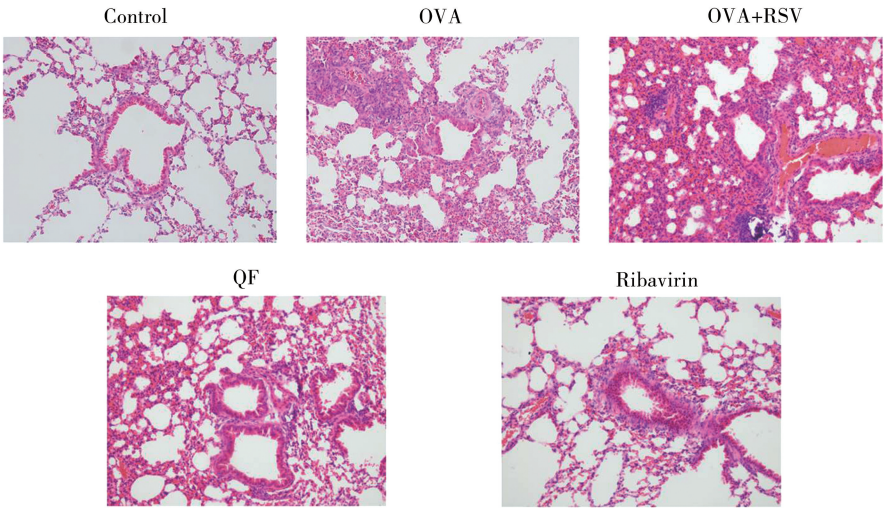


Figure 2. Histomorphological analysis on lung tissue of mice (HE staining, ×200).

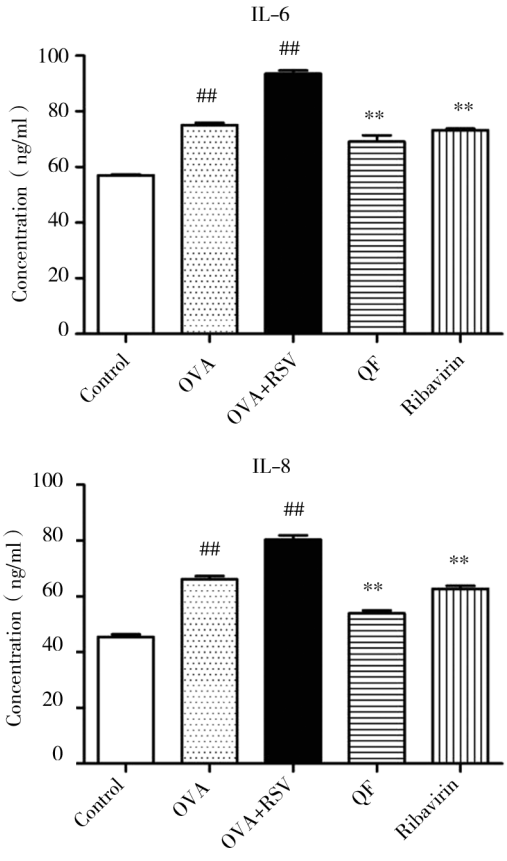


Figure 3. Serum IL-6 and IL-8 levels in different groups by ELISA. Data are presented as mean ± SD, $n = 6$. ##$P<0.01$, compared with Control group; **$P<0.01$, compared with OVA+RSV group.

Effects of QF on Bax, Bcl-2, Atg5 proteins expressions

Atg5 plays an important role in the airway inflammation of asthma. We investigated the effects of QF on Bax, Bcl-2 and Atg5 protein expressions in mice lung tissue. As shown in Figure 4, RSV-infected asthmatic mice showed Atg5 and Bax proteins expressions were significantly increased and Bcl-2 protein expression was decreased, compared with those in the control group ($P<0.01$). Both QF and Ribavirin could down-regulate Atg5 and Bax proteins expressions and up-regulate Bcl-2 protein expression in RSV-infected asthmatic mice ($P<0.01$), but the reversal effects of QF were more significant than those of Ribavirin ($P<0.01$).

Discussion

The incidence, prevalence and disability-adjusted life years of asthma in Chinese children continue to increase[20-21]. A survey by the National Pediatric Asthma Collaborative Group suggested that the prevalence of childhood asthma in our country showed a significant rise[22], which was a serious threat to children's health. According to epidemiological studies in testing cities in China, the prevalence of asthma among children aged 0-14 was 0.91% in 1990[23], 1.50% in 2000[23] and 3.02% in 2010[22], respectively. Studies have shown that RSV is closely related to asthma and can cause acute asthma attacks and severe wheezing[24].

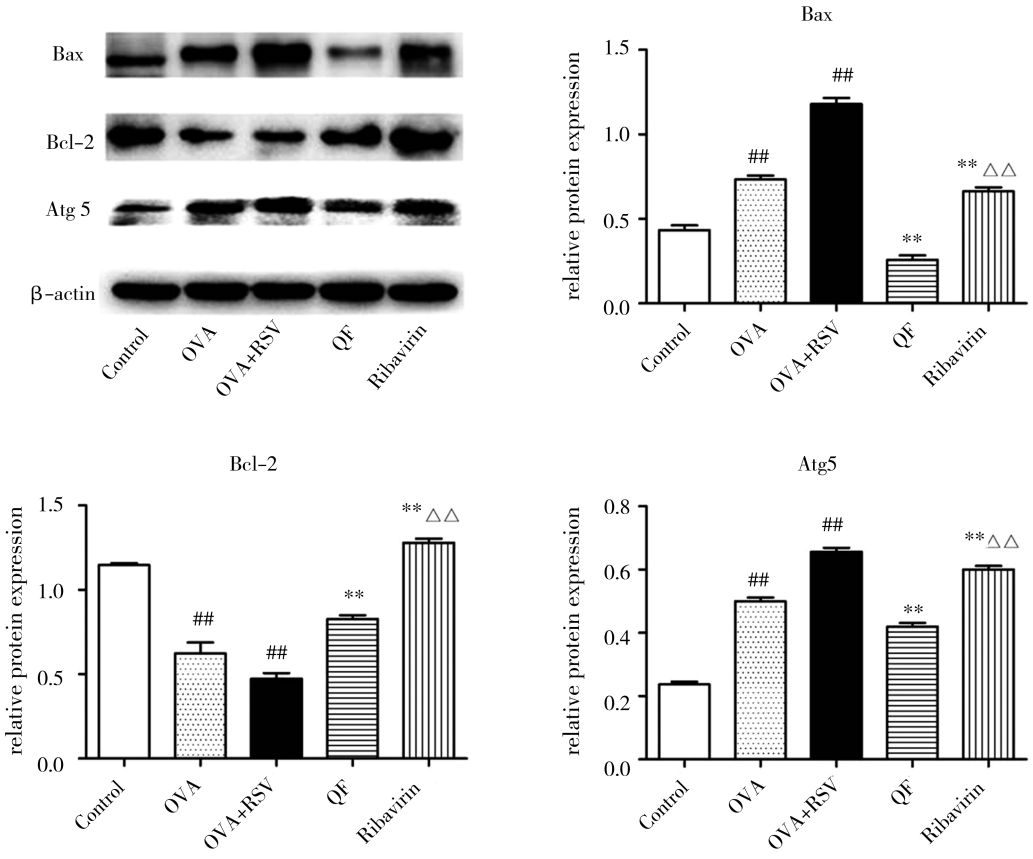


Figure 4. Expressions of Bax, Bcl-2 and Atg5 proteins in lung tissue by Western blot. Data are presented as mean±SD, $n=3$. ##$P<0.01$, compared with Control group; **$P<0.01$, compared with OVA + RSV group; △△$P<0.01$, compared with QF group.

In this study, we established the OVA plus RSV challenged severe asthma model, which showed more severe airway inflammation and infiltration compared with the OVA-challenged mice. Airway inflammation of asthma has long been recognized as a pathologic feature[25-27]. Our research revealed that QF significantly reduced the inflammatory cell infiltration around the airway and the interstitial tissue compared with other groups in histological analysis.

Severe asthma induced by RSV causes many cytokines secretion, such as IL-6 and IL-8, which plays an important role in inflammatory reaction of asthma. QF could decrease IL-6 and IL-8 levels in serum of RSV-infected asthmatic mice. QF could alleviate airway inflammation by inhibiting Atg5 protein expression, as well as significantly up-regulating Bcl-2 expression and down-regulating Bax expression in model mice. These results suggested that autophagy and apoptosis might greatly affect RSV-infected asthma.

In summary, our research demonstrated a role of autophagy and apoptosis in severe allergic asthma and provided a few evidence for the association between autophagy and apoptosis in RSV-infected asthma. Therapeutic strategies targeting autophagy and apoptosis may provide new approach to treat RSV-infected severe asthma. Further study would focus on the mechanism of RSV-infected asthma *in vitro* and *in vivo* and the relationship between autophagy and apoptosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest on the study or preparation of the manuscript.

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Reference

[1] GLOBAL INITIATIVE FOR ASTHMA. Global Strategy for Asthma Management and Prevention [EB/OL]. (2020-05-17). http://www. ginasthma.org.

[2] GLOBAL INITIATIVE FOR ASTHMA. Global Strategy for Asthma Management and Prevention [EB/OL]. (2019-06-27). http://www. ginasthma.org.

[3] NYIRO J U, MUNYWOKI P, KAMAU E, et al. Surveillance of respiratory viruses in the outpatient setting in rural coastal Kenya: baseline epidemiological observations [J]. Wellcome Open Res, 2018, 3: 89.

[4] REZAEE F, LINFIELD D T, HARFORD T J, et al. Ongoing developments in RSV prophylaxis: a clinician's analysis[J]. Curr Opin Virol, 2017, 4: 70-78.

[5] OMARI K E, DHALIWAL B, REN J, et al. Structures of respiratory syncytial virus nucleocapsid protein from two crystal forms: details of potential packing interactions in the native helical form[J]. Acta Crystallogr Sect F Struct Biol Cryst Commun, 2011, 67(Pt 10): 1179-1183.

[6] WU P, HARTERT T V. Evidence for a causal relationship between respiratory syncytial virus infection and asthma[J]. Expert Rev Anti Infect Ther, 2011, 9(9): 731-745.

[7] RITCHIE A I, FARNE H A, SINGANYAGAM A, et al. Pathogenesis of Viral Infection in Exacerbations of Airway Disease [J]. Ann Am Thorac Soc, 2015, 12 (Suppl 2):S115-S132.

[8] FOOLAD F, AITKEN S L, SHIGLE T L, et al. Oral Versus Aerosolized Ribavirin for the Treatment of Respiratory Syncytial Virus Infections in Hematopoietic Cell Transplant Recipients [J]. Clin Infect Dis, 2019, 68(10): 1641-1649.

[9] SHIN Y, LIU J N , CHOI Y , et al. The Role of Autophagy in Allergic Inflammation: A New Target for Severe Asthma[J]. Journal of Allergy & Clinical Immunology, 2016, 137(2): AB70.

[10] LI M, LI J, ZENG R, et al. Respiratory Syncytial Virus Replication Is Promoted by Autophagy-Mediated Inhibition of Apoptosis[J]. J Virol, 2018, 92(8): e02193.

[11] CHOPRA M, REUBEN J S, SHARM A C. Acute lung injury: apoptosis and signaling mechanisms[J], Exp Biol Med, 2009, 234(4): 361-371.

[12] POON A H, EIDELAN D, LAPRISE C, et al. ATG5, autophagy and lung function in asthma [J]. Autophagy, 2012, 8 (4): 694-695.

[13] NAIK S P , MAHESH P A , JAYARAJ B S , et al. Evaluation of inflammatory markers interleukin-6 (IL-6) and matrix metalloproteinase-9 (MMP-9) in asthma[J]. J Asthma, 2017, 54 (6): 584-593.

[14] NAKAGOME K, NAGATA M. Involvement and Possible Role of Eosinophils in Asthma Exacerbation [J]. Front Immunol, 2018, 9: 2220.

[15] ZHANG J, BAI C. The Significance of Serum Interleukin-8 in Acute Exacerbations of Chronic Obstructive Pulmonary Disease[J]. Tanaffos, 2018, 17(1): 13-21.

[16] ZANG N, XIE X H, DENG Y, et al. Effects of resveratrol on pulmonary virus titer and airway inflammation in mice infected with respiratory syncytial virus [J]. J Third Mil Med Univ, 2011, 33 (18): 1924-1927.

[17] ZHANG Y Y, LUO S N, YANG J, et al. Impact of Jiang Xinxia Formulae on Expression of HIF-1α and VEGF in Airway Remodeling of Asthmatic Rats [J]. Chinese Journal of Experimental Traditional Medical Formulae, 2014, 20 (11): 131-134.

[18] LIANG X X, DAI Q G, WANG S C, et al. Regulation of Jinxin Oral Liquid on Expression of IFN-α in BALB/c Mice Infected by Respiratory Syncytial Virus [J]. Chinese Journal of Experimental Traditional Medical Formulae, 2013, 19 (12): 200-203.

[19] WANG S C, SUN Y Q, BIAN G B, et al. Clinical study on Qingfei oral liquid treating 507 cases of phlegm-heat obstructing lung syndrome in children virus pneumonia [J]. World Chin Med, 2016, 11(9): 1649-1653.

[20] MATTIUZZI C, LIPPI G. Worldwide asthma epidemiology: insights from the Global Health Data Exchange database [J]. Int Forum Allergy Rhinol, 2020, 10(1):75-80.

[21] HONG J G, BAO Y X, CHEN A H, et al. Chinese guidelines for childhood asthma 2016: Major updates, recommendations and key regional data [J]. J Asthma, 2018, 55(10): 1138-1146.

[22] THE NATIONAL COOPERATIVE GROUP ON CHILDHOOD ASTHMA, CHINESE CENTER FOR DISEASE CONTROL AND PREVENTION. Third nationwide survey of childhood asthma in urban areas of China [J]. Chin J Pediatr, 2013, 51 (10): 729-735.

[23] NATIONAL COOPERATION GROUP ON CHILDHOOD ASTHMA. Comparative analysis of the state of asthma prevalence in children from two nation-wide surveys in 1990 and 2000 year [J]. Chinese Journal of Tuberculosis and Respiratory Diseases, 2004, 27 (2): 112-116.

[24] MOHAPATRA S S, BOYANALLE S. Epidemiologic, experimental, and clinical links between respiratory syncytial virus infection and asthma[J]. Clin Microbiol Rev, 2008, 21(3): 495-504.

[25] KAY A B. The role of eosinophils in the pathogenesis of asthma [J]. Trends Mol Med, 2005, 11(4): 148-152.

[26] POON A H, EIEDLAN D H, MARTIN J G, et al. Pathogenesis of severe asthma[J]. Clin Exp Allergy, 2012, 42(5): 625-637.

[27] WANG J, WU J F, KONG L W, et al. BuShen YiQi Formula strengthens Th1 response and suppresses Th2-Th17 responses in RSV-induced asthma exacerbated mice [J]. J Ethnopharmacol, 2014, 154(1): 131-147.

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