

Original Article

Effect of Yiqi Wenyang Huwei decoction on ovalbumin-induced bronchial asthma in rats

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Abstract: Yiqi Wenyang Huwei decoction (YWHD) has been a commonly used traditional medicine for bronchial asthma. In this study, the therapeutic effects of YWHD in rats with ovalbumin-induced bronchial asthma were investigated. Sprague-Dawley rats were randomly divided into normal control, model, low dose YWHD, medium dose YWHD, and high dose YWHD groups (n=12 in each group). The asthma model was established and validated by hematoxylin-eosin staining. Tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) expression was determined by ELISA assay. The mRNA and protein expression of human beta-defensin (HBD)-1, HBD-2 and HBD-3 was determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and Western blot. The model group showed a large number of inflammatory cells infiltration in bronchial submucosa and around the bronchial wall, wall thickening, narrowing of lumen, epithelial hyperplasia, smooth muscle hypertrophy, and thickening and irregular basement membrane. Compared with the model group, inflammatory cell infiltration and wall thickening in the low dose YWHD group showed no significant changes, inflammatory cell infiltration around the bronchioles decreased in the medium dose YWHD group, while wall thickening were significantly improved in the high dose YWHD group. At week 1 and 4, the TNF- α and IL-1 β expression in the model group was significantly increased compared with normal control ($P<0.05$). At week 1 and 4, TNF- α expression in all YWHD groups was significantly decreased compared with the model group ($P<0.05$). At week 1, IL-1 β expression in the medium and high dose YWHD groups was significantly decreased ($P<0.05$), while at week 4, the expression in all YWHD groups was significantly decreased compared with the model group ($P<0.05$). At week 1 and 4, expression of human beta-defensin (HBD)-1, HBD-2 and HBD-3 in the model group was significantly increased compared with normal control ($P<0.05$) while expression in all YWHD groups was significantly decreased compared with the model group ($P<0.05$). The effect of YWHD on asthma prevention was thus achieved through regulation of TNF- α , IL-1 β and defensin expression.

Keywords: Yiqi Wenyang Huwei decoction, bronchial asthma, human beta-defensin

Introduction

Bronchial asthma (BA) is a chronic inflammatory disease of the airway in which many cells and cellular elements play a role, including eosinophils, mast cells, T-lymphocytes, neutrophils and epithelial cells [1]. Its incidence and mortality remain high and poses a great threat to human health. Despite continuous advancement in modern medicine and in prevention and treatment of asthma, however, no breakthrough progress has been made. Current therapy with inhaled corticosteroids and long-acting β_2 -agonists is highly effective, safe, and relatively inexpensive, but for many patients, their disease remains poorly controlled. Many

new treatments are specific, targeting a single mediator or receptor, are unlikely to have a major clinical effect. Drugs with more widespread effects, such as kinase inhibitors, might be more effective but have a greater risk of side effects. New treatments targeting the underlying allergic/immune process would treat concomitant allergic diseases and improved immunotherapy approaches might have the prospect of disease modification [2].

Traditional Chinese Medicine (TCM) has a good therapeutic effect for the prevention and treatment of BA with no obvious side effects. There has been increasing scientific evidence supporting the use of TCM in asthma treatment

[3-5]. One advantage of Chinese herbal medicine therapeutics is multiple components in the herbal formula often play a synergistic role which is greater than individual drug. TCM also has the advantage in treatment during remission period, which has become an important part in asthma prevention and treatment [6].

Yiqi Wenyang Huwei decoction (YWH) which comprised of Yupingfeng (YPF) powder, *Ramulus Cinnamomi* decoction, *Rhizoma Curculiginis* and *Herba Epimedii*, has been a commonly used traditional medicine formula for BA in China [7]. It can improve immune regulation ability and enhance the adaptability of respiratory tract to stimulating factors in the environment [7].

YPF powder is comprised of *Astragalus membranaceus* (Fisch.) Bunge, *Atractylodes macrocephala* (Koidz) and *Saposhnikovia divaricata* (Turcz.) Schischk. Clinically, it is mainly adopted in the treatment and prevention of immunodeficiency associated inflammation such as cold, respiratory infections, allergic rhinitis, chronic bronchitis and BA [8, 9], and has been used traditionally for allergic diseases and reduce relapses. Its polysaccharides have been proven to possess antioxidant activity [10] while glucosidic extract has anti-inflammatory and immunoregulation action [8]. Previous studies showed that YPF powder have anti-inflammatory and immune regulatory effects on immunosuppressive mice [11], rat models of chronic bronchitis [12] and colonic inflammation [13].

Ramulus Cinnamomi (Gui Zhi) extract has been reported to exert anti-inflammatory and neuroprotective effects on rats [14]. Cinnamaldehyde, its major active ingredient, is known for its sedative, antioxidant, and anti-neuroinflammation activity [15-19]. Several other active components have been reported to have neuroprotective effects [20, 21].

Rhizoma Curculiginis (Xianmao) has shown to possess anti-inflammation effect on cell *in vitro* and collagen induced arthritis *in vivo* [22] and reduce the contents of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β) and interleukin (IL)-6 in hippocampus and cortex [23]. Its isolated compounds have a wide spectrum of pharmacological activities, including neuro-protective, anti-oxidant, anti-inflammatory, antibacterial,

anti-osteoporotic and estrogenic effects [24, 25]. In China, it is often used together with *Epimedii Folium* as herb pairs in formula to obtain a synergistic effect for reinforcing [23].

Herba Epimedii has been used in various traditional Chinese formulations for thousands of years. Icariin, the most abundant constituent, is pharmacologically bioactive and has osteoprotective, neuroprotective, cardiovascular protective, anti-cancer, anti-inflammation, and immunoprotective effects, and reproductive function [26]. It could enhance the production of antibodies and cytokines in mice, with more marked effects when the mice were immunized with ovalbumin (OVA), suggesting that it is effective on Th cell functions and has protective effects against immune diseases [27].

Previous studies have shown that YWH can regulate T helper (Th)1/Th2 cell subsets by inhibiting mRNA expression of trans-acting T-cell-specific transcription factor (GATA3) [28], IL-4 and signal transducer and activator of transcription 6 (STAT6) [29], and increasing mRNA expression of IL-12 and STAT4 in asthmatic rats [30]. It can alleviate the infiltration and activation of eosinophil (EOS) in airway of guinea pigs with asthma [31] and regulate Th1/Th2 cell subsets by decreasing the level of IL-5, EOS and eosinophil cationic protein (ECP), and increasing the level of interferon- γ (IFN- γ) in patient's sputum [32]. It increases IFN- γ mRNA in asthmatic rats [33] and can effectively alleviate the clinical symptoms and signs in patients with chronic obstructive pulmonary disease [34].

Defensins are endogenous antimicrobial peptides which are widely distributed in organisms and have a unique stable structure. Defensins possess antimicrobial and immune activity. They not only can resist the invasive pathogenic microorganisms through direct bactericidal action, but also play an important role in mediating acquired immune responses, regulating inflammatory responses, and wound healing [35].

In this study, the effect of YWH on rats with ovalbumin-induced BA was evaluated. The hypothesis that YWH could be a potential treatment for BA by regulating the expression of TNF- α , IL-1 β and human beta-defensins was tested in this study.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1996).

Experimental animal

Sixty 4-week-old healthy male and female Sprague-Dawley (SD) rats, weighed 90 ± 4.6 g, purchased from Hunan SJA Laboratory Animal Co., Ltd., Changsha, Hunan, P.R. China (License Number (SCXK (Xiang) 2016-0002) were used in this study. All rats were kept in a specific-pathogen-free (SPF) animal room, maintained at a constant temperature and humidity under a 12-hour light/dark cycle, and supplied with aseptic nutritional pellet feed and sterile water *ad libitum*.

Main reagents and instruments

Ovalbumin (Sigma-Aldrich Corp., St. Louis, MO, USA); Aluminum hydroxide (Damao Chemical Reagent Factory, Tianjin, P.R. China); TNF- α ELISA detection kit, IL-1 β ELISA detection kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, P.R. China); TRLzon reagent, Ultrapure RNA extraction kit, HiFiScript first strand cDNA synthesis kit, UltraSYBR Mixture (Beijing CW Biotech Co., Ltd., Beijing, P.R. China); rabbit polyclonal human beta-defensin (HBD)-1 antibody (1:500), rabbit polyclonal HBD-2 antibody (1:1000), rabbit polyclonal HBD-3 antibody (1:500) (Shanghai Sixin Biotechnology Co., Ltd., Shanghai, P.R. China); radioimmunoprecipitation assay buffer (RIPA) lysis buffer (Applygen Technologies Inc., Beijing, P.R. China); ultra-sensitive enhanced chemiluminescent (ECL), hematoxylin and eosin (HE) stain (Thermo Fisher Scientific Inc., Waltham, MA, USA); polyvinylidene fluoride (PVDF) membrane (MilliporeSigma Corp., St. Louis, MO, USA); vertical protein electrophoresis apparatus (Beijing Liuyi Instrument Factory, Beijing, P.R. China); Chemi Doc™ XRS + Ultra high sensitivity chemiluminescence imaging system, CFX Connect™ Real-Time polymerase chain reaction (PCR) detection system (Bio-Rad Laboratories, Shanghai, P.R. China); microplate reader

(Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, P.R. China).

Preparation of YWHD

The quality of each crude drug was tested in accordance with the pharmacopoeia of China. The formula is a mixture of ten medicinal herbs: *Radix Astragali*, *Rhizoma Atractylodis Macrocephalae*, *Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Radix Paeoniae Alba*, *Zingiber Officinale* Roscoe, *Fructus Ziziphi Jujubae*, *Radix Glycyrrhizae Preparata*, *Rhizoma Curculiginis*, *Epimedii Folium* were mixed in a ratio of 2:1.5:1.5:1:1:1:1:0.5:1:1.5. Volatile oil was extracted from *Rhizoma Atractylodis Macrocephalae*, *Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Radix Paeoniae Alba* and *Zingiber officinale* Roscoe. The herbal remedies were then immersed in 8 times its amount of cold water for half an hour and fried for 1 hour after boiling. Then, this was added with 6 times the amount of water and fried for 40 minutes. The decoction was filtered and concentrated. The extract was mixed and adjusted to 2 g/ml of crude drug. Each concentrated solution was sealed in a sterile volumetric flask.

Establishment of asthma rat model

The rats were sensitized with 1 ml intraperitoneal injection of freshly prepared ovalbumin aluminum hydroxide solution (containing 10 mg ovalbumin (OVA) and 200 mg aluminum hydroxide) on day 1. Injections were repeated once on day 8. From day 15 onwards, 2% OVA inhalation was given to induce asthma, once per day, 30 minutes each time, and continued for 1 and 4 weeks.

Experimental grouping

The rats were randomly divided into: (1) normal control group: ovalbumin for sensitize and induce asthma was replaced with physiological saline; (2) model group: similar as airway remodeling in rat asthma model; (3) low dose YWHD group; (4) medium dose YWHD group; (5) high dose YWHD group. Group (3), (4) and (5) were sensitized and induced as the model group. From day 16 onwards (after sensitization), 0.36 g/kg, 0.72 g/kg and 1.44 g/kg of YWHD respectively was given by intra-gastric administration 1 hour before OVA inhalation each day.

Table 1. Primer sequences

Primer	Sequence (5'-3')	Product size (bp)
HBD-1 F	CTGGGTGCTGGCATTCT	141
HBD-1 R	ACAGTTGGGCTTATCTGGTTT	
HBD-2 F	TCCTTTTCTCCTATGCCACTC	104
HBD-2 R	AGCATTGAGACAGAACCCTTT	
HBD-3 F	TGCTGTCGCCCTTTCT	122
HBD-3 R	AGTGCCACAAGTCCAATC	
GAPDH F	TACCCACGGCAAGTTCAA	141
GAPDH R	ACCAGCATCACCCCATTT	

Each group consisted of 12 rats, with equal number of male and female. The YWHD dosage were converted in reference to the adult dosage: the medium dose was calculated in accordance to the adult dose, the high dose was doubled the medium dose while the low dose was half the medium dose.

H&E staining

At the end of week 1 and week 4, 6 rats from each group were sacrificed by cervical dislocation. The lungs of the rats were removed by thoracotomy. The lung tissues were fixed with 4% formalin (preservative) and kept at room temperature (RT). The formalin-fixed lung tissue was washed in running water for several hours and subjected to gradient dehydration with 70%, 80%, 90% ethanol solution followed by clearing with alcohol/xylol 1:1, xylol I and xylol II solution for 15 minutes each. Then this was placed in xylene/paraffin 1:1 solution for 15 minutes and in paraffin I and II, each for 50-60 minutes. The paraffin embedded tissue was sliced, and the sections were heated, dewaxed and hydrated. The sections were then placed in distilled water and hematoxylin aqueous solution, and stained for 3 minutes. This was followed by ethanol hydrochloride differentiation for 15 seconds, rinsed mildly, added with Bluing solution for 15 seconds, washed with running water, eosin stained for 3 minutes, washed with running water, dehydrated, cleared, mounted with neutral resin, and examined under microscope.

Detection of TNF- α and IL-1 β expressions with enzyme-linked immunosorbent assay (ELISA)

All reagents and components were brought to RT. Replicate wells were prepared for stan-

dards, controls and samples. The working solution of various components of the kit were prepared according to the kit instructions. The required microplates were removed from aluminum foil bags and the remaining were kept in self-sealing bags and put back in the fridge. For standards, 50 μ L with different concentrations of standards were added to the wells. For blank control, samples and enzyme-labeled reagents were not added, while other steps remained the same. For samples, 40 μ L of diluent was added to the enzyme-labeled coated microplate, followed by 10 μ L samples (the final dilution was 1:5). The samples were added to the bottom of the well without touching the side wall, gently shaken, and mixed. 100 μ L of enzyme-labeled reagent was added to each well, except blank control. This was sealed with microplate sealing film and incubated at 37°C for 60 min. Then, 20-fold concentrated washing solution was diluted 20 times with distilled water and reserved for use. The sealing film was carefully removed. The liquid was discarded and dried. Each well was filled with washing solution, let stand for 30 seconds, discarded, repeated 5 times and patted dry. Then, 50 μ L chromogenic reagent A was added to each well followed by 50 μ L chromogenic reagent B, gently shaken and mixed, and developed at 37°C for 15 min. 50 μ L stop solution was added to terminate the reaction (blue color turned yellow). With the blank control wells adjusted to zero, the absorbance (optical density, OD) of each well was measured sequentially at 450 nm wavelength.

Detection of HBD-1, HBD-2 and HBD-3 mRNA expression with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA was extracted from the tissues of each group. cDNA was synthesized according to the reverse transcription kit. Using cDNA as template, detection was performed with qRT-PCR. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference, the relative mRNA expression of HBD-1, HBD-2 and HBD-3 in the tissues of each group was determined. Primers use are as shown in **Table 1**.

The reaction system included: RNase Free dH₂O 9.5 μ L, cDNA/DNA 1 μ L, upstream primer 1 μ L, downstream primer 1 μ L, 2 \times ultraSYBR mixture 12.5 μ L. The reaction procedure was performed

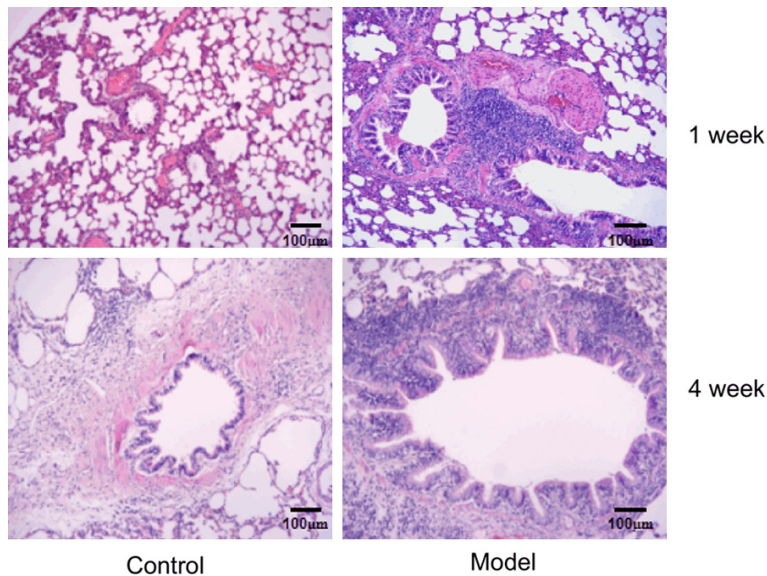


Figure 1. Determination of the successful establishment of asthma rat model with H&E staining.

according to the three-step protocol. The system underwent pre-denaturation at 95°C for 10 minutes, followed by denaturation at 95°C for 10 seconds, annealing at 57.5°C for 30 seconds and extension at 72°C for 30 seconds. The number of cycles was 40.

The relative mRNA expression of the target gene indicated multiple of the expression of the control group and was calculated by the formula:

$$\Delta\Delta Ct = \Delta Ct \text{ test sample} - \Delta Ct \text{ control}$$

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

With $P < 0.05$, $2^{-\Delta\Delta Ct} < 1$ indicated downregulation of expression in the experimental group, while $2^{-\Delta\Delta Ct} > 1$ indicated upregulation of expression. Otherwise, the expression was not significantly different.

Detection of HBD-1, HBD-2 and HBD-3 protein expression with Western blot

The tissue was added with corresponding lysis buffer, lysed at 4°C for 30 minutes at 1000 rpm/min. The supernatant was carefully extracted for total protein. Protein concentration was determined with bicinchoninic acid (BCA) kit. This was followed by protein denaturation, loading, and electrophoresis for 1-2 h followed by wet transfer for 30-50 minutes. Then this was incubated with primary antibodies at 4°C overnight and secondary antibodies at RT for 1-2 hours. ECL exposure solution was added to

the membrane and exposed. The gray value of each antibody strip was analyzed using "Quantity One" software.

Statistical analysis

Data were analyzed with SPSS 19.0 (SPSS Inc, Chicago, Illinois, USA) and all data were expressed as mean \pm standard deviation. One-way analysis of variance (ANOVA) was used for comparison between groups. $P < 0.05$ was considered as statistically significant.

Results

Determination of the successful establishment of asthma rat model with H&E staining

Compared with the normal control group, the lung tissue of the asthma model group has a large number of inflammatory cell infiltration in the bronchial submucosa and around the wall, and the wall thickening was obvious, indicating that the model was established successfully (**Figure 1**).

Improvement of histopathological changes in the medium and high dose YWHD groups

The model group showed a large number of inflammatory cells infiltrating in the bronchial submucosa and around the bronchial wall, thickening of the wall, narrowing of lumen, epithelial hyperplasia, smooth muscle hypertrophy, and thickening and irregular basement membrane. Compared with the model group, inflammatory cell infiltration and wall thickness in the low dose YWHD group showed no significant changes, inflammatory cell infiltration around the bronchioles was decreased in the medium dose YWHD group, while wall thickening in the high dose YWHD group were significantly improved. No pathological changes as above were observed in the normal control group (**Figure 2**).

TNF- α and IL-1 β expression in each group

Compared with the normal control group, the expression of TNF- α and IL-1 β in the model group increased significantly at week 1 and 4 ($P < 0.05$). Compared with the model group,

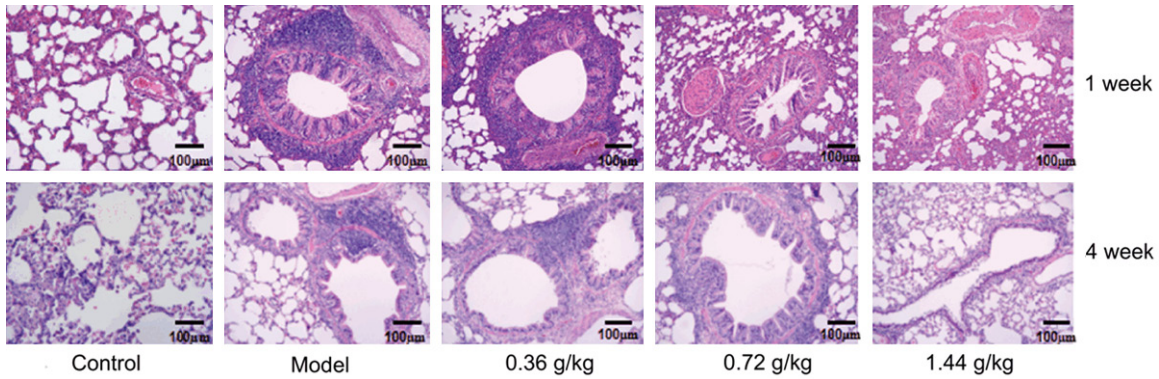


Figure 2. Results of H&E staining in each group.

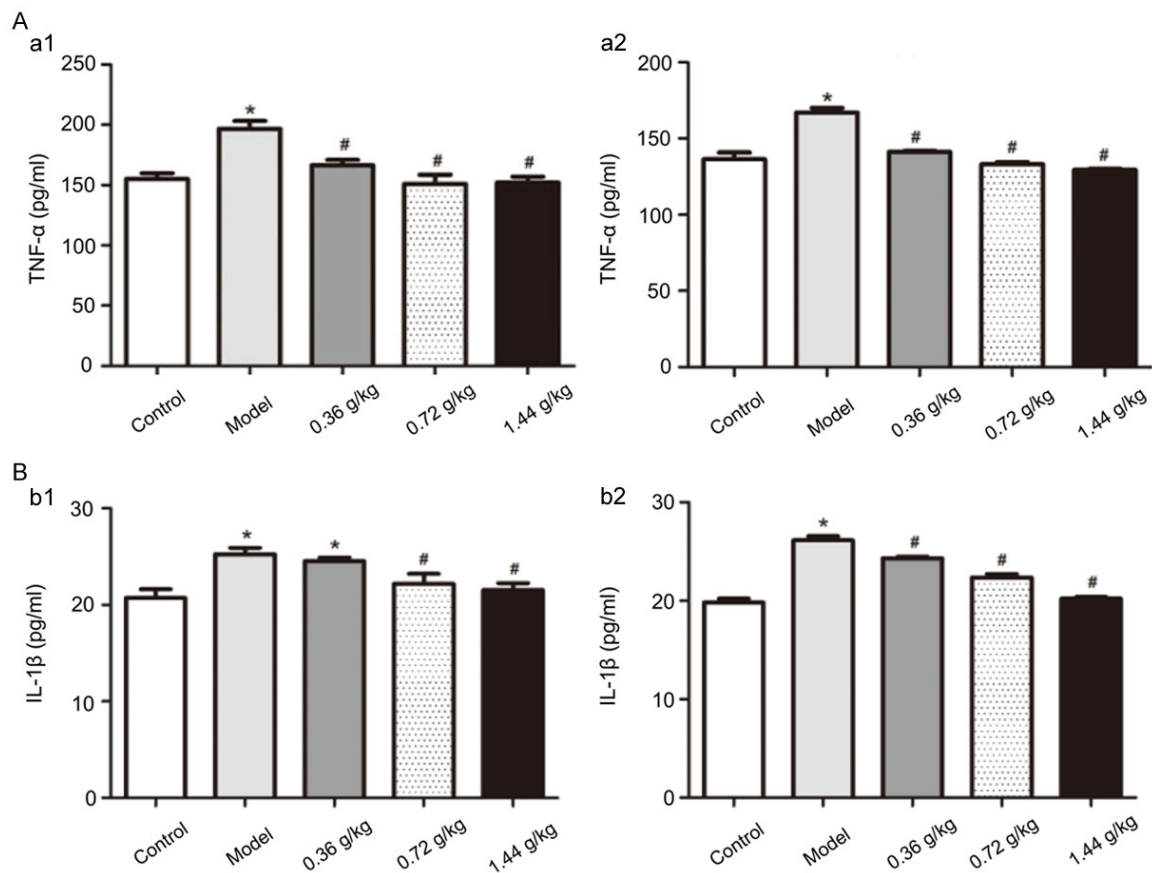


Figure 3. TNF- α and IL-1 β expression in each group. (A) TNF- α (a1) week 1 (a2) week 4. (B) IL-1 β (b1) week 1 (b2) week 4. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the model group.

expression of TNF- α in the low dose YWHD group, medium dose YWHD group and high dose YWHD group were significantly decreased at week 1 and 4 ($P < 0.05$). Compared with the model group, expression of IL-1 β in the medium dose YWHD group and high dose YWHD group were significantly decreased at week 1 ($P < 0.05$), and expression was significantly decreased in all YWHD treatment groups at week 4 ($P < 0.05$) (Figure 3).

HBD-1, HBD-2 and HBD-3 relative mRNA expression in each group

Compared with the normal control group, HBD-1, HBD-2 and HBD-3 mRNA expression in the model group was significantly increased at week 1 and 4 ($P < 0.05$). Compared with the model group the HBD-1, HBD-2 and HBD-3 mRNA expression in the low dose YWHD group, medium dose YWHD group and high dose

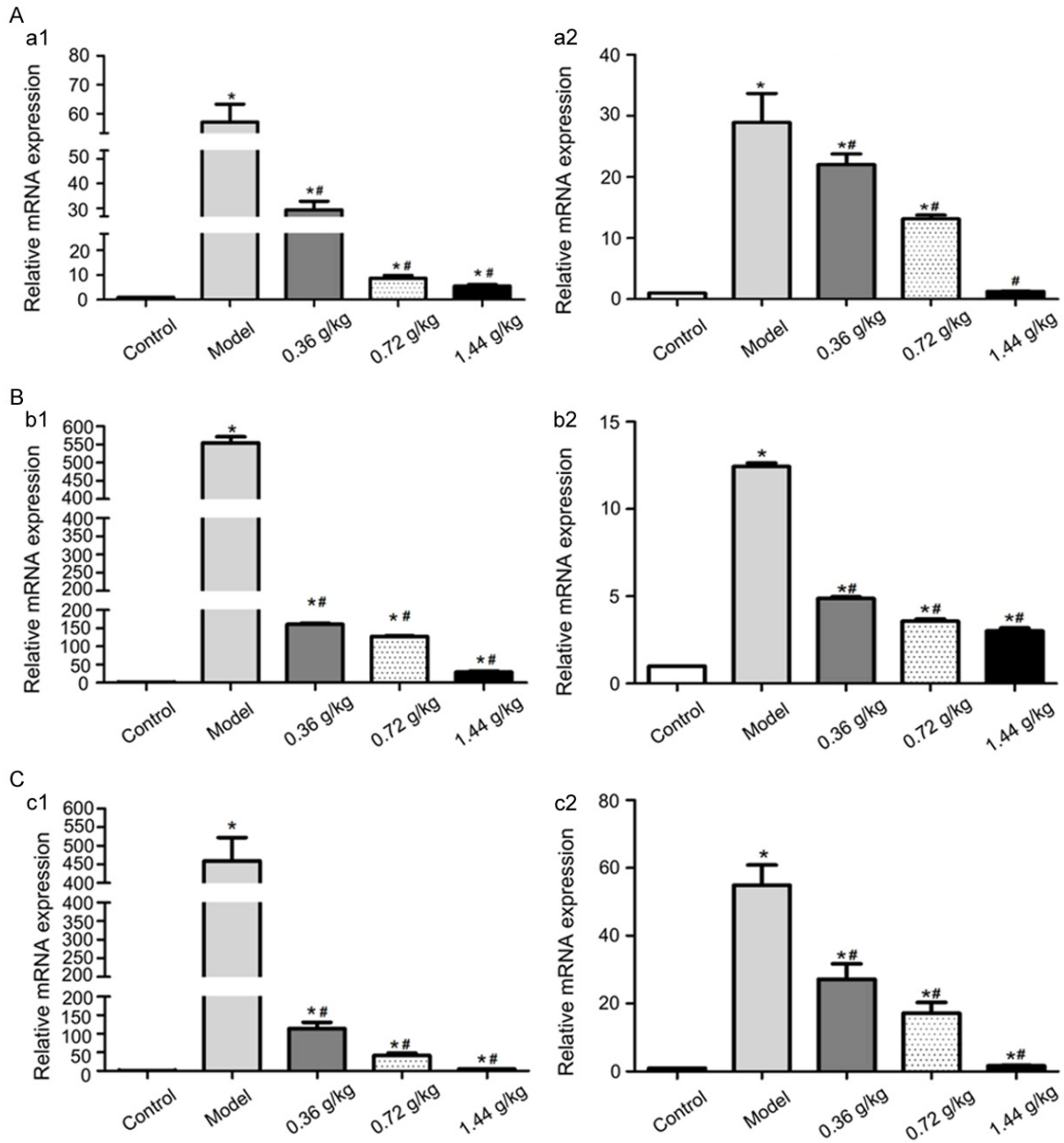


Figure 4. HBD-1, HBD-2 and HBD-3 mRNA expression in each group. (A) HBD-1 (a1) week 1 (a2) week 4. (B) HBD-2 (b1) week 1 (b2) week 4. (C) HBD-3 (c1) week 1 (c2) week 4. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the model group.

YWHD group was significantly decreased at week 1 and 4 ($P < 0.05$) (Figure 4).

HBD-1, HBD-2 and HBD-3 protein expression in each group

Compared with the normal control group, HBD-1, HBD-2 and HBD-3 protein expression in the model group was significantly increased at week 1 and 4 ($P < 0.05$). Compared with the model group, HBD-1, HBD-2 and HBD-3 protein

expression in the low dose YWHD group, medium dose YWHD group, and high dose YWHD group was significantly decreased at week 1 and 4 ($P < 0.05$) (Figure 5).

Discussion

BA is a disease commonly encountered in clinical practice. Through YWHD herbal formula, the respiratory defense function and immune-regulatory ability can be enhanced, the airway

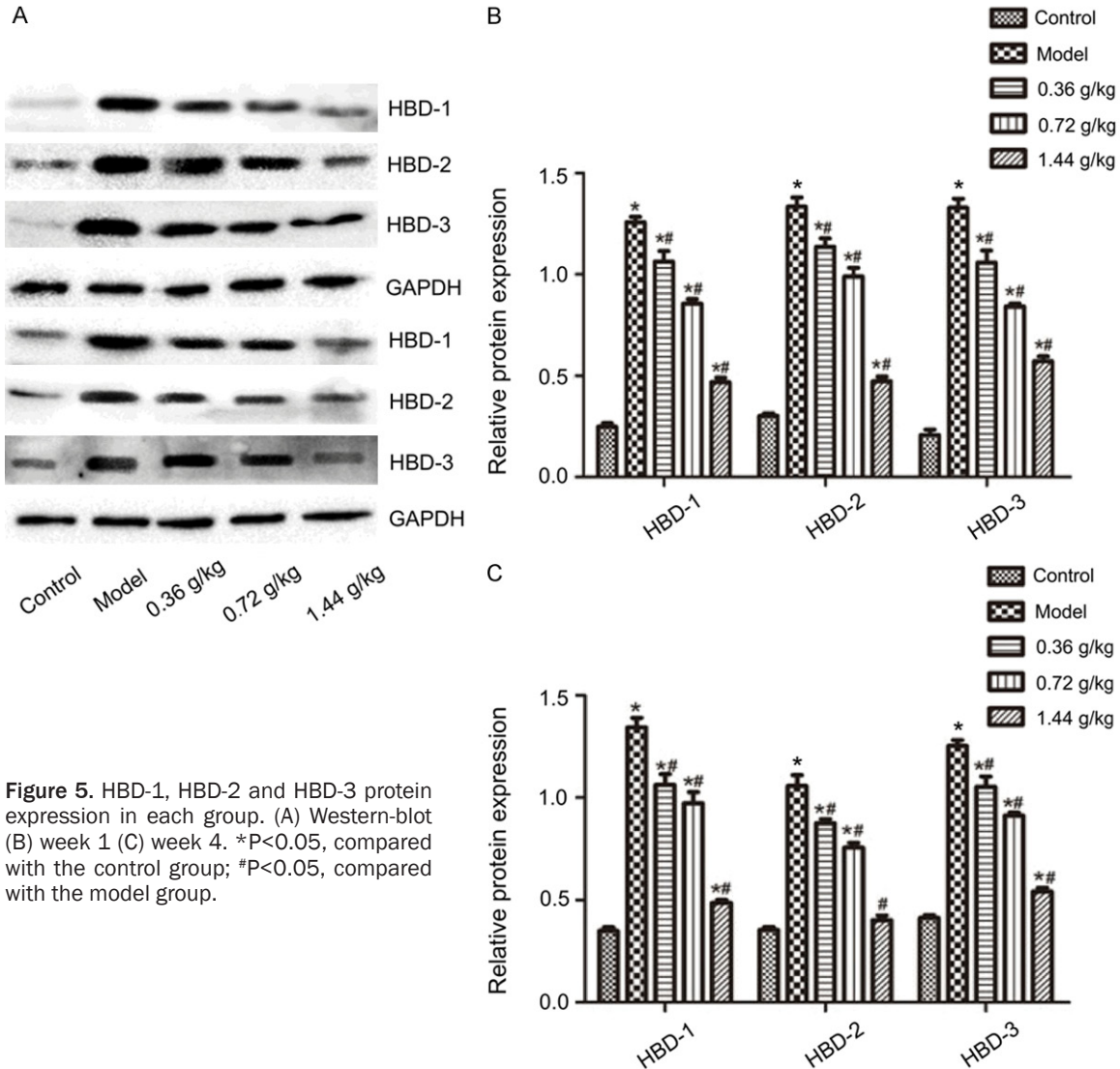


Figure 5. HBD-1, HBD-2 and HBD-3 protein expression in each group. (A) Western-blot (B) week 1 (C) week 4. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the model group.

inflammation and responsiveness can be reduced and asthmatic attacks can be reduced or controlled. The goal of preventing recurrent asthma attacks is achievable [7].

To date, therapeutic studies have shown that Chinese medicine and modern medicine have reached a consensus on certain commonly used methods of treatment. With the development of modern medicine, the pharmacology and mechanism of action of many Chinese herbs have been determined. YPF powder, which is known as TCM immune-modulator is among the ancient herbal formulas that form consensus with modern medicine [36].

In this study, the effect of YWHD on an asthma rat model was studied. HE staining showed successful establishment of ovalbumin-induced

asthma rat model. The HE pathological results showed that the infiltration of inflammatory cells around the bronchus was decreased in the medium dose YWHD group while the thickening of wall was obviously improved in the high dose YWHD group. The results show that YWHD has a therapeutic effect on BA. Pro-inflammatory cytokines IL-1 β and TNF- α are involved in initiating or acceleration allergic inflammation, and both are equally important in the cytokine interaction network. Previous study showed that blockade of IL-1 β and TNF- α can alleviate pathological allergic inflammatory reactions and reduce inflammatory cell infiltration in the nasal mucosa and lung tissues in OVA-induced allergic rhinitis guinea pigs [37]. In addition, studies have shown that IL-1 β increased TNF- α mRNA expression while anti-

TNF- α antibody markedly suppressed IL-1 β -induced up-regulation of bradykinin B1 and B2 in mouse tracheal smooth muscle [38]. The present results show that the expression of IL-1 β and TNF- α increased significantly after establishment of asthma model, and decreased after treatment with YWHD, indicating that the effect of YWHD on asthma treatment might be achieved via down-regulating the IL-1 β and TNF- α expression.

Defensins possess antimicrobial activity and can play an important role as the first natural barrier against microbial invasion. HBDs play a role in pathogenesis of asthma. Understanding the mechanism can help in the development of novel therapeutic strategies for this disease [39]. HBD-1 and HBD-2 can destroy *Escherichia coli* and other Gram-negative bacteria at a certain concentration, but the bactericidal effect on Gram-positive bacteria is relatively weak. HBD-3 has strong inhibitory and killing effects on Gram-positive and Gram-negative bacteria [40]. Defensins also possess antiviral activity [41]. They can prevent entry of viruses at different stages of invasion, as well as blocking the replication of viruses in cells. Previous studies found that human epithelial cells HBD-2 and HBD-3 can inhibit HIV-1 replication via direct interaction with virions and through modulation of the CXCR4 co-receptor [42, 43]. BA is a reversible, regulatable, multifactorial and polygenic inheritance chronic inflammatory disorder of the airways characterized by airflow obstruction, bronchial hyper-reactivity and underlying inflammation [44-46]. Since defensins may induce histamine release from mast cells and increase airway hyper-responsiveness to histamine, it is speculated that they may be involved in the inflammatory process of asthma [34, 47]. A previous study showed that the antimicrobial activity of airway epithelial cells exposed to Th2 cytokines was decreased, and at the same time HBD-2 was inhibited at the mRNA level [48]. The results of this study show that expression of defensins HBD-1, HBD-2 and HBD-3 increased upon the establishment of asthma model, and decreased after treatment with YWHD, which was consistent with the trend of expression of inflammatory cytokines.

In conclusion, the results from the present study show that the effect of YWHD on asthma prevention and treatment can be achieved by regulating the expression of TNF- α , IL-1 β and

defensins. The findings further improve the level of understanding of the theory of asthma prevention and treatment in TCM and are worth further exploration.

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Disclosure of conflict of interest

None.

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