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Astragalus membranaceus improves therapeutic efficacy of asthmatic children by regulating the balance of Treg/Th17 cells

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[ABSTRACT] *Astragalus membranaceus* may be a potential therapy for childhood asthma but its driving mechanism remains elusive. The main components of *A. membranaceus* were identified by HPLC. The children with asthma remission were divided into two combination group (control group, the combination of budesonide and terbutaline) and *A. membranaceus* group (treatment group, the combination of budesonide, terbutaline and *A. membranaceus*). The therapeutic results were compared between two groups after 3-month therapy. Porcine peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by using density gradient centrifugation on percoll. The levels of FoxP3, EGF-*β*, IL-17 and IL-23 from PBMCs and serum IgE were measured. The relative percentage of Treg/Th17 cells was determined using flow cytometry. The main components of *A. membranaceus* were calycosin-7-*O*-glucoside, isoquercitrin, ononin, calycosin, quercetin, genistein, kaempferol, isorhamnetin and formononetin, all of which may contribute to asthma therapy. Lung function was significantly improved in the treatment group when compared with a control group ($P < 0.05$). The efficacy in preventing the occurrence of childhood asthma was higher in the treatment group than the control group ($P < 0.05$). The levels of IgE, IL-17 and IL-23 were reduced significantly in the treatment group when compared with the control group, while the levels of FoxP3 and TGF-β were increased in the treatment group when compared with the control group ($P < 0.05$). *A. membranaceus* increased the percentage of Treg cells and reduced the percentage of Th17 cells. *A. membranaceus* is potential natural product for improving the therapeutic efficacy of combination therapy of budesonide and terbutaline for the children with asthma remission by modulating the balance of Treg/Th17 cells.

[KEY WORDS] *Astragalus membranaceus*; Asthma; Children; Treg/Th17; Budesonide; Terbutaline

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Introduction

The prevalence, incidence and mortality of asthma have risen sharply in recent years, especially in childhood asthma [1]. Childhood asthma is a respiratory system disease with inflammation and airway hyperresponsiveness [2-3]. The disease is mainly characterized as eosinophilic infiltration into human airway, subcutaneous fibrosis and smooth muscle proliferation and airway remodeling caused by collagen deposition [4]. Childhood asthma can be affected not only by environment, climate and allergic factors, but also a genetic predisposition and the heritability up to from 70% to 90% [5]. At present, childhood asthma are still not cured, and long-term standard strategy is needed to control the disease. Inhalation of glucocorticoid and bronchodilators commonly is often used in the treatment of childhood asthma. Inhaled budesonide suspension is the only corticosteroid (ICS) approved by the Food and Drug Administration (FDA) for the therapy of children asthma[6]. It is a non-halogenated glucocorticoid that has no adverse effects on glucocorticoid. The chemical structure of ICS differs from systemic corticosteroids (SCS) in that its steroid structure incorporates lipophilic groups at C16a, or C17a of the D ring, with increased lipophilicity, affinity with tissue cells and GR, and its anti-inflammatory activity increases. Budesonide suspension exerts therapeutic effects through both classical and non-classical pathways. The classical pathway acts via the action of glucocorticoid receptors in the cytoplasm, reduces airway inflammation and inhibits the release of inflammatory mediators; Non-classical pathway inhibits calcium channels, and allows airway vasoconstriction to reduce cell secretion

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and rapidly improve symptoms [7].

Terbutaline is a short-acting β 2 receptor agonist that acts on the small airways to relax bronchial smooth muscle, decrease vascular permeability and improve lung function, but long-term use decreases the number of receptors. The main side effects include heart palpitations, tachycardia and chest pain [8-9]. Daily application of P-receptor agonists can cause severe acute asthma and sudden death [10]. Salpeter found that LABA reduced the severity of asthma and asthma-related hospitalization. P receptor agonists in the treatment of childhood asthma status is still controversial [11]. Budesonide suspension combined with terbutaline has poor control of asthma at night, and the symptoms are easily repeated.

In the remission of asthma, the treatment is still difficult due to the mild symptoms and long incubation time. Patients with remission asthma are difficult to be cured because the clinical symptoms of asthma are often not obvious or even disappear, and the diagnosis and treatment of asthma can be ignored. Studies have shown that patients with asthma at remission stage are still at risk of recurrence, and no clear risk factors have been found to prompt asthma recurrence [12-13]. Type I allergy has been recognized as the pathogenesis of allergic asthma when allergens enter into the body with the differentiation of T cells and B cells into plasma cells, resulting in the production of IgE antibodies. Allergens again enter into the body and antigen-antibody binding, resuling in the degranulation of mast cells and causing asthma [14].

Recent studies suggest that the imbalance between Th1 and Th2 in the two subtypes of helper T lymphocytes (Th) is a mechanism of asthma [15-16]. Traditional Thl/Th2 imbalance theory cannot fully explain the mechanism for causing asthma. Recent data suggest that Th cells are plastic, and new Th cells can also trigger and expand the inflammatory response of asthma. Regulatory T cells (Treg cells) are a subset of T cells that function to control immune responses and have been widely reported as drivers of immunosuppression [17-19]. Th17 cells are a distinct lineage of effector $CD4^+$T cells, and characterized by interleukin (IL)-17. The imbalance of Treg/Th17 cells will result in the riks of asthma and attracts many researches for exploring its moleculer mechanism [20-21]. The dysfunction of Treg is closely related to the development of asthma [22]. Th17 cells can secrete IL-17, which can recruit neutrophils and promote local tissue to produce neutrophil-related chemokines, thus aggravating the local inflammatory response [23].

More and more clinical studies have shown that combined treatment of asthma can improve the efficacy and reduce adverse reactions [24-26]. ICS combing with short acting beta agonists (SABA) inhalation is often clinically used to treat childhood asthma [27], but its therapy for nocturnal asthma is poor with repeated symptoms. Futhermore, long-term inhalation of SABA easily leads to cardiovascular side effects, hypokalemia, receptor sensitivity and hormone resistance [28]. The aerosol inhalation therapy of budesonide with terbutaline has been proved to be effective and safe to asthmatic children [29]. However, considering the side effects of budesonide [30] and terbutaline [31], it is necessary to find novel medicine with few side effects to reduce the their dosage. *Astragalus membranaceus* has been used in Traditional Chinese Medicine (TCM) for thousands of years. Astragalus belongs to the leguminous plant and its main chemical composition includes astragaloside, astragalus polysaccharide, astragalus flavonoids, selenium and other trace elements [32]. Modern research shows that it has some regulation on immunity [33]. It also has antibacterial [34], anti-virus [35], anti-oxidation [36], anti-aging [37], and lung protection [38] properties. Thus, *A. membranaceus* may be used in the prevention of childhood asthma.

The type and dose of asthma medicine is often used according to age. However, the same age group differences in the weight of astham children because of their rapid growth and development. Thus, weight administration will be more reasonable. Therefore, the dosage was administrated according to children weight. We explored the effects of *A. membranaceus* on childhood asthma remission combining with budesonide plus terbutaline. To explore the related molecular mechanism, the changes of Th17 and Treg cells in peripheral blood mononuclear cells (PBMCs), and related molecules were measured.

Materials and Methods

Reagents, materials and instruments

Acetonitrile, methanol, formic acid and the standards (calycosin-7-*O*-glucoside, isoquercitrin, stilbene, calycosin, quercetin, genistein, kaempferol, isorhamnetin, and monghonolicin) were purchased from Sigma (Louis, MO, USA). *A. membranaceus* pills were produced by Sichuan Qi Li Pharmaceutical Co., Ltd. (production No. National Medicine Zhunzi Z20090313, approved by State Food and Drug Administration at http://app2.sfda.gov.cn/datasearchp/gzcxSearch.do?formRender=cx&page=1) and purchased from the Chinese Medicinal Herbs Company (Beijing, China). The quality of *A. membranaceus* was detected by Beijing Tongke Yuanda Pharmaceutical Technology Co., Ltd. according to the number of Huangqi batch number "Huangyi Decoction Piece Quality Standard" STP-C/019-00, and inspection Record No. was R-SOP-ZLC/019-01. Budesonide and terbutaline were purchased from AstraZeneca (Shanghai, China). FoxP3 (Biorbyt, Cambridge, UK), TGF-β (R&D Systems, Boston, MA, USA), IL-17 (R&D Systems) and IL-23 (R&D Systems) antibodies were purchased from corresponding companies. Fluorescein isothiocyanate (FITC)-CD4, phycoerythrin (PE)-CD25 and IL-17 antibodies, and corresponding controls were purchased from R&D system (Boston, MA, USA). FOXP3 PerCP-Cy5.5 antibody was from eBioscience (clone PCH101, San Diego, CA, USA). Percoll was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). FC500 flow cytometer was purchased from Beckman Coulter (Brea, CA, USA). A 0.22-μm microporous membrane was purchased from Millipore

(Temecula, CA, USA). 1100 HPLC system and SB-C_{18} column (250 mm × 4.6 mm, 5 μm) were purchased from Agilent Technologies (Palo Alto, CA, USA). An automatic blood biochemical analyzer was purchased from Medsoul (Beijing, China). A Cobas E601 automatic biochemical analyzer and Viaan IgE ELISA kit were purchased from Diagnostics Ltd Roche Diagnostics (Shanghai, China). Software SPSS 20.0 was purchased from SPSS, Inc. (Chicago, IL, USA).

HPLC analysis of the components of A. membranaceus

The following chromatographic conditions were applied for the analysis of the components of *A. membranaceus*: column, Agilent SB-C_{18}; mobile phase: acetonitrile (A)–0.3% formic acid solution (B), gradient elution (0 to 15 min, 5% ding control and 30 min, 20% acetonitrile (A)–0.3% formic acid solution (B); detection wavelength: 254 nm; column temperature: 35 °C and injection volume: 10 μL. For the sample solution, 5-g samples were ground and passed through No. 4 sieve, and placed in a stoppered Erlenmeyer flask. Fifty-mL of methanol was added and stored overnight, at 50 °C. Ultrasound treatment was performed for 1 h, filtered, concentrated, dried and dissolved in 5-mL methanol, filtered through a 0.22 μm microporous membrane, and filtrated solution was collected. The samples from 6 batches were analyzed. The concentrations of calycosin-7-*O*-glucoside, isoquercitrin, spinosa, calycosin, quercetin, genistein, kaempferol, isorhamnetin, and mongholicin were 2.274, 0.073, 1.578, 1.448, 0.210, 0.005, 0.069, 0.014, and 0.114 mg·g^{-1}, respectively. The relative standard deviations (RSDs) of nine kinds of the compounds were 0.13%, 1.75%, 0.06%, 0.07%, 0.61%, 1.87%, 0.96%, 0.64%, and 0.46% (n = 6), indicating that this method has good repeatability.

Patients

Before the present study, all procedures were approved by the Ethical Human Research Committee of Department of Pediatric, Affiliated Hospital of Changchun University of Traditional Chinese Medicine (Approval No. CCZYFYLL2015, Changchun, China). From December 2015 to August 2016, the children with asthma remission were determined at Affiliated Hospital of Changchun University of Traditional Chinese Medicine and recruited. All enrolled patients submitted signed informed consent.

Inclusion criteria

The following asthma children were included: the children with asthma remission were diagnosed by asthma experts according to Global Strategy for Asthma Management and Prevention [39]; all patients had dyspnea and wheezing in the past; the main symptoms were cough, and asthma attack was accompanied by the symptoms anxiety and irritability, and shortness of breath; bronchial breath sounds were confirmed by respiratory auscultation; The peak expiratory flows (PEF) values would be 60%–80% after taking P2 receptor agonist.

Exclusion criteria

The following asthma children were excluded: the asthma was not caused by respiratory disease; the children had chest injury and other chronic diseases including heart, liver, and kidney diseases; the children had severe bacterial infection; the children did not use glucocorticoid within one month. According to clinical manifestations, the children were with acute exacerbation or chronic persistent asthma.

Patients grouping

The selected children were randomly divided into two groups according to the random number table: the control group and the treatment group (n = 74 for each group). In the control group, budesonide, terbutaline and placebo were administrated via nebulized inhalation. In the treatment group, budesonide, terbutaline and *A. membranaceus* were administrated via nebulized inhalation. For the children with weight < 20 kg in the control group, the combination (budesonide suspension 1.0 mg + terbutaline solution 2.5 mg + 0.25 mg dried chickweed herb placebo) was used; for the children with weight > 20 kg: the combination (budesonide suspension 1.0 mg + terbutaline nebulizer 5.0 mg + 0.5 mg dried chickweed herb placebo) was used. For the children with weight < 20 kg in the treatment group, the combination (budesonide suspension 1.0 mg + terbutaline solution 2.0 mg + *A. membranaceus* 0.25 mg) was used; For the children with weight > 20 kg in the treatment group, the combination (budesonide suspension 1.0 mg + special Bupropion nebulizer 2.5 mg + *A. membranaceus* 0.5 mg) was used. The efficacy and safety of *A. membranaceus* were evaluated in a blinded fashion by four independent investigators. The drugs were administered every 6 h, and one week was regarded as a course of treatment proceeds.

Evaluation of therapeutic efficacy

The efficacy criteria were defined as significantly effective, effective and ineffective. Significantly effective: clinical symptoms disappeared after 3-day treatment, including cough, sputum, wheezing, dyspnea and wheeze. The quality of life and sleep was improved; effective: clinical symptoms were ameliorated after 3-day treatment, including asthma, cough, breathing and wheeze; invalid: there was no improvement in clinical symptoms after 3-day treatment. After the treatment of systemic glucocorticoid or globulin, clinical symptoms were repeated with wheeze, asthma and cough. The combination of significantly effective percentage and effective percentage were regarded as the total effective percentage.

Clincial observation

The diappearnce time of clinical symptoms and duration of hospital stay was recorded. The clinical manifestations (asthma, cough, wheeze) were observed after 3-month treatment. The clinical symptom were scored after 3-month treatment. The scoring criteria of clinical symptoms were used as follows: score 0, neither cough nor wheeze; score 1: minor cough and or mild wheeze; score 2: obvious cough and moderate wheeze, daily activity was not affected; score 3: a serious cough and loud wheeze, daily activity was significantly affected.

Lung function index

Before and after treatment, lung function index was

measured by using the following parameters: predicted value of forced vital capacity (FVC) in the first second (FEV1) %), Peak expiratory flow (PEF%), and FEV1/FVC%. Above pulmonary function tests need to be completed under the guidance of professional and technical personnel.

Laboratory indicators of asthma

Two-mL venous blood was obtained from each subject and eosinophile granulocyte% (EOS%) were measured by using an automatic blood analyzer (Hitachi, Japan). The concentration of C reactive protein (CRP) was measured by using an automatic blood biochemical analyzer. Two-mL venous blood was placed at room temperature for 1 h, serum was prepared via the centrifuge at 2000 *g* for 10 min. The serum level of immunoglobulin E (IgE) was measured by using a Cobas E601 automatic biochemical analyzer via an IgE ELISA kit.

Measurement of FoxP3, TGF-β, IL-17 and IL-23

PBMCs were prepared from 2-mL venous blood by using density gradient centrifugation on percoll. The cells were washed twice with PBS buffer and resuspended in 500 μL of PBS. Before the measurement of IL-17, PBMCs were stimulated by phorbol myristate acetate (PMA) (5 μg·mL^{-1}) and Ionomycin (5 μg·mL^{-1}) for 5 h and brefeldin-A (BFA) (10 μg·mL^{-1}) for 4 h [40]. PBMCs were fixed with 4% paraformaldehyde for half an hour, washed two times in PBS, permeabilised by 0.1% Triton X-100 plus 0.1% sodium citrate for 2 min at 4 °C, and washed two times in PBS with 1% BSA. The cells were then labeled with FoxP3, TGF-*β*, IL-17 and IL-23 antibodies, respectively.

Measurment of the percentage of CD25$^+$FOXP3 PerCP-Cy5.5 Treg and CD4$^+$IL-17$^+$Th17 cells in PBMCs

The PBMCs were divided into two sections and incubated with FITC-CD4, IL-17-PE-A and PE-CD25 antibodies for 30 min at room temperature in the dark. PBS The percentage of CD4$^+$IL-17$^+$Th17 and CD25$^+$FOXP3 PerCP-Cy5.5 Treg cells were measured by using FC500 flow cytometer.

Statistical analysis

Qualitative data were expressed as mean values ± standard deviation (SD). The test of independent samples was used to compare the differences between two groups, and χ^2 test was used to compare the significant difference for count data. Statistical analysis was performed by using software SPSS 20.0. $P < 0.05$ was considered as statistically significant.

Results and Discussion

The main components of A. membranaceus

HPLC analysis showed that the main components of *A. membranaceus* were 1. calycosin-7-*O*-glucoside; 2. isoquercitrin; 3. ononin; 4. calycosin; 5. quercetin; 6. genistein; 7. Kaempferol; 8. isorhamnetin; 9. formononetin (Fig. 1B) according to the standards (Fig. 1A). Furthermore, the components of *A. membranaceus* were stable from different batches (Fig. 1B).

Demographic characteristics

The statistical differences for the age, gender distribution, body mass index (BMI, weight (kg) /height (m^2)) and disease duration were insignificant between the two groups ($P > 0.05$, Table 1).

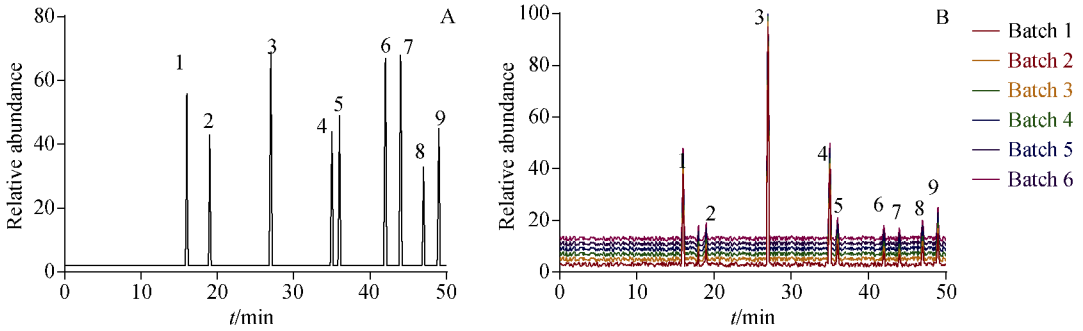

Fig. 1 HPLC analysis of the components of *Astragalus membranaceus*. A, mixed standards; B. test samples; 1. calycosin-7-*O*-glucoside; 2. isoquercitrin; 3. ononin; 4. calycosin; 5. quercetin; 6. genistein; 7. Kaempferol; 8. isorhamnetin; 9. formononetin

A. membranaceus treatment significantly improved therapeutic efficacy

After 7-day treatment, the significant effective rates were of 75.0% and 76.2% in the children from treament group with weight less than 20 kg and more than 20 kg, respectively, which were higher than 35.0% and 29.4% in the control group ($P < 0.05$). The total effective rate was 93.8% and 95.2% in the children from treatment group with weight less than 20 kg and more than 20 kg, respectively, which were higher than 50.0% and 58.8% in the control group ($P < 0.05$, Table 2). The findings suggest that *A. membranaceus* treatment significantly improves therapeutic efficacy of children asthma.

A. membranaceus treatment reduces the disappearance time of cough, hoarse breathing and wheeze, and hospital stay

The time for the diappearnce time of main clinical symptoms such as cough, hoarse breathing, wheeze and hospital stay were longer in the control group than those in the treatment group. The trends were same between the weight more than or less than 20 kg ($P < 0.05$, Table 3). The findings suggest that *A. membranaceus* treatment reduces the disappearance time of cough, hoarse breathing and wheeze, and hospital stay.

Table 1 Demograhic characteristics between control and treatment groups

	Male/Female	Age	BMI	Disease duration
		< 20 kg		
Control group	24/16	6.87 ± 2.21	22.47 ± 3.06	3.52 ± 2.46
Treatment group	18/14	7.15 ± 2.43	21.14 ± 2.91	3.36 ± 2.38
χ^2 or *t* values	0.103	0.231	0.742	0.127
P values	0.784	0.459	0.165	0.658
		> 20 kg		
Control group	20/14	6.98 ± 1.97	22.59 ± 2.75	2.94 ± 2.21
Treatment group	20/22	7.23 ± 2.82	23.08 ± 3.24	3.47 ± 3.17
χ^2 or *t* values	0.946	0.267	0.171	0.984
P values	0.331	0.391	0.562	0.072

Note: BMI, weight (kg) /height (m^2). The stastistical difference was significant if $P < 0.05$

Table 2 Comparision of therapeutic efficacy between two groups

	Significant effective, *n* (%)	Effective, *n* (%)	No effective, *n* (%)	Total effective, *n* (%)
		< 20 kg		
Control group	14 (35.0)	6 (15.0)	20 (50.0)	20 (50.0)
Treatment group	24 (75.0)	6 (18.8)	2 (6.3)	30 (93.8)
χ^2	16.676		16.036	
P values	0.000		0.000	
		> 20 kg		
Control group	10 (29.4)	10 (29.4)	14 (41.2)	20 (58.8)
Treatment group	32 (76.2)	8 (19.0)	2 (4.8)	40 (95.2)
χ^2	20.127		14.991	
P values	0.000		0.000	

Note: the stastistical difference was significant if $P < 0.05$

Table 3 Comparison of the disappearance time of clinical symptoms and length of hospital stay (d)

	Cases	Cough disappearance	Hoarse breathing diappearnce	Wheeze disappearance	Hospital stay
< 20 kg					
Control group	40	7.25 ± 1.29	5.25 ± 1.6 9	6.46 ± 1.75	7.71 ± 1.35
Treatment group	32	5.60 ± 1.37	3.58 ± 1.96	3.79 ± 1.21	5.84 ± 1.24
t values		123.56	69.82	218.63	98.54
P values		0.000	0.000	0.000	0.000
> 20 kg					
Control group	34	7.34 ± 1.65	4.81 ± 1.52	5.84 ± 1.83	7.75 ± 1.29
Treatment group	42	5.67 ± 1.53	3.81 ± 1.34	3.91 ± 0.94	5.62 ± 1.58
t values		91.73	86.59	247.69	148.2
P values		0.000	0.001	0.000	0.000

Note: the stastistical difference was significant if $P < 0.05$

Comparison of lung function

FVC is an important indicator of lung function and used to determine the presence of respiratory resistance. FEV1 was used to examine the presence of obstructive lesions. PEF reflects the maximum flow rate and was used to detect airway obstruction. FEV1/FVC/% was clinically used to determine the severity of asthma and airway obstruction. There was no significant difference for lung function between the two groups before treatment ($P > 0.05$). After treatment, the FVC% (Fig. 2A), FEV1% (Fig. 2B), PEF% (Fig. 2C) and FEV1/FVC% (Fig. 2D) of children in the treatment group were higher than those in the control group ($P < 0.05$).

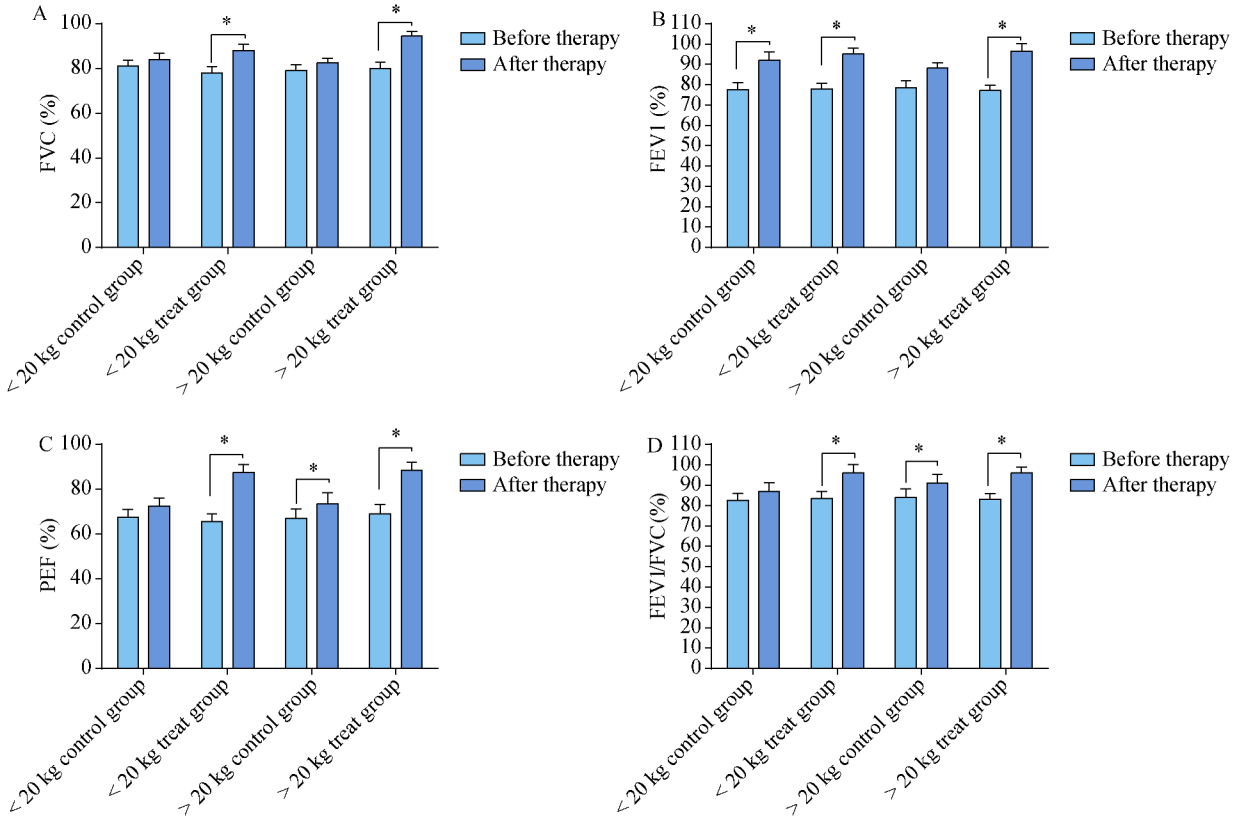

Fig. 2 Comparison of lung function between control and treatment groups. A, FVC%, the rate of forced vital capacity. B, FEV1%, the rate of forced Expiratory Volume in the first second. C, PEF%, the rate of peak expiratory flow. D, FEV1/FVC%. *n* = 40 and 34 for control group with < 20 kg and > 20 kg, respectively. *n* = 32 and 42 for treatment group with < 20 kg and > 20 kg, respectively. The stastistical difference was significant if $^{*}P < 0.05$

IgE is an immunoglobulin in human plasma and positively correlated with children asthma [14]. CRP, also known as C-reactive protein, is a clinical indicator of inflammation that can be used as an important indicator of treatment outcome in children asthma [41]. EOS refers to eosinophils, which are the major effector cells in the inflammatory response to asthma and are closely related to the severity and severity of asthma attacks [42-43]. There was no significant difference between the two groups before treatment ($P > 0.05$). After treatment, the laboratory parameters, such as IgE (Fig. 3A) and CRP (Fig. 3B), were significantly lower ($P < 0.05$) than those in the same quality control group except for EOS% (Fig. 3C).

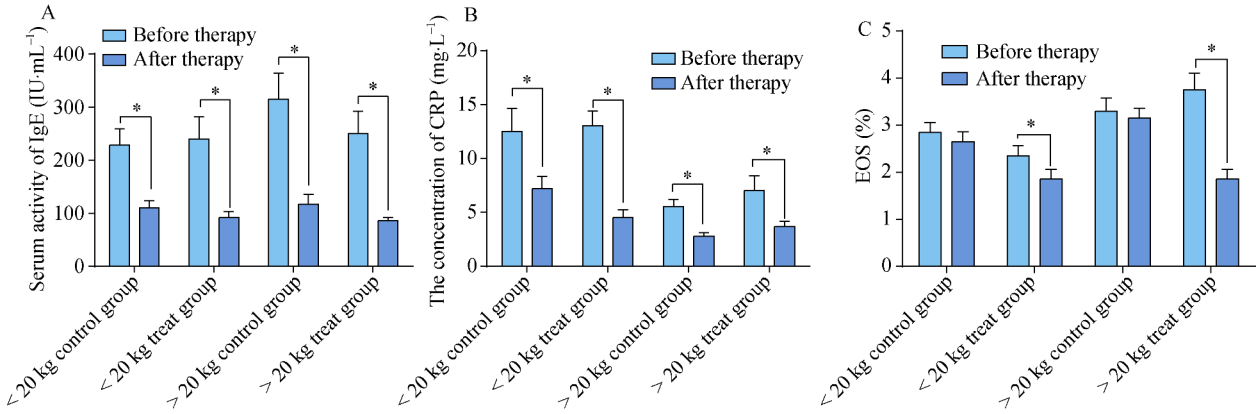

Fig. 3 Laboratory parameters between control and treatment groups. A, serum levels of IgE. B, the concentration of C reactive protein (CRP). C, the percent of eosinophilic granulocyte (EOS%). *n* = 40 and 34 for control group with < 20 kg and > 20 kg, respectively. *n* = 32 and 42 for treatment group with < 20 kg and > 20 kg, respectively. The statistical difference was significant if $^{*}P < 0.05$

The levels of FoxP3, TGF-β, IL-17 and IL-23

Before the treatment, the statistical differences for the levels of FoxP3 (Fig. 4A), TGF-*β* (Fig. 4B), IL-17 (Fig. 4C) and IL-23 (Fig. 4D) were insignificant between control and treatment groups. After treatment, the levels of FoxP3 (Fig. 4A) and TGF-*β* (Fig. 4B) were higher in the treatment group than control group, and IL-17 (Fig. 4C) and IL-23 (Fig. 4D) were lower in the treatment group than control group. *A. membranaceus* treatment increased the levels of FoxP3 and TGF-*β* and reduced the levels of IL-17 and IL-23 in the children with the weight < 20 kg or > 20 kg.

Measurment of the percentage of $CD4^+IL\text{-}17^+Th17$ and $CD4^+CD25^+$Treg cells in peripheral blood

After treatment, the statistical differences for the percentage of Treg cells in the treatment group (Fig. 5A) and control group (Fig. 5B) with < 20 kg were significant ($P < 0.05$). Comparatively, the percentage of Treg cells was higher in the treatment group (Fig. 5C) than the control group (Fig. 5D) with < 20 kg ($P < 0.05$). After treatment, the statistical differences for the percentage of Th17 cells in the treatment group (Fig. 6A) and control group (Fig. 6B) with < 20 kg were insignificant. Similarly, the percentage of Th17 cells was higher in the treatment group (Fig. 6C) than control group (Fig. 6D) with > 20 kg. *A. membranaceus* treatment increased the percentage of Treg cells (Fig. 5E) and reduced the percentage of Th17 cells (Fig. 6E).

For the children with asthma remission, the study was based on budesonide suspension and terbutaline, and combining with the inhalation of *A. membranaceus*. The combination could make up the shortcoming of the combination of two drugs. The results showed that the total effective rates of the treatment group were higher than the control group ($P < 0.05$, Table 2); After the treatment, wheeze disappeared in all children. The disappearance time of each clinical symptom and hospital stay in the treatment group were shorter than those from the control group ($P < 0.05$, Table 3), indicating that *A. membranaceus* improved the therapeutic results of budesonide suspension and terbutaline.

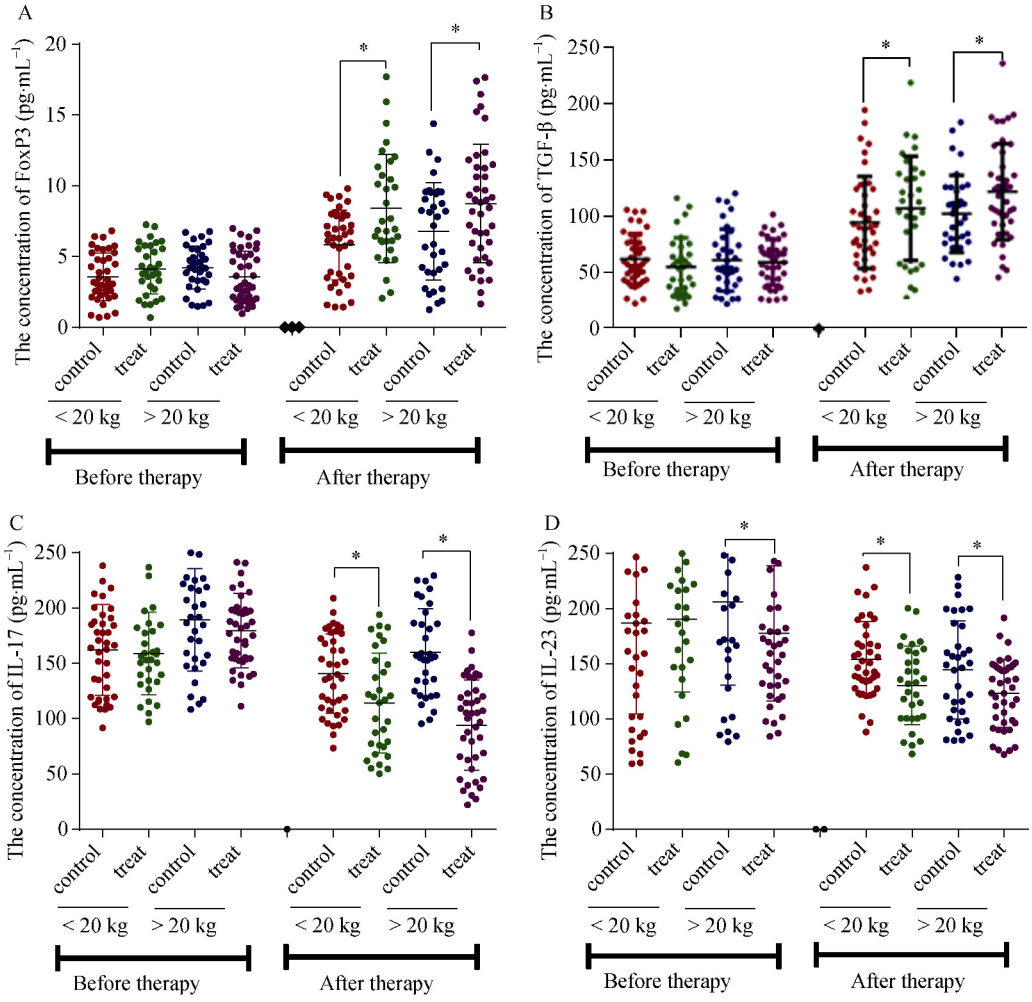

Fig. 4 The levels of transcription factors and cytokines between control and treatment groups. A, the levels of FoxP3. B, the levels of TGF-*β*. C, the levels of IL-17. D, the levels of IL-23. *n* = 40 and 34 for control group with < 20 kg and > 20 kg, respectively. *n* = 32 and 42 for treatment group with < 20 kg and > 20 kg, respectively. The statistical difference was significant if $^*P < 0.05$

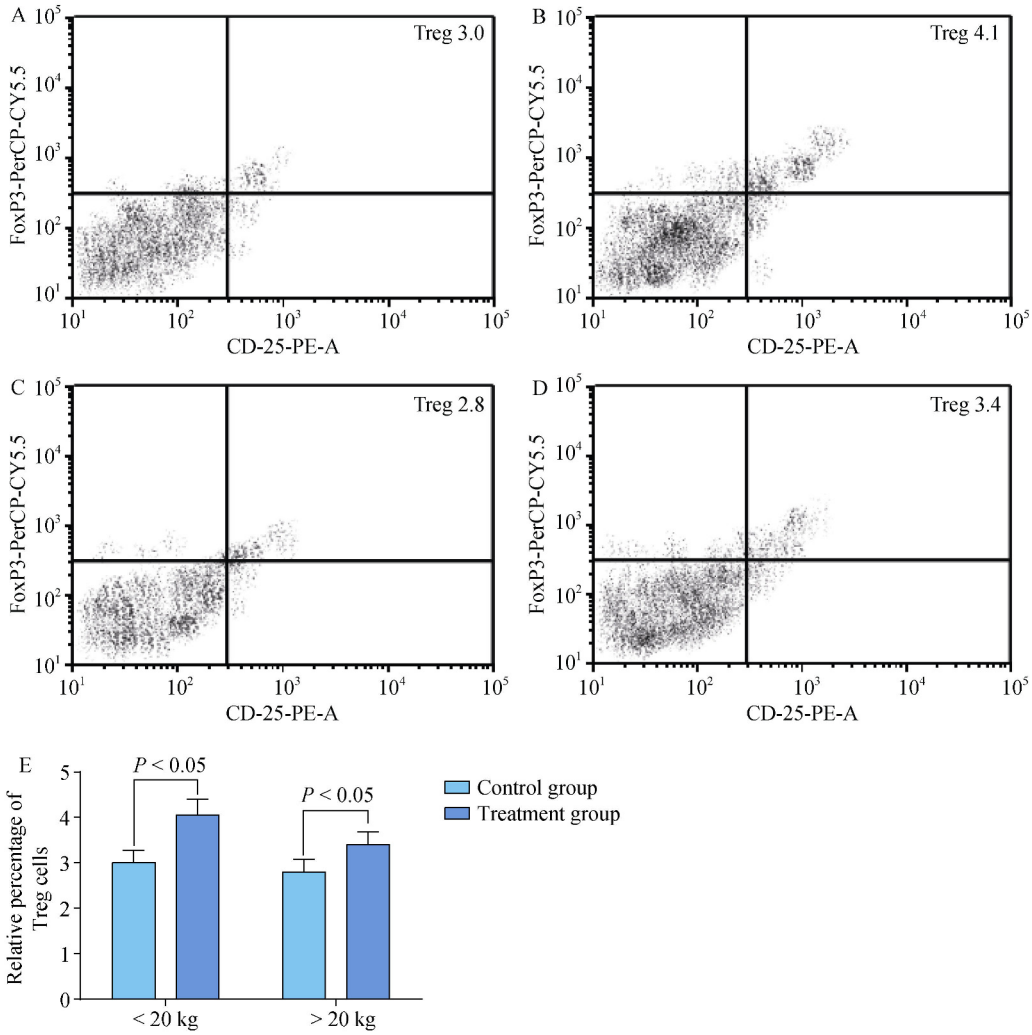

Fig. 5 The percentage of Treg cells in PMBCs between control and treatment groups. A, the percentage of Treg cells in PMBCs in the control group with < 20 kg. B, the percentage of Treg cells in PMBCs in the treatment group with < 20 kg. C, the percentage of Treg cells in PMBCs in the control group with > 20 kg. D, the percentage of Treg cells in PMBCs in the treatment group with > 20 kg. E, the mean and SD values of percentage of Treg cells in PMBCs between two groups. *n* = 40 and 34 for control group with < 20 kg and > 20 kg, respectively. *n* = 32 and 42 for treatment group with < 20 kg and > 20 kg, respectively

Lung function tests play an important role in the diagnosis of asthmatic children with airway lesions and obstruction, and are also important methods to evaluate therapeutic effect of asthma [44]. FVC mainly reflects the lung capacity of children; FEV1 is an index reflecting the resistance of expiratory airway, which can indirectly reflect the change of the airway diameter; PEF reflects the change of the lung gas flow rate and the obstruction of the airway; FEV1/FVC% < 80% predicted values, can be used as indicators of airway obstruction [45]. The results showed that the FEV1%, FVC%, PEF% and FEV1/FVC% of the treatment group were significantly higher than those of the control group ($P < 0.05$, Fig. 2). The present findings showed that the combination of three drugs in the treatment group was superior to the control group in improving lung function and the therapeutic efficacy of the combination of the two drugs.

Indicators of airway inflammation include eosinophil count and percentage of peripheral blood and induced sputum, exhaled nitric oxide, and CRP. Asthma patients have clinical features such as elevated serum IgE, CRP and EOS% [46]. CRP can be used clinically as an objective biochemical marker to evaluate the severity of asthma [47]. IgE has a pro-cellular activity that binds to mast cells and basophils in vivo and is a type I allergic mediator. The combination of IgE expression and FEV1 value can provide important values for the early diagnosis of lung disease [48]. The results of this study showed that children in the treatment group had lower levels of CRP, IgE and EOS% than the control group ($P < 0.05$, Fig. 3). The present findings indicate that treatment group is more effective than the control group for lowering the risk of asthma.

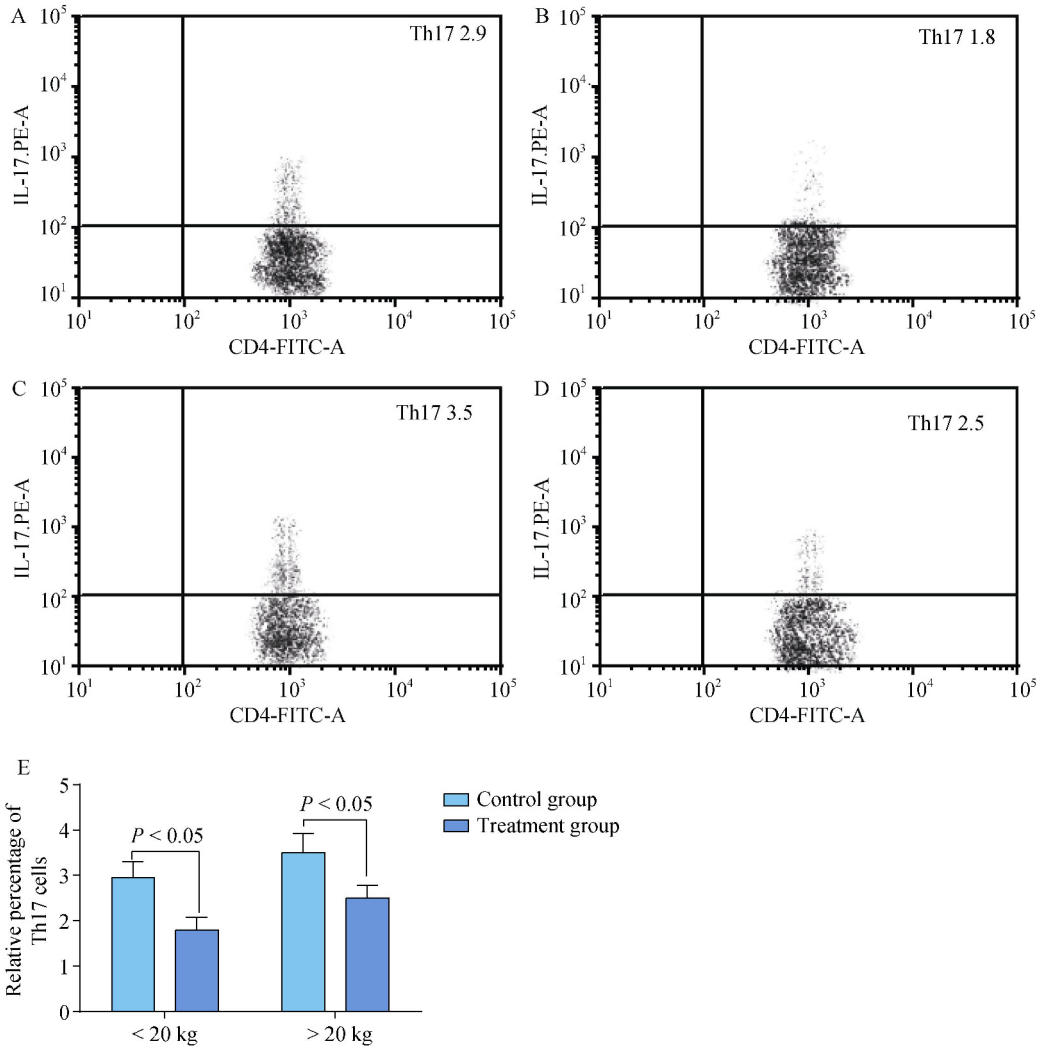

Fig. 6 The percentage of Th17 cells in PMBCs between control and treatment groups. A, the percentage of Th17 cells in PMBCs in the control group with < 20 kg. B, the percentage of Th17 cells in PMBCs in the treatment group with < 20 kg. C, the percentage of Th17 cells in PMBCs in the control group with > 20 kg. D, the percentage of Th17 cells in PMBCs in the treatment group with > 20 kg. E, the mean and SD values of percentage of Th17 cells in PMBCs between two groups. *n* = 40 and 34 for control group with < 20 kg and > 20 kg, respectively. *n* = 32 and 42 for treatment group with < 20 kg and > 20 kg, respectively

This study found that the combinations (budesonide, terbutaline and *A. membranaceus*) effectively increased the percentage of Treg cells and reduced the percentage of Th17 cells. The statistical difference was significant between two groups (Figs. 4 and 5). Currently, budesonide suspension and terbutaline are safe and effective in the prevention and treatment of asthma. However, asthma is still easy to relapse. The present findings demonstrated that the treatment group could prevent the recurrence of asthma better, and the total effective rate was higher than the control group. Meanwhile, the therapeutic parameters in the treatment group were improved significantly when compared with the control group. *A. membranaceus* was added to inhale glucocorticoid and used to the therapy of asthma remission by modulating regulated the imbalance of Treg/Th17 cells. The study provided a molecular basis for clinical therapy of the childhood asthma using *A. membranaceus*.

The main components of *A. membranaceus* were calycosin-7-*O*-glucoside, isoquercitrin, ononin, calycosin, quercetin, genistein, kaempferol, isorhamnetin and formononetin. Calycosin and formononetin were reported to suppress chronic airway inflammation and maintain Th17/Treg cell balance in a mouse ovalbumin asthma model [49]. Calycosin controlled allergic dermatitis by improving epithelial tight junctions via the down-regulation of HIF-1alpha [50]. Formononetin treatment lowered the levels of serum low-density lipoprotein (LDL) cholesterol [51]. Isoquercitrin and quercetin exerted anti-asthma effects [52]. Modified isoquercitrin therapy reduced the serum level of oxidized low-density lipoprotein (LDL), thymus and activation-regulated chemokine in the patients with 20 subjects with Japanese cedar pollinosis [53]. LDL modification was also involved in immune system, allergy and asthma [52].

Quercetin could be well tolerant and had anti-inflammatory properties by improved category III chronic prostatitis [54]. Ononin and calycosin treatment ameliorated asthma by affecting Th2-Th17 responses in RSV-induced asthma model [55]. Genistein was demonstrated to prevent airway inflammation in an asthma animal model [56]. Quercetin had antiasthmatic potential with immunomodulatory and bronchodilatory properties [57]. kaempferol and isorhamnetin were potential drugs for treating asthma and chronic bronchitis [58]. Kaempferol had antioxidant property in the thalassemia patient with high-level superoxide and hydroxyl radicals [59]. Kaempferol has anti-allergic properties by controlling airway hyperplasia and hypertrophy in an allergic asthma model [60]. Isorhamnetin treatment reduced LPS-induced inflammatory response by downregulating NF-*κ*B signaling, whereas NF-*κ*B is associated with the pathogenesis of asthma [61]. All these components may contribute to the asthma therapy in children patients.

There were some limitations to the present study. The detail components of *A. membranaceus* were not analyzed in the present experiment. Thus, the exact bioactive component for improving the therapeutic efficacy of combination therapy of budesonide and terbutaline for asthmatic children during remission remained unknown. The experimental duration was short and long-term efficacy of three combination therapies was still unknown. To make sure the exact molecular mechanism, specific gene silence or overexpression should be performed in an animal model. Thus, further work is still highly demanded to understand the present results better.

Conclusions

The efficacy of inhaled budesonide and terbutaline combined with *A. membranaceus* was better than budesonide combined with terbutaline in children with asthma during remission. The disappearance time of symptoms shortened, pulmonary function was significantly improved, and airway inflammation and adverse reactions were reduced. At the same time, three combination therapies could shorten the time of hospital stay. *A. membranaceus* improves therapeutic efficacy of the combination therapy of budesonide and terbutaline for asthmatic children during remission by regulating the balance of Treg/Th17 cells in PMBCs. All the main components of *A. membranaceus* contribute to the asthma therapy in children patients. The three combination therapies for the childhood asthma have higher efficacy and few side effects for asthmatic children.

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