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Wuhu Decoction Regulates Dendritic Cell Autophagy in the Treatment of Respiratory Syncytial Virus (RSV)-Induced Mouse Asthma by AMPK/ULK1 Signaling Pathway

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Background:

Dendritic cell autophagy plays a pivotal role in asthma. Wuhu decoction can significantly improve respiratory syncytial virus (RSV) bronchiolitis and delay its development into asthma. The aim of the present study was to explore the therapeutic effect and mechanism of Wuhu decoction on RSV-induced asthma in mice.

Material/Methods:

Establishment of asthmatic mice model was induced by RSV. Hematoxylin-eosin staining, periodic acid-Schiff (PAS) staining, and Masson trichrome staining were performed to observe pathological changes in the lungs. The levels of CD4⁺ T, CD8⁺ T, and CD4⁺ CD25⁺ T in blood were analyzed by flow cytometry. The contents of interleukin (IL)-4, interferon-gamma (IFN- γ), IL-10, and IL-13 in serum were measured by enzyme-linked immunosorbent assay (ELISA). The number of autophagosomes in dendritic cells (DCs) of lung tissue was observed by transmission electron microscope. The DCs of lung tissue were isolated by magnetic bead sorting. The levels of LC3-II, Beclin-1, and LC3-I in DCs and MMP-9, TIMP-1, AMPK, p-AMPK, ULK1, and LK1 expression in lung tissues were detected by western blot. Real-time polymerase chain reaction (PCR) detected the expression of AMPK and ULK1 genes.

Results:

Wuhu decoction can effectively alleviate chronic airway inflammation and airway remodeling and reduce airway hyperresponsiveness. Moreover, Wuhu decoction can significantly enhance the level of autophagy in DCs of lung tissue and promote the expression of AMPK and ULK1 in lung tissue.

Conclusions:

Wuhu decoction may improve the RSV-induced asthmatic symptoms by enhancing autophagy of DCs in lung tissue dependent on the AMPK/ULK1 signaling pathway.

MeSH Keywords:

Asthma • Autophagy • Dendritic Cells • Respiratory Syncytial Viruses

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Background

A common airway chronic inflammatory disease, asthma has been increasing in recent years. Respiratory syncytial virus (RSV) infection is the main factor in inducing acute asthma attacks in children, and 85% of acute asthma attacks are associated with RSV infection [1]. At present, as the main drugs for the prevention and treatment of RSV-induced asthma, glucocorticoids and leukotriene modifiers have certain effects on controlling asthma symptoms and reducing the number of acute asthma attacks. However, these medicines do not completely cure asthma, and there are various side effects to their use. Therefore, there is an urgent need for safe and effective clinical treatment for asthma.

Autophagy is a process by which cells use lysosomes to degrade their damaged organelles and macromolecules. Recent studies have emphasized the important role of autophagy in the development of asthma [2]. Ban et al. [3] confirmed that the levels of autophagy in sputum granulocytes, peripheral blood cells, and peripheral blood eosinophils in patients with severe asthma were higher than those in patients with non-severe asthma and healthy controls. Kim et al. [4] found that budesonide can exert antiviral effects on human rhinovirus (HRV) by mediating autophagy, thus effectively preventing asthma exacerbation. Using chloroquine treatment to inhibit autophagy significantly reduced the antiviral and anti-inflammatory effects of budesonide toward HRV, suggesting that autophagy may be a new strategy for the prevention and treatment of asthma. However, how autophagy affects the development of asthma remains to be elucidated. DCs, which are currently known as the most powerful antigen-presenting cells (APCs), act at the initial stage of asthma. Reed et al. found that DC autophagy induces DC differentiation and maturation and play a role in effective antiviral adaptive immune response during RSV infection [5]. Therefore, enhancing autophagy in DCs by drugs or other methods may be a novel therapeutic target for inhibiting RSV-induced asthma progression and reducing the frequency of its acute attacks.

Traditional Chinese medicine has been used for the prevention and treatment of asthma for thousands of years, and its effect has been gradually affirmed. The traditional ancient Wuhu decoction can significantly reduce the development of RSV bronchiolitis into typical asthma. Previous studies have confirmed that Wuhu decoction can downregulate the expression of interleukin (IL)-13 in lung tissue and reduce the deposition of collagen fibers, thereby inhibiting airway remodeling in asthma and alleviating the symptoms of asthma [6,7]. However, the specific mechanism of Wuhu decoction on the therapeutic effect of asthma is still unclear. Therefore, this study established an asthma model by RSV nasal drops combined with chicken egg albumin (OVA) sensitization, and different

concentrations of Wuhu decoction were used to explore the treatment of asthma with Wuhu decoction and its potential molecular mechanism, aimed to provide a new theoretical and experimental basis for the mechanism of Wuhu decoction in preventing and treating asthma.

Material and Methods

Main reagents

Wuhu decoction is made of ephedra, almond, raw gypsum, raw licorice, and fine tea. The Wuhu decoction for this experiment was produced in the preparation room of the First Affiliated Hospital of Hunan University of Chinese Medicine, 100 mL per bottle, containing 24.6 g of crude drug. RSV long strain and human laryngeal carcinoma epithelial cells (Hep-2) were provided by the Institute of Virology, Wuhan University Medical School. OVA was purchased from Beijing Solarbio; acetylcholine chloride was produced by Selleck, USA (batch number: 2260-50-6); dexamethasone was supplied by Sigma (article number D1756); rapamycin was provided by Selleck (item no. S1039); metformin was supplied by Selleck (item no. S1950). Hematoxylin-eosin staining solution was manufactured by Auragene (Cat. No. P032IH). Lung Dissociation Kit (mouse), CD11c MicroBeads UltraPure (mouse), and autoMACS Running Buffer were produced by Mirin Biotech, Germany (numbers: 130-095-927, 130-108-338, 130-091-221). Fitcanti-mouse CD3, percp-cy5.5 anti-mouse CD4, pe anti-mouse CD8, apc anti-mouse CD25, and hemolysin were produced by BioLegend, USA (batch numbers 100203, 100539, 100707, 102011, A11895, respectively). IL-4, IL-10, IL-13, IFN- γ , and enzyme-linked immunosorbent assay (ELISA) kit were from Shanghai Jingtian Biotechnology Co., Ltd. (production, batch number: E20181109001, E20181109017, E20181109019, E20180713006).

Main instruments

Ultrasonic nebulizers were purchased from Nanjing Daofen Electronics Co., Ltd. (model: S888E). The small animal ventilator and airway resistance and lung compliance analysis software were purchased from BUXCO, USA, model DHX-50, WESTERN BLOT. The biological tissue spreader is produced in Hubei Xiaogan, model YT-6C. Flow cytometry was purchased from Beckman Gallios, USA. The microplate reader was purchased from Thermo Corporation (model MK3). MACS SmartStrainers (70 μ m) and LS Columns (sorting columns) were purchased from Mirin Biotech, Germany (numbers 130-098-462 and 130-042-401). The transmission electron microscope was purchased from Hitachi High-Technologies Corporation (model HT7700). The fluorescence quantitative PCR instrument was purchased from ABI (Model 7300). The PCR instrument was purchased from Axygen Corporation (model number THERM-1000). The nucleic

acid quantitation instrument was purchased from Thermo Corporation (model NanoDrop Lite).

Animals

We used 180 SPF (specific pathogen free) female BALB/c mice, 6 to 8 weeks of age, weighing 18 to 20 g, were purchased from Hunan Slack Laboratory Animal Co., Ltd., animal certificate number: SCXK (Xiang) 2016-0002.

The experiment was started after 1 week of normal feeding. Mice were under controlled standard conditions (12-hour light/dark cycle, humidity $55\pm5\%$, temperature $22\pm2^{\circ}\text{C}$) with free access to food and water. Mice with successful modeling randomly divided into 7 groups ($n=20$ in each group): model group (the same volume of normal saline), Wuhu decoction low-dose group (0.2 mL at 5 mL/kg Wuhu decoction treatment+the same volume of normal saline), Wuhu decoction middle-dose group (0.2 mL at 10 mL/kg Wuhu decoction treatment+the same volume of normal saline), Wuhu decoction high-dose group (0.2 mL at 20 mL/kg Wuhu decoction treatment+the same volume of normal saline), positive drug control group (0.2 mL at 1.82 mg/kg dexamethasone+the same volume of normal saline), rapamycin (autophagy inducer) group (0.2 mL at 1 mg/kg rapamycin treatment+the same volume of normal saline), and metformin group (0.2 mL at 250 mg/kg metformin treatment+the same volume of normal saline). The control group were gastric administration and intraperitoneal injection with the same volume of normal saline once a day for 14 days. At 24 hours after the last treatment, all mice were anesthetized by intraperitoneal injection of 5% chloral hydrate (400 mg/kg, mice without any discomfort) for the airway reactivity determination and then collection of 0.8 mL blood and finally sacrificed with cervical dislocation and then collection of lung tissue. This study was approved by the Ethics Committee of the first Affiliated Hospital of Hunan University of Traditional Chinese Medicine (approval 20180810-02).

Mouse RSV-induced asthma model establishment

Consulting references [8] and based on appropriate improvement, an RSV-induced mouse asthma model was established. Here were 180 SPF-level BALB/c mice randomly divided into a control group, a model group, and a control group. The mice were injected with 2.4×10^6 PFU RSV 0.1 mL/nose in the nasal cavity on the first and second days of the experiment. At the same time, an intraperitoneal injection of 1% OVA 0.25 mL/sensitization was administered. The control group was given an equal volume of Hep-2 cells nasal drops and an intraperitoneal injection of physiological saline 0.25 mL. From the 9th day of the experiment, mice other than the control group were placed in a nebulizer for inhalation of 1% OVA to stimulate asthma. Each time 1% OVA 20 mL was added, 10 times each time.

The control group was given an equal volume of normal saline. The nebulization started at 9: 00 am, the atomization time was fixed at 30 minutes to ensure that each mouse received the same stimulation of concentration, dose and time, once every other day for 2 weeks. After the end of the atomization, the asthma model group showed obvious nodular wheezing, abdominal muscle convulsions, and even unstable standing and thumping, which indicated an acute asthma attack. Ten randomly selected mice from the control group and the model mouse group were measured for airway reactivity. It was observed that the airway responsiveness of the mice after modeling was significantly higher than that of the control group, indicating successful modeling.

Airway reactivity determination

At 24 hours after the last administration, 5% chloral hydrate (400 mg/kg) was intraperitoneally injected into the mice, and the trachea was cut, intubated, and fixed. The mice were placed in a closed body drawing box, using an invasive method, the tracheal intubation was connected, and the mice were mechanically ventilated. The ventilator parameters were adjusted to a respiratory rate of 75 times/minute and a tidal volume of 8 mL/kg. The changes in airway pressure, airway flow rate, and tidal volume were recorded. After the airway pressure in the mice was stabilized, nebulization was performed with different concentrations of acetylcholine (0, 6.25, 12.5, 25, and 50 $\mu\text{g/mL}$) at 0.1 mL. After inhalation of acetylcholine, data from 5 seconds to 1 minute after inhalation were collected, and the maximum lung resistance (RL) was calculated using software.

Hematoxylin-eosin (HE), Masson trichrome, and periodic acid-Schiff (PAS) staining

The lung tissue of mice was removed, and 1 mL of paraformaldehyde was injected into the trachea; then, the whole lung was immersed in 4% paraformaldehyde for 24 hours. Conventional paraffin embedding, sectioning, HE staining, Masson trichrome staining, and periodic acid-Schiff (PAS) staining were performed to observe gross pathological changes in the lungs, collagen deposition in bronchial epithelial cells, and mucus secretion by goblet cells. The results of Masson trichrome staining were quantitatively analyzed by Image ProPlus 6.0 system. The positive area was blue. The percentage of blue positive staining area in the range of 20 μm under endothelial cells was calculated as the percentage of total statistical area, which was the result of Masson trichrome staining. The results of PAS staining were statistically analyzed using the Image ProPlus 6.0 system to quantify the blue stain-positive area and calculate the airway mucus reserve index.

Transmission electron microscopy

Lung tissue was taken and fixed with 3% glutaraldehyde and 0.22 mmol/L sucrose phosphate buffer for 4 hours, rinsed 3 times with 0.1 M phosphoric acid rinsing solution for 10 to 15 minutes, and fixed with 1% citric acid for 2 hours. Next, stepwise ethanol dehydration, followed by epoxy resin embedding, overnight in a 30°C oven, then in a 60°C oven for 12 hours. Using an ultra-thin slicer, 50- to 100-nm-thick slices were cut. Using uranium acetate and lead nitrate double staining, a Hitachi H7700 transmission electron microscopy was used to examine autophagosomes in lung tissue DCs.

Flow cytometry

Using the peripheral blood of mice, EDTA anticoagulation was performed. After mixing, 100 μ L was taken for each sample, and 5 μ L of corresponding detection antibody was added. After vortexing and mixing, samples were left at room temperature for 20 minutes, 1 mL diluted hemolysin was added, and the samples were again mixed. After leave at room temperature for 15 minutes, then mixing well, samples were centrifuged at 400 g for 5 minutes. Next, the supernatant was discarded, leaving 0.1 mL of sample underneath. PBS solution (2 mL) was added to each tube, then mixed, and centrifuged at 400 g for 5 minutes. The supernatant was discarded, and a sample of 0.1 mL was left at the bottom. The liquid above was further supplemented with 100 μ L of PBS, mixed, and detected by a machine.

ELISA

For the ELISA assay, 0.1 mL of plasma was added to the coated reaction wells, and blank wells, negative control wells, and positive control wells were incubated at 37°C for 1 hour, then washed. 0.1 mL of freshly diluted enzyme-labeled antibody was added to each reaction well, and the mixture was incubated at 37°C for 0.5 to 1 hour, and then washed. 0.1 mL of the temporarily prepared TMB substrate solution was added for color development at 37°C for 10 to 30 minutes. The reaction was stopped by adding 0.05 mL of 2M sulfuric acid. The results of the enzyme-based colorimeter were used to measure the optical density (OD) value of each well at a wavelength of 450 nm, and the linear regression equation of the standard curve was calculated according to the concentration of the standard product and the corresponding OD value, and then according to the sample. The corresponding sample concentration is calculated on the regression equation of the OD value.

Magnetic bead sorting method to separate DCs from lung tissue

The process was as follows: prepare a mixed enzyme suspension using buffer S, enzyme D, and enzyme A in the lung dissociation box of MACS mice; perfuse the lungs through the right ventricle to remove red blood cells. cut the lungs into small pieces, rinse them with phosphate-buffered saline (PBS), add the lungs to the mixed enzyme suspension, incubate for 40 minutes at 37°C in a shaker, and briefly centrifuge. Next, resuspend the sample and filter it. Then, collect the cell suspension, and rinse and discard the filter. Centrifuge the cell suspension at 300 g for 5 minutes, completely discard the supernatant, resuspend to obtain a single cell suspension, centrifuge the cell suspension at 300 g for 10 minutes, and completely aspirate the supernatant. Resuspend 10^8 total cells in 400 μ L of buffer. Add 100 μ L of CD11c MicroBeads UltraPure per 10^8 total cells. Mix well and incubate in the refrigerator (2°C to 8°C) for 10 minutes in the dark. Wash cells with 10 mL of buffer per 10^8 cells, and after centrifugation at 300 g for 10 minutes, the supernatant is completely aspirated and resuspended in 500 μ L of the buffer. Then, place the column in the magnetic field of the MACS separator, rinse the LS column with an appropriate amount of buffer, inject the cell suspension into the column, wait for the liquid to empty, wash the column with the appropriate amount of buffer three times, and take the chromatogram from the separator. Take away the column and place it on a suitable collection tube. Pipette 5 mL of buffer into the column and immediately push the plunger firmly into the column and rinse the magnetically labeled cells. The magnetic separation step was repeated twice using a new column to obtain DCs.

Western blot

The process was as follows: extract total protein from lung tissues, using the BCA (bicinchoninic acid) method to determine protein concentration. Each group takes 50 μ g of total protein and adds sodium dodecyl sulfate (SDS) loading buffer in a ratio of 1: 4; heat in a boiling water bath for 5 minutes, add sample to pre-made glue hole, proceed to SDS-PAGE electrophoresis, transfer film, block with 5% milk for 1 hour. Next, ULK1 (ab167139, Abcam, UK), TIMP-1 (ab61224, Abcam, UK), and MMP9 (ab38898, Abcam, UK) were added respectively. AMPK (#5831, Cell Signaling Technology, USA), p-AMPK (Thr172; #50081, Cell Signaling Technology, USA), p-ULK1 (Ser555; #5869, Cell Signaling Technology, USA), LC3-I (#4599, Cell Signaling Technology, USA), LC3-II (#3868, Cell Signaling Technology, USA), Beclin-1 (#3495, Cell Signaling Technology, USA), rabbit anti-polyclonal antibody, and internal reference β -actin were incubated overnight. TBST was washed 3 times for 10 minute each time, horseradish peroxidase-labeled secondary antibody was added, the membrane was incubated

Figure 1. Wuhu decoction improves airway hyperresponsiveness in a mouse model of RSV-induced asthma. **(A)** Flow chart for establishing an asthma mouse model using OVA and RSV. **(B)** Detection of airway reactivity and verification of successful modeling. **(C)** Airway responsiveness of each group of mice challenged with different concentrations of acetylcholine was determined. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the asthma group. Mean $\pm$ standard deviation, $n = 10$. OVA – with chicken egg albumin; RSV – respiratory syncytial virus.

with membrane for 1 hour at room temperature, and TBST was washed 3 times for 10 minutes each time. The gel imaging system was exposed to light. After scanning, the OD was calculated by Quantity One grayscale analysis software. The ratio of the gray value of the target protein band to the gray value of the internal reference β -actin was used as the index of expression of the corresponding target protein.

Real-time polymerase chain reaction (RT-PCR)

The process was as follows: extract the RNA of the cell sample with TRIzol, take 1 μ L of the sample, and reverse-transform 1 μ g of RNA into cDNA in a 20- μ L system. Using this as a template, the setting procedure is a 2-step method of real-time PCR quantification. With pre-denaturation at 95°C, for 3 minutes, each step is denatured at 95°C, for 10 seconds, with 60°C annealing extended for 30 seconds, a total of 40 cycles for each time in the extension phase to read the fluorescence value. Quantitative primers for amplification:
AMPK upstream primer sequence
5'-AGGTGGACATCTGGAGCA-3',
downstream primer sequence
5'-GGCTGATTACTGAAGGGT-3';

ULK1 upstream primer sequence
5'-TCATTAGGGACAGACAGTTCATTG-3',
downstream primer sequence
5'-GAGTTGGGAGGTGGAGACA-3';
 β -actin upstream primer sequence
5'-AGGGGCCGGACTCGTCATACT-3',
downstream primer sequence
5'-GGCGGCACCACCATGTACCCT-3',
Synthesized by Hunan Qingke Biotechnology Co., Ltd. The results were measured by observing the dissolution curve and the amplification curve, and by using β -actin as an internal reference. The results were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All measurement data were presented as mean $\pm$ standard deviation (SD), and a normal distribution test was performed using SPSS version 16.0. If the data obeyed normal distribution, then the comparisons between any 2 groups of means were performed using one-way analysis of variance (ANOVA). A least significant difference (LSD) test is appropriate to equal variance one, and the Dunnett T3 test is the opposite. P value < 0.05 was considered statistically significant.

Figure 2. Wuhu decoction improved airway inflammation in asthma mouse model induced by RSV. **(A)** Representative images of HE staining sections of mouse lung tissue, and the nuclei were stained blue with hematoxylin. The cytoplasm is stained with eosin in varying shades of pink to deep red. **(B, C)** The number of CD4⁺ T, CD8⁺ T and CD4⁺ CD25⁺ T in the blood of each group was detected by flow cytometry, and the CD4⁺ T/CD8⁺ T ratio was calculated. **(D)** Serum levels of IL-4, IFN-γ, IL-10, and IL-13 were determined by ELISA. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the asthma group, mean $\pm$ standard deviation, $n = 10$. RSV – respiratory syncytial virus; HE – hematoxylin-eosin; ELISA – enzyme-linked immunosorbent assay.

Results

Wuhu decoction improves airway hyperresponsiveness in asthmatic mice

Under the stimulation of each concentration gradient acetylcholine, the airway responsiveness of the model group was significantly higher than that of the control group ($P < 0.01$) (Figure 1). Wuhu decoction, dexamethasone, and rapamycin can significantly reduce airway hyperresponsiveness in asthmatic

mice and reduce lung resistance (RL) in mice after acetylcholine challenge ($P < 0.01$).

Wuhu decoction improves airway inflammation and improves Airway Remodeling in asthmatic mice

There was no abnormality in the bronchial and alveolar structures in the control group, and there was almost no inflammatory cell infiltration. In the asthma group, there was significant thickening of the alveolar septum, pulmonary capillary

Figure 3. Wuhu decoction improved airway remodeling in asthma mouse model induced by RSV. **(A)** Representative images of PAS and Masson staining sections of mouse lung tissue. The cytoplasm of PAS staining bronchial epithelial cells was red, and the nucleus was blue. The nuclei are stained black from Masson staining; muscle fibers are red; and collagen fibers are blue. **(B)** The positive area of Masson staining was blue, PAS staining results showed that the positive areas were red, and the Image ProPlus 6.0 system was used for quantitative analysis to calculate the collagen area under the airway basement membrane and the index of airway mucus reserve. **(C)** Western blot analysis of the expressions of AMPK and p-AMPK in lung tissues of mice in each group. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the asthma group. mean $\pm$ standard deviation, $n = 3$. RSV – respiratory syncytial virus, PAS – periodic acid-Schiff.

edema, increased bronchial mucosal folds, rupture, and a large number of inflammatory cell infiltration changes around the bronchi and blood vessels; compared with the control group, Wuhu decoction treatment significantly reduced lung lesions, bronchial and perivascular inflammatory cell infiltration, airway mucosal congestion, and edema (Figure 2A). Compared with the control group, the number of CD4⁺ T and CD4⁺CD25⁺ T cells in the model group decreased, the number of CD8⁺ T cells increased significantly, and the ratio of CD4⁺ T/CD8⁺ T decreased significantly ($P < 0.05$). The CD4⁺ T/CD8⁺ T ratio of the Wuhu decoction group was significantly improved compared with the model group, and the level of CD4⁺ CD25⁺ T cells was significantly increased ($P < 0.01$). The effect of Wuhu decoction middle dose group was the most obvious one, and shows no significant difference when compared with the dexamethasone group ($P > 0.05$), as shown in Figure 2B and 2C. Compared with the control group, IL-4 and IL-13 in the serum of the model group were significantly increased ($P < 0.01$), whereas INF- γ and IL-10 were significantly decreased ($P < 0.01$). After treatment with dexamethasone and rapamycin, the ratio of INF- γ /IL-4 was increased ($P < 0.01$), the expression of IL-13 was significantly downregulated, and the level of IL-10 was significantly

increased ($P < 0.01$) (Figure 2D). These results indicated that Wuhu decoction can alleviate airway inflammation in RSV-induced asthmatic mice. Furthermore, PAS staining and Masson staining showed that the number of airway cup epithelia in asthmatic mice increased significantly, and extensive collagen deposition appeared in the airway epithelium, suggesting that the airway wall of the model mice had severe airway remodeling, which may be the main cause of high airway reactivity in model mice. The administration of Wuhu decoction can significantly reduce the infiltration of lung inflammatory cells and reduce the accumulation of collagen and airway mucus secretion in airway epithelial cells, significantly improving asthma lesions (Figure 3A). Quantitative analysis of the blue-stained area showed that there was a significant difference between the Wuhu decoction group and the model group ($P < 0.01$), as shown in Figure 3B. The effects of Wuhu decoction on the expression of MMP-9 and TIMP-1 in lung tissue of asthmatic mice were determined by western blot. MMP-9, TIMP-1 expression, and MMP-9/TIMP-1 were significantly higher in the model mice than in the control group ($P < 0.01$). The expression levels of MMP-9 and TIMP-1 and MMP-9/TIMP-1 in the Wuhu decoction middle- and high-dose groups and the rapamycin

Figure 4. The effect of Wuhu decoction on the autophagy level of DCs in the lung tissue of asthmatic mice induced by RSV. (A) observation of autophagosomes in DCs of lung tissues of each group by transmission electron microscopy. (B) Western blot was used to detect the expressions of LC3-I, LC3-II, and Beclin-1 in the lung DCs of mice in each group separated by magnetic beads, and the values of LC3-II/LC3-I were determined. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the asthma group. Mean $\pm$ standard deviation, $n = 3$. DCs – dendritic cells; RSV – respiratory syncytial virus

group were significantly lower than those in the model group ($P < 0.01$), as shown in Figure 3C.

Effect of Wuhu decoction on autophagy of DCs in lung tissue of asthmatic mice

The results of transmission electron microscopy showed that the number of autophagosomes in the control group was small. Compared with the control group, the number of autophagosomes in the DCs of the asthma group increased significantly. The number of autophagosomes in the DCs of the low, middle, and high doses of Wuhu decoction and rapamycin were significantly increased compared with the model group (Figure 4A). The results of western blot analysis showed that the ratio of LC3-II, Beclin-1, and LC3-II/LC3-I in the control group was low. Compared with the control group, the expression level of autophagy protein in the asthma group was significantly increased ($P < 0.01$). Compared with the asthma group, the expression levels of LC3-II and LC3-II/LC3-I in the Wuhu decoction group and the rapamycin group were significantly increased ($P < 0.01$);

however, there was no significant difference in the dexamethasone group ($P > 0.05$). Compared with the asthma group, the expression level of Beclin-1 in the Wuhu decoction group was significantly increased ($P < 0.01$), as shown in Figure 4B. This finding proves that the autophagy level of DCs in lung tissue of asthma mice is increased. After treatment with Wuhu decoction and rapamycin, the level of autophagy in DCs is further increased, indicating that Wuhu decoction has the effect of upregulating the level of autophagy in DCs.

Wuhu decoction Regulates AMPK/ULK1 pathway-related proteins in lung tissue of asthmatic mice

Compared with the control group, the levels of p-AMPK/AMPK in the lung tissue of the asthma group were significantly lower ($P < 0.01$). Compared with the asthma group, the p-AMPK/AMPK in the Wuhu decoction low-dose group and the dexamethasone group increased ($P < 0.05$). The p-AMPK/AMPK of the Wuhu decoction middle-dose group and metformin group were significantly upregulated ($P < 0.01$). There was no significant

Figure 5. Effect of Wuhu decoction on the AMPK/ULK1 pathway-related proteins in lung tissues of asthmatic mice induced by RSV. (A) Western blot was used to detect the expressions of AMPK and p-AMPK in the lung tissues of mice in each group. (B) Expression of ULK1 and p-ulk1 in lung tissues of mice in each group was detected by Western blot. (C) Real-time PCR was performed to detect the expressions of AMPK and ULK1 genes in the lung tissues of mice. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the asthma group. Mean $\pm$ standard deviation, $n = 3$. RSV – respiratory syncytial virus; PCR – polymerase chain reaction.

difference between p-ULK1/ULK1 in the asthma group and the control group ($P > 0.05$). Compared with the asthma group, the p-ULK1/ULK1 in the Wuhu decoction middle-dose group and the metformin group were significantly upregulated ($P < 0.01$). The p-ULK1/ULK1 of the rapamycin group was significantly

decreased ($P < 0.01$), and the QPCR data were basically consistent with the results, as shown in Figure 5.

Discussion

Asthma is a common global respiratory disease with significant morbidity and mortality [9]. Wuhu decoction is a traditional and classic Chinese medicine for clearing the lungs and removing phlegm, relieving cough, and relieving asthma. Clinical research has confirmed that Wuhu decoction can significantly improve RSV bronchiolitis and delay its development into asthma. It is an effective prescription for the treatment of RSV-induced asthma. However, its specific mechanism is not yet clear. This experiment shows that Wuhu decoction can improve the asthmatic symptoms of mice by reducing chronic airway inflammation and airway remodeling in RSV-induced asthmatic mice, and more importantly, our research found that this role may be related to the regulation of the AMPK/ULK1 signaling pathway and the enhancement of autophagy in DCs in lung tissue.

In this experiment, mouse models of asthma were successfully established by RSV nasal infusion combined with OVA sensitization. The asthma group showed obvious signs of asthma behavior such as scratching the nose and sneezing. Pathology shows that there are a large number of inflammatory cell infiltration of eosinophils and lymphocytes around the blood vessels and trachea of the lung tissue, and airway hyperresponsiveness occurs, as shown in Figure 1B and 1C, indicating that the model was successfully established. At the same time, we found that Wuhu decoction, dexamethasone, and rapamycin can effectively reduce the airway reactivity under different concentrations of stimulation. The Wuhu decoction middle-dose group had the best effect, followed by the dexamethasone group. The CD4⁺/CD8⁺ T cell ratio plays an important role in maintaining the stability of the body environment. When the ratio of the 2 is imbalanced, it is easy to cause a disorder of the immune system, affecting the balance regulation of T lymphocytes and causing the onset of asthma [10,11]. CD4⁺ CD25⁺ regulatory T cells (CD4⁺ CD25⁺ Treg) play an important role in the development of bronchial asthma [12]. Recent studies have shown that by promoting the expansion and activity of lung CD4⁺ CD25⁺ T cells, mice Th2 cell responses can be down-regulated and immunogenic maturation of lung DCs can be reduced [13]. This study found that compared with the control group, the CD8⁺ T cells in the asthma group were significantly elevated, and the number of CD4⁺ CD25⁺ T cells was significantly downregulated. After treatment with Wuhu decoction and dexamethasone, the ratio of CD4⁺/CD8⁺ T cells in the blood of mice was significantly improved, and the level of CD4⁺ CD25⁺ T cells was significantly increased as well. TH1/TH2 cell imbalance is recognized as an important pathogenesis leading to airway inflammation in asthma. IFN- γ /IL-4 is a pair of important antagonistic cytokines in TH1/TH2 cell balance. Excessive proliferation of TH2 cells and excessive secretion of inflammatory factors such as IL-4 cause bronchial

mast cell degranulation, histamine release, and eosinophil infiltration. This study showed that Wuhu decoction can significantly inhibit Th2 cytokines. For example, the secretion of IL-4 and IL-13 increases the expression level of Th1 cytokines, such as IFN- γ and the asthma protective factor IL-10, and regulates the TH1/TH2 cell balance. Chronic inflammation of the airway is repeatedly damaged and repaired, eventually leading to airway remodeling. Airway remodeling is the main cause of airway hyperresponsiveness and recurrence of asthma. MMP-9 and TIMP-1 are key factors in airway remodeling. MMP-9 and TIMP-1 induces cell proliferation and differentiation by up-regulating matrix-associated growth factors such as TGF- β 1, accelerates collagen deposition, and promotes new blood vessels and epithelial formation, leading to airway fibrosis [14]. This study found that the expression of MMP-9 and TIMP-1 in the lung tissue of mice with asthma induced by RSV decreased after treatment with Wuhu decoction, suggesting that Wuhu decoction may regulate the levels of MMP-9 and TIMP-1 in lung tissue to play its therapeutic role.

Autophagy is an intracellular waste treatment process. Baseline autophagy can maintain cell homeostasis and degrade cytoplasmic components for the body's energy supply. Autophagy plays a role in innate and adaptive immune responses and B lymphocyte development. Antigen presentation is associated with antiviral immunity [15,16]. In RSV infection, autophagy is essential for DC activation and maturation, activation of T lymphocytes, and regulation of TH1/TH2 cytokines [4]. Western blot analysis and electron microscopy showed that the ratio of LC3-II/LC3-I and Beclin-1 in lung tissue of RSV-infected mice increased significantly, and the number of autophagosomes in lung tissue increased significantly compared with the control group, suggesting DCs. Autophagy participates in the formation of asthma induced by RSV. At the same time, we found that after treatment with Wuhu decoction, the level of autophagy in DCs was significantly higher than that in asthma group, and the enhancement effect of Wuhu decoction on autophagy is consistent with the therapeutic effect of Wuhu decoction, suggesting that Wuhu decoction can induce DC autophagy. DC autophagy may play a protective role in RSV-induced asthma. The autophagy activator rapamycin intervention also has a positive effect on airway hyperresponsiveness and airway inflammation in asthmatic mice, which also proves that autophagy plays a protective role in asthma. However, some studies [17] have shown that increased autophagy may be a factor in the development of allergic asthma and reducing autophagy in lung tissue can improve asthma. We speculate that the main reason for this difference may be related to whether RSV induction is used when the asthma model is established and the double complex effect of autophagy in the body. According to Reed et al. [5], autophagy participates in the immune mechanism of RSV-induced asthma. RSV infection can induce autophagy in DCs. DC autophagy can activate

a variety of antiviral adaptive immune responses, resist virus damage, and protect the body. Our research results are consistent with this.

Autophagy is activated by AMPK-mediated phosphorylation or inhibited by mTORC1 phosphorylation. The Unc. like kinase 1 (ULK1) complex is at the central bifurcation of the autophagy regulatory pathway, and the AMPK/ULK1 signaling Pathway is one of the important pathways for the regulation of autophagy at present. Multiple experiments have shown that AMPK/ULK1-dependent autophagy is involved in the regulation and transformation of various diseases [18,19], but there are no reports on the role of AMPK/ULK1-dependent autophagy in RSV-induced asthma. AMPK is a heterotrimeric protein complex that promotes Thr172 site phosphorylation of the alpha subunit N-terminal kinase domain by upstream kinases as a prerequisite for AMPK activation [20]. More and more studies have shown that AMPK plays an important role in many respiratory inflammatory diseases. Lee et al. [21] found that AMPK α 1-deficient mice have increased susceptibility to pulmonary inflammation and emphysema, and they have demonstrated that AMPK can reduce the production of IL-8 to reduce lung inflammation and emphysema. Park et al. [22] found that the expression of fibronectin and collagen in lung tissue of an asthma mouse model was significantly increased by knocking out the AMPK gene. This *in vitro* study also confirmed that AMPK's effective activator metformin can effectively promote AMPK α 1 activation in fibroblasts, and metformin can effectively reduce eosinophilic inflammation in asthmatic mice, thereby improving airway inflammation. In our study we found similar results. We found that Wuhu decoction treatment can significantly promote the phosphorylation of AMPK. ULK1 is an important downstream protein of AMPK. When the body energy is insufficient, AMPK is activated by phosphorylation; p-AMPK

further phosphorylates ULK1, and positively regulates the amino acid-serine 555 (Ser555) site at position 555, which induces autophagy. When the phosphorylation of the ULK1 Ser757 site is activated, the role between ULK1 and AMPK is disrupted, and the occurrence of autophagy is inhibited [23,24]. Our study found that the AMPK/ULK1 signaling pathway is involved in the regulation of autophagy in DCs in the lung tissue of mice with RSV-induced asthma. In addition, we demonstrated that Wuhu decoction treatment significantly upregulated p-AMPK and autophagy, accompanied by a significant upregulation of p-ULK1 (555), and the trends were basically consistent. Based on these data, it is suggested that AMPK/ULK1 may be an important molecular signaling pathway for Wuhu decoction to exert autophagy regulation on DCs and therapeutic effects on RSV-induced asthma.

Conclusions

Wuhu decoction can reduce the airway hyperresponsiveness in asthmatic mice, regulate the balance of TH1/TH2 in asthmatic mice induced by RSV, reduce the infiltration of airway inflammation cells, and improve the symptoms of airway inflammation. It can also reduce airway collagen deposition, and goblet epithelial hyperplasia, effectively reduce the levels of MMP-9 and TIMP-1 in mouse lung tissue induced by RSV, and improve airway remodeling in asthma. These effects may be achieved by regulating autophagy in lung tissue DCs dependent on the AMPK/ULK1 signaling pathway.

Conflict of interest

None.

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