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Research Article
Effect and Mechanism of Reduning Injection on Respiratory Syncytial Virus (RSV)-Induced Airway Inflammation in Mice

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Abstract

Background and Objective: Respiratory Syncytial Virus (RSV) can cause severe pneumonia and bronchiolitis, leading to symptoms such as wheezing and dyspnea and inducing heart failure in severe cases. Reduning injection demonstrates notable efficacy in treating conditions like influenza, acute bronchitis, respiratory infections and cough resulting from an invasion of external wind-heat factors. To explore the effect of Reduning injection on the Respiratory Syncytial Virus (RSV)-induced airway inflammation in mice and analyze its mechanism of action. **Materials and Methods:** Thirty mice were divided into a control group, a model group and Reduning group according to the random number table method, with 10 mice in each group. The model group and Reduning group were given nasal dripping of the RSV virus to establish an RSV infection model and the blank control group received nasal dripping of an equal amount of normal saline. After successful modeling, 0.2 mL/kg of Reduning injection was intraperitoneally injected in the Reduning group and an equal amount of normal saline was given intraperitoneally in the control group and model group and the treatment was continuous for 7 consecutive days. **Results:** Compared with the model group, the Reduning group displayed higher levels of IL-2 and IFN-γ but lower levels of IL-6, IL-17 and IL-23 ($p<0.05$); when compared with the model group, the Reduning group showed lower levels of MDA and NO but higher levels of SOD ($p<0.05$). **Conclusion:** Reduning injection can effectively reduce RSV-induced airway inflammation and improve immune function in mice and its mechanism of action may be related to the inhibition of oxidative stress and the regulation of JAK1, STAT6 and NGF protein expression.

Key words: Respiratory syncytial virus, mice, Reduning injection, airway inflammation, mechanism of action

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Respiratory Syncytial Virus (RSV) is a common pathogenic microorganism that causes lower respiratory tract infections in humans, which can induce severe pneumonia and capillary bronchitis, resulting in symptoms such as dyspnea and respiratory distress and can lead to heart failure in severe cases, posing a significant threat to the health of the patients[1,2]. According to statistics, globally, the number of patients suffering from lower respiratory tract infections caused by RSV infection exceeds ten million, including several hundred thousand severe cases[3]. Presently, there is no specific drug for preventing or treating RSV infections in clinical practice. The administration of RSV-specific F protein humanized monoclonal antibodies stands as a common approach to prevent RSV infections[4]. However, this approach still falls short of entirely preventing respiratory system infections and its high cost poses challenges for widespread adoption.

According to traditional Chinese medicine (TCM), respiratory diseases associated with RSV infection belong to the category of "epidemic disease", which is caused by external pathogenic factors and can be spread and prevalent within a certain range. The TCM for the treatment of respiratory tract infections caused by RSV has antiviral and immune-regulating effects and it has fewer adverse effects and possesses the advantage of being inexpensive compared with western anti-infective drugs[5]. Reduning injection is a proprietary Chinese medicine injection, with the main ingredients of *Artemisia annua*, honeysuckle, gardenia, etc., which has the effects of heat-clearing, detoxification and wind-dispersing properties, exhibiting notable therapeutic benefits against conditions such as colds, acute bronchitis, respiratory infections and coughs caused by external pathogenic factors of wind-heat[6,7]. Based on this, the present study analyzed the effect of Reduning injection on RSV infection-induced airway inflammation in mice and observed the changes in lung index, lung tissue morphology, immune function and other indicators after the administration of the drug in animals to explore the mechanism of action of Reduning injection, thus providing a basis for the clinical application of this drug. The results are reported as follows.

MATERIALS AND METHODS

Study area: The animal experiments of this study were conducted in Fuyang Vocational and Technical College from May 2022 to October 2022.

Materials

Animal: Thirty Balb/c mice of SPF grade, aged 6-8 weeks, weighing 18-20 g, regardless of gender, were purchased from Guangdong Experimental Animal Center. Feeding conditions: Mice were raised under simulated natural light, with one change of illumination state every 12 hrs, humidity of 55-65% and temperature of 25°C. The experimental procedures commenced after 1 week of acclimatization feeding, during which an autonomous respiratory pathway, provision of water and sterilized feed, were implemented.

Virus: The RSV long strain, provided by Wuhan National Typical Culture Collection Center.

Ethical consideration: All animal experimental procedures complied with the Regulations on the Management of Laboratory Animals and were approved by the Laboratory Animal Ethics Committee of Guangzhou University of Chinese Medicine.

Drugs, reagents and instruments: Reduning injection was purchased from Jiangsu Kanion Pharmaceutical Co. Ltd., batch number 200721, specifications: 10 mL/dose, in a package of 6 doses; The 402AI Ultrasonic Nebulizer was supplied by Yuwell-Jiangsu Yuyue Medical Equipment and Supply Co. Ltd. The Optical Microscope is a product of Olympus Corporation from Japan, model: CKX41-A32PH; The Flow Cytometer is a product of Beckman Coulter Inc. from the United States, model: CytoFLEX LX; The Microplate Reader is a product of BioTek Instruments, Inc. from the United States, model: Synergy 2; The Centrifuge is a product of Eppendorf AG from Germany, model: CX-41; The Fully Automated Modular Hematology Analyzer is a product of Sysmex Corporation from Japan, model: XN-1000; The ATP700 (ST) Computerized Automated Dehydrator is a product of Gestion Pty Ltd., from Australia; The PowerPac Basic Electrophoresis System is a product of Bio-Rad Laboratories, Inc. from the United States; The malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) concentration assay kits are products of Nanjing Jiancheng Bioengineering Research Institute; The Enzyme-Linked Immunosorbent Assay (ELISA) kits for mouse Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-17 (IL-17), Interleukin-23 (IL-23) and Interferon-Gamma (IFN-γ) are products of Wuhan Boster Biological Technology Co., Ltd.

Grouping, modeling and drug administration

Grouping: Mice were divided into a control group, a model group and Reduning group according to the random number table method, with 10 mice in each group.

Establishment of RSV infection model: In the model group and Reduning group, mice were slowly dripped with RSV long strain viral solution, 50 µ/each, through the nostrils under light anesthesia with ether. The control group, under similar conditions, received an equal volume of virus-free normal saline via nasal drip once a day for three consecutive days. On the 3rd day after infection, administering capsaicin nebulization resulted in increased nasal and oral secretions, frequent sneezing, mouth gaping and diminished vitality in mice, indicating the successful establishment of the RSV infection model.

Drug administration: Each group was treated with drug administration on the 4th day after infection. The Reduning group was intraperitoneally injected with 0.2 mL/kg of Reduning injection and the control group and model group were intraperitoneally given an equal amount of normal saline. The treatment was continuous for 7 consecutive days.

Observational indicators and methods

Body weight and lung index of mice: Mice were weighed and recorded before administration at 10:00 am daily; on the 8th day of administration after RSV virus modeling, the lungs of mice were removed, weighed and the lung index was calculated:

$$\text{Lung index } (\%) = \frac{\text{Total lung weight of mice}}{\text{Body weight of mice}} \times 100$$

The body weight and lung index of mice on the 8th day of drug administration were compared among the three groups.

Lung tissue morphology: After execution of mice, lung tissues were taken and fixed in 4% paraformaldehyde for 24 hrs. A section of 3-5 mm in thickness, including the left lobe of the lung with the left main bronchus, was transversely cut, dehydrated, transparent, paraffin embedded, was sliced into 3 µm paraffin sections and stained with Hematoxylin-Eosin (H&E). The pathological injury of the lung tissue was observed under light microscope (Olympus CKX41-A32PH, Tokyo, Japan). Pulmonary interstitial lesion score: Based on the inflammatory cell infiltration in the small bronchi, around the venules and within the alveolar walls, a 5-point Likert scale was employed, with a score of 0-4 indicating ranging from absence to a significant amount of inflammatory cell infiltration, respectively. Bronchial epithelial lesion score: According to the degeneration and necrosis of the small bronchial epithelium, a 5-point Likert scale was used, with a score of 0-4 indicating ranging from absence to a substantial shedding of epithelial cells, respectively.

Immune function indices: As 300 µL of blood was collected from the three groups of mice via the eye socket, centrifuged at 5000 r/min for 10 min at room temperature and the supernatant was extracted. Flow cytometry was used to determine $CD3^+$, $CD4^+$ and $CD8^+$ and the $CD4^+/CD8^+$ values were calculated.

Inflammatory response indices: Following euthanasia of the mice, blunt dissection of skin tissue was performed, exposing the trachea. Subsequently, 1 mL of pre-chilled phosphate-buffered saline (PBS) was injected into the lungs using a syringe for 15 seconds, ensuring a recovery rate exceeding 80%. This procedure was repeated three times for thorough lavage. The collected bronchoalveolar lavage fluid was centrifuged at 2500 r/min for 5 min to obtain the supernatant and the levels of IL-2, IL-6, IL-17, IL-23 and IFN-γ were quantified according to the ELISA instructions.

Oxidative stress indices: Blood specimens were taken and the levels of MDA, SOD and NO were measured according to the ELISA instructions.

Cell counts: Blood specimens were taken and the fully automated modular hematology analyzer was used to determine the white blood cell count, neutrophil count, monocyte count, lymphocyte count and eosinophil count.

Western blot method for the determination of related proteins in lung tissue: Mouse lung tissue was taken and lysed in lysis buffer for 30 min at 4°C, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was collected and subjected to protein quantification using a blood cell analysis method. Subsequently, it was added with protein loading buffer and denatured in boiling water for 5 min. The denatured proteins were then subjected to 12% SDS-PAGE, transferred onto a membrane, stained with Ponceau S Staining Solution and blocked with defatted milk for 120 min. Primary antibodies including Janus Kinase 1 (JAK1), Signal Transducer and Activator of Transcription 6 (STAT6) rabbit anti-mouse polyclonal antibody (1:200) and Nerve Growth Factor (NGF) (1:1000) were added to incubate overnight at 4°C. The membrane was washed, followed by incubation with secondary antibodies for 60 min, further washing, exposure to a chemiluminescent substrate and subsequent imaging using film exposure. The β-actin was used as an internal reference and image analysis software

(Photoshop) was used to analyze the grayscale values of protein bands. The relative expression level of JAK1, STAT6 and NGF proteins was the ratio between the grayscale value of JAK1, STAT6 and NGF protein bands and the β-actin band.

Statistical analysis: The SPSS 23.0 statistical analysis software was used. Shapiro-Wilk was used to determine whether the data were normally distributed. The measurements that conformed to normal distribution were expressed as $\bar{\chi}\pm S$. Comparisons between multiple groups were made using one-way ANOVA and further pairwise comparisons were made using the LSD-t test. The $p<0.05$ was considered as the statistically significant difference.

RESULTS

Comparison of body weight and lung index among the three groups of mice: Compared with the control group, the mice in the model and Reduning groups had reduced body weight (Fig. 1a) and increased lung index (Fig. 1b) ($p<0.05$). Compared with the model group, the mice in the Reduning group exhibited higher body weight and lower lung index ($p<0.05$) (Fig. 1).

Comparison of lung tissue morphology among the three groups: The pulmonary alveolar structure of rats in the control group, as shown in Fig. 2a, exhibited clarity with thin walls devoid of congestion and the pulmonary interstitium showed an absence of inflammatory cell infiltration. In contrast, the model group revealed a diffuse thickening of alveolar septa and disruption of alveolar walls. There was varying degrees of inflammatory cell infiltration around the trachea and blood vessels. Notably, the aforementioned inflammatory conditions in the Reduning group were markedly alleviated. Compared with the control group, the model group and the Reduning group exhibited a significant elevation in pulmonary interstitial lesion scores (Fig. 2b) and bronchial epithelial lesion scores (Fig. 2c) ($p<0.05$). In comparison with the model group, the Reduning group showed lower pulmonary interstitial lesion scores and bronchial epithelial lesion scores ($p<0.05$) (Fig. 2).

Comparison of immune function indices among the three groups: Compared with the control group, $CD3^+$, $CD4^+$ and $CD4^+/CD8^+$ levels decreased, while $CD8^+$ levels were elevated in the model group and Reduning group ($p<0.05$). Compared with the model group, the Reduning group had higher $CD3^+$, $CD4^+$ and $CD4^+/CD8^+$ levels but lower $CD8^+$ levels ($p<0.05$) (Table 1).

Comparison of inflammatory response indices among the three groups: Compared with the control group, IL-2 (Fig. 3a) level was decreased, IL-6 (Fig. 3b), IL-17 (Fig. 3c) and IL-23 (Fig. 3d) levels were increased and IFN-γ (Fig. 3e) level was decreased in the model group and the Reduning group ($p<0.05$). In comparison with the model group, the Reduning group displayed higher level of IL-2 (Fig. 3a), lower levels of IL-6 (Fig. 3b), IL-17 (Fig. 3c), IL-23 (Fig. 3d) and higher level of IFN-γ (Fig. 3e) ($p<0.05$) (Fig. 3).

Comparison of oxidative stress indices among the three groups: Compared with the control group, MDA and NO levels were elevated, while SOD levels were decreased in the model group and the Reduning group ($p<0.05$). Compared with the model group, the Reduning group showed lower levels of MDA and NO but higher levels of SOD ($p<0.05$) (Table 2).

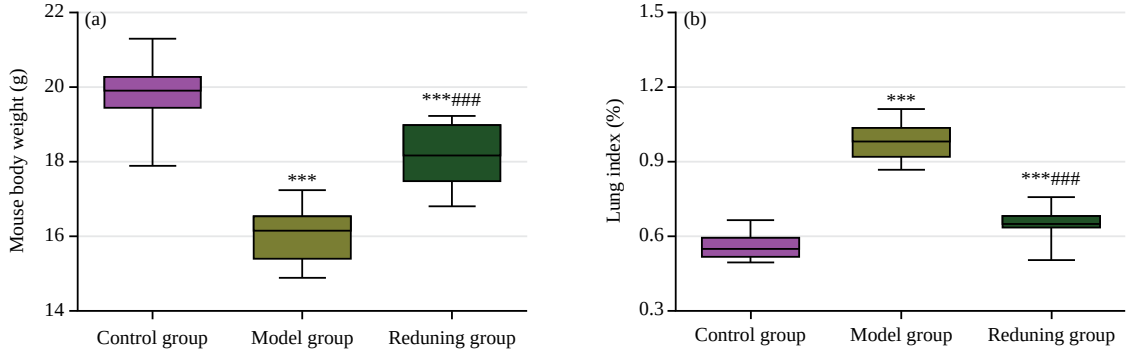

Fig. 1(a-b): Comparison of body weight and lung index among the three groups of mice, (a) Body weight and (b) Lung index
Compared with the control group, **p<0.01, ***p<0.001 and compared with the model group, ###p<0.001

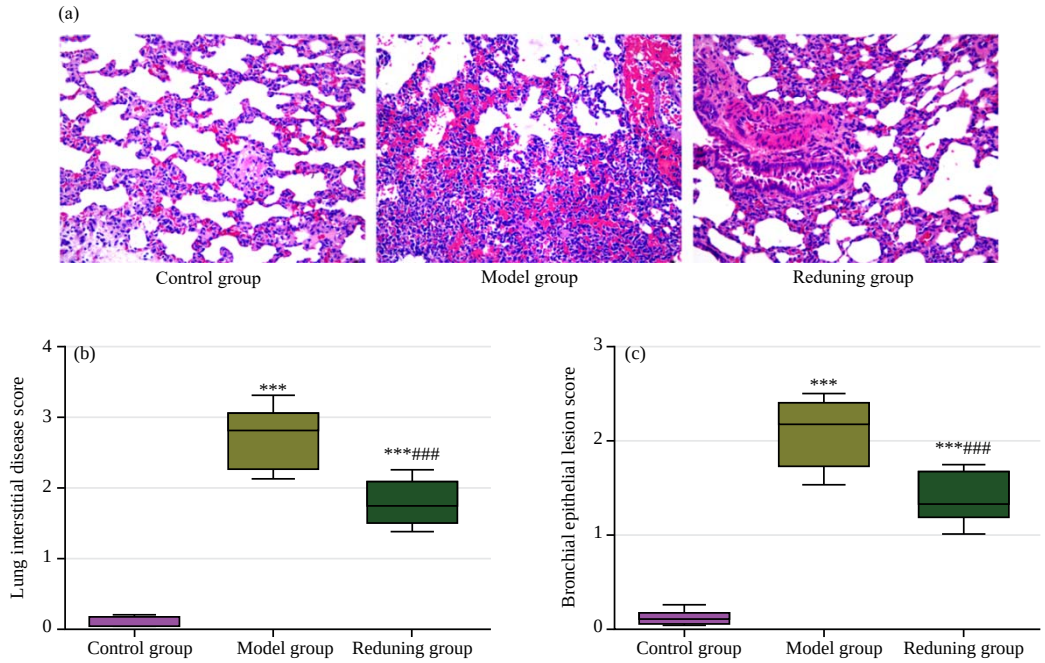

Fig. 2(a-c): Comparison of lung tissue morphology among the three groups, (a) Lung histopathological changes, (b) Pulmonary interstitial lesion score and (c) Bronchial epithelial lesion score
Compared with the control group, ***p<0.001 and compared with the model group, ###p<0.001

Table 1: Comparison of immune function indices among the three groups ($\bar{\chi}\pm S$)

Group	Number	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4^+/CD8^+$
Control group	10	53.79±4.58	48.69±7.14	21.54±1.82	2.26±0.28
Model group	10	42.03±6.12***	36.25±6.72***	29.01±2.13***	1.25±0.24***
Reduning group	10	49.28±5.34***#	42.39±5.21*#	24.84±1.73***###	1.74±0.25***###

Compared with the control group, *p<0.05, ***p<0.001 and Compared with the model group, ###p<0.001

Table 2: Comparison of oxidative stress indices among the three groups ($\bar{\chi}\pm S$, μmol/mL)

Group	Number	MDA	SOD	NO
Control group	10	15.86±2.43	502.64±83.76	24.92±3.79
Model group	10	27.21±4.68***	236.58±58.92***	63.52±9.46***
Reduning group	10	18.72±2.85*###	391.43±67.49**###	41.25±6.73***###

MDA: Malondialdehyde, SOD: Superoxide dismutase, NO: Nitric oxide, Compared with the control group, *p<0.05, **p<0.01, ***p<0.001 and Compared with the model group, ###p<0.001

Table 3: Comparison of cell counts among the three groups ($\bar{\chi}\pm S$)

Group	Number	White blood cell count ($\times 10^8$/L)	Neutrophil count (%)	Monocyte count (%)	Lymphocyte count (%)	Eosinophil count (%)
Control group	10	4.83±1.04	2.11±0.89	0.32±0.10	2.89±0.70	0.10±0.04
Model group	10	6.26±2.39	2.61±1.02	0.37±0.14	2.64±0.67	0.14±0.05
Reduning group	10	5.61±1.40	2.36±0.76	0.34±0.12	2.93±0.78	0.11±0.05

Comparison of cell counts among the three groups: There were no significant differences in white blood cell count, neutrophil count, monocyte count, lymphocyte count and eosinophil count among the three groups (p>0.05) (Table 3).

Comparison of JAK1, STAT6 and NGF proteins among the three groups: In comparison to the control group, the model group exhibited elevated levels of JAK1 (Fig. 4a-b), STAT6 (Fig. 4a and c) and NGF (Fig. 4a and d), while the NGF levels in the Reduning group also showed an increase (p<0.05). The levels of JAK1 and STAT6 in the Reduning group were lower than those in the model group (p<0.05), with no statistically significant differences observed in comparison with the control group (p>0.05) (Fig. 4).

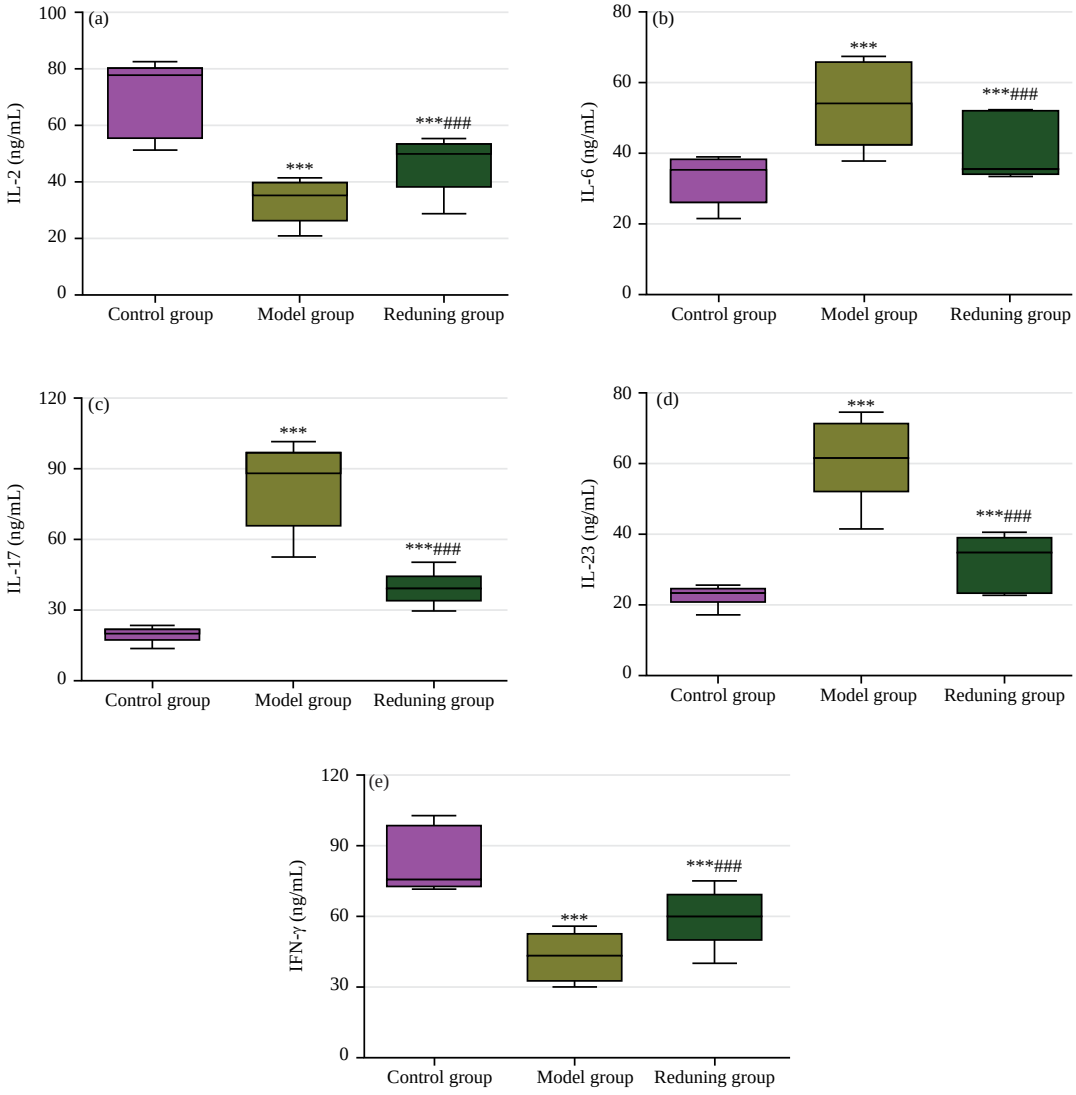

Fig. 3(a-e): Comparison of inflammatory response indices among the three groups, (a) IL-2, (b) IL-6, (c) IL-17, (d) IL-23 and (e) IFN-γ

Compared with the control group, ***p<0.001 and compared with the model group, ###p<0.001

DISCUSSION

The findings of this study demonstrated that the Reduning injection could effectively alleviate RSV-induced airway inflammation in mice and enhance immune function. Xiao-Min *et al.*[8] have pointed out that the Reduning injection is an efficacious and safe medication for treating acute upper respiratory tract infections, which is similar to the findings of the present study.

The RSV virus is a member of the negative-sense single-stranded RNA viruses, highly contagious and it invades the human respiratory epithelial cells mainly through the joint action of its G protein and F protein, disrupting normal immune function and causing disturbances in T cell subsets[9,10]. Modern medicine believes that RSV infection can lead to airway epithelial damage, airway allergic inflammation and airway hyper responsiveness[11]. According to the theory of TCM, the main pathogenesis of RNA infection is the obstruction of the lungs by exogenous evil, the interconnection of phlegm, heat and stasis and the loss of lung ventilation and purification. The lung governs all vessels; if Lung Qi stagnates, it leads to blood vessel congestion. Severe coughing may even injure the veins, resulting in blood stasis to obstruct the airways, leading to blockages in bodily fluids, the generation of phlegm, alignment with exogenous evil and resulting in disorder of Qi movement, leading to

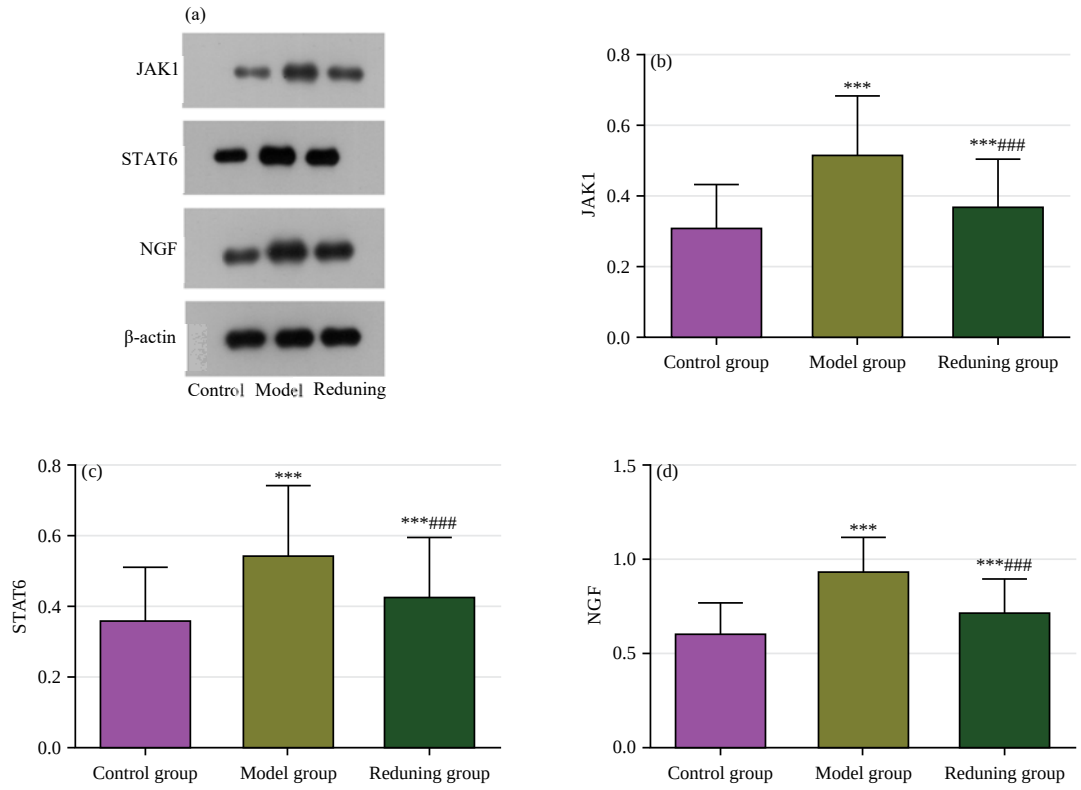

Fig. 4(a-d): Comparison of JAK1, STAT6 and NGF proteins among the three groups, (a) Western blot images, (b) JAK1, (c) STAT6 and (d) NGF

Compared with the control group, **p<0.01, ***p<0.001 and compared with the model group, ###p<0.001

coughing, wheezing and breathlessness[12]. The treatment of such diseases by Chinese medicine is manifested in the aspects of circumventing the evil Qi and protecting the vital Qi, with the advantages of improving immunity, overall regulation and multi-target action.

Reduning injection is composed of *Artemisia annua*, honeysuckle and gardenia, among which *Artemisia annua* is the sovereign drug, possessing the efficacy of diffusing the superficial layer of the muscles, clearing heat and cooling the blood and preventing malaria; honeysuckle is the minister drug, which can disperse the wind and dissipate the heat, detoxify and stop dysentery; gardenia is the assistant drug, which can play the role of clearing heat and promoting diuresis, purging fire and relieving dysphoria, cooling blood and detoxication; the combination of the three herbs exerts the role of clearing heat, cooling blood and combating viruses[13]. The present study established an RSV infection model and observed that mice infected with the RSV virus exhibited reduced body weight, substantial infiltration of inflammatory cells in the airway mucosa and a decline in immune function compared to the normal control group, similar to previous research[14]. The administration of Reduning injection on the 4th day after infection resulted in an increase in body weight and a decrease in the pulmonary interstitial lesion scores/bronchial epithelial lesion scores of the mice on the 8th day of administration, suggesting that Reduning injection can improve lung lesions in RSV-infected mice. Modern pharmacological studies have shown that artemisinin, an extract of *Artemisia annua* in Reduning injection, has a significant antimalarial effect, capable of exterminating the erythrocytic phase of plasmodium, inhibiting the growth of *Plasmodium falciparum* and at the same time, it has various degrees of bacteriostatic activity against *Staphylococcus epidermidis*, *Corynebacterium diphtheriae*, *Micrococcus catarrhalis*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* etc[15,16]. Chlorogenic acid compounds in honeysuckle have a certain inhibitory effect on a variety of pathogenic bacteria such as *Staphylococcus aureus*, dysentery bacillus, hemolytic *Streptococcus*, *Vibrio cholerae* and they also have a certain efficacy against the influenza virus and both the injectable and oral forms of honeysuckle solution demonstrate varying

degrees of antipyretic properties[17]. Gardenia mainly contains diterpenoids, flavonoids, triterpenoids, sterols and other related compounds with good antibacterial and antiviral effects[18].

It has been pointed out that RSV infection can cause disturbances in T cell subsets and reduce the body's immune function[19]. The $CD3^+$, $CD4^+$ and $CD8^+$ are important components of T-lymphocytes and the assessment of the number of immune cells can accurately reflect the immune status of the body[20]. The RSV infection also causes an increase in IL-17 and IL-23, which mediate the body's inflammatory response; Immune dysfunction may also disrupt the balance between Th1 and Th2 cells, leading to an imbalance in the secretion of their cytokines, in which Th1 cells mainly secrete IFN-γ and IL-2 and Th2 cells secrete IL-6, all of which have a variety of biological activities and whose level disruption can adversely affect the body's immune response[21,22]. In this study, it was found that T cell subsets and various inflammatory cytokines were abnormally altered in mice infected with RSV virus, which were gradually normalized after treatment with Reduning injection, suggesting that Reduning injection can be able to regulate the immune function of the body and alleviate the inflammatory response. Artemisinin can regulate Th1/Th2 cell differentiation, inhibit T-lymphocyte proliferation and antibody production and enhance immunity; geniposide can directly neutralize endotoxin, reduce the damage to cells, protect cell integrity and has anti-microbial, anti-infection efficacy, effectively resisting inflammation; the organic acids and iridoid glycosides in the honeysuckle exhibit specific immunosuppressive activity, mitigating the invasive potential of various upper respiratory tract infection viruses and demonstrating an anti-inflammatory effect.

Relevant studies have indicated that RSV infection can lead to cellular damage through oxidative stress, causing physiological and pathological changes[23]. The MDA, SOD and NO are common indicators for clinical assessment of oxidative stress and the observation of changes in their levels can effectively reflect the degree of oxidative stress in the body. In this study, RSV-infected mice had elevated levels of MDA and NO and reduced levels of SOD, which were significantly improved after treatment with Reduning injection, indicating that RSV virus can cause cellular damage by enhancing oxidative stress in the body, whereas Reduning injection can scavenge excessive oxygen free radicals and NO in the body and enhance the activity of SOD, thus alleviating cellular damage. Studies on immune mechanisms have shown that Th1/Th2 cytokine imbalance after RSV infection activates the JAK1/STAT6 signaling pathway, which mediates biological effects in the body, causing inflammatory responses, as well as affecting cell differentiation, proliferation, apoptosis and immune regulation[24]. Activation of the JAK1/STAT6 signaling pathway plays an important role in goblet cell metaplasia, leading to excessive mucus secretion of the body and inducing respiratory diseases[25]. The NGF is a bridge between the immune system and the nervous system, which can regulate neuronal cell differentiation and growth, as well as modulate the inflammatory network and the dynamic balance of the immune system[26]. The results of this study showed that the expression of JAK1, STAT6 and NGF was elevated in the model group, indicating that RSV virus can up-regulate JAK1, STAT6 and NGF proteins, activate the related signaling pathways and induce the disease. The expression of JAK1, STAT6 and NGF was reduced after treatment with Reduning injection, indicating that Reduning injection can inhibit the activation of JAK1/STAT6 signaling pathway and the expression of NGF and reduce the pathological damage of lung tissues, showcasing its promising therapeutic effects on respiratory diseases.

CONCLUSION

The Reduning injection can effectively reduce RSV-induced airway inflammation and improve immune function in mice and its mechanism of action may be related to the inhibition of oxidative stress and the regulation of JAK1, STAT6 and NGF protein expression.

SIGNIFICANCE STATEMENT

The Respiratory Syncytial Virus (RSV) is capable of inducing severe pneumonia and bronchiolitis, leading to symptoms such as wheezing and dyspnea in patients. In critical cases, it can induce heart failure, posing a threat to the health of the affected individuals. This study revealed that the Reduning injection can effectively alleviate RSV-induced airway inflammation in mice and enhance immune function. The findings provide an experimental basis for the clinical application of Reduning injection.

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