Protective Effects of Astragaloside IV Combined with Budesonide in Bronchitis in Rats by Regulation of Nrf2/Keap1 Pathway

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Background: This study was conducted to evaluate the effects of astragaloside IV and budesonide on bronchitis in rats and to explore the mechanism involved.

Material/Methods: Eighty Sprague-Dawley (SD) rats were randomly divided into 5 groups, including a Bronchitis model group (BM), a Budesonide group (BG), an Astragaloside IV group (AG), an Astragaloside IV combined with Budesonide group (CG), and a blank control group (BC). Lung tissue was stained with hematoxylin and eosin (H&E). The activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were detected by enzyme-linked immunosorbent assay (ELISA). The nuclear factor erythroid 2 [NF-E2]-related factor 2 (Nrf2), Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1 (Keap1), BTB and CNC homology 1 (Bach1), B-cell lymphoma-2(Bcl-2), and BCl-2-associated X protein (Bax) mRNA and protein were examined by RT-PCR and Western blot, respectively.

Results: Compared with the Bronchitis model group, the lung tissue lesions in the Budesonide group, Astragaloside IV group, and Astragaloside IV combined with Budesonide group were effectively ameliorated and the airway resistance was significantly decreased. The activities of SOD, GSH-Px, and CAT were increased after treatment with drugs, while the content of MDA was decreased. The levels of Nrf2, Keap1, and Bcl-2 proteins were increased and the levels of Bach1 and Bax were decreased after treatment with Budesonide and Astragaloside IV.

Conclusions: Astragaloside IV combined with budesonide can ameliorate the lesions caused by bronchitis in rats through activating the Nrf2/Keap1 pathway, which plays a protective role on anti-oxidative stress injury.

MeSH Keywords: **Bronchitis • Oxidative Stress • Signal Transduction**

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Background

Bronchitis is a condition where there is swelling and irritation in air passages. For both acute bronchitis and chronic bronchitis, medications such as antibiotics, anticholinergic agents, and bronchodilators are commonly used [1]. However, due to the distinct etiology, there is still a need find new drugs to cure bronchitis.

Astragalus has been used as a remedy for asthma, as well as in immune enhancers, hepatoprotectives, diuretics, expectorants, anti-diabetic analgesics, and sedatives [2,3]. Astragaloside IV (As-IV; 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl cycloastragenol) is a natural saponin purified from the plant *Astragalus membranaceus.* Studies have shown that astragaloside IV has anti-oxidant, anti-inflammatory, and anti-apoptotic effects [4–7]. In addition, astragalus affects the apoptosis and anti-oxidation pathways in rats with lung disease [8], mainly by regulating the expression of B-cell lymphoma-2 (Bcl-2) and BCl-2-associated X protein (Bax) [9].

Budesonide, a member of a class of drugs known as corticosteroids, is commonly used in preventing symptoms (e.g., wheezing and shortness of breath) caused by asthma. Studies have shown that budesonide can achieve anti-inflammatory effects through inhibiting basement membrane thickening, inflammatory cell aggregation and activation, and the release of inflammatory mediators [10–12]. It protects the lungs, making breathing easier by reducing irritation and swelling of the airways. However, patients with bronchial asthma are prone to recurrent asthma after being treated only with glucocorticoids.

Previous studies have shown that treatment using astragalus combined with budesonide nebulization inhalation significantly reduced the inflammatory factors of interleukin-6 (IL-6) and interleukin-17 (IL-17), while interleukin-10 (IL-10), which is an inhibition of inflammatory response, was significantly increased. Those results indicate that the combination of astragalus and budesonide is effective in improving the airway inflammatory response of these patients [13]. However, the oxidative stress and apoptosis reactions after administering the combination of astragaloside IV and budesonide in treatment of bronchitis have not been reported. The present study investigated the effects of combination therapy on oxidative stress and apoptosis and may provide a reliable basis for clinical treatment.

Material and Methods

Ethics statement

All animal experiments were performed in accordance with the Institutional Animal Care Committee of Guangdong Provincial Center for Disease Control and Prevention.

Animals

Eighty specific pathogen-free (SPF) Sprague-Dawley (SD) rats (40 males and 40 females, 6–8 weeks old, body weight 230±20 g) were provided by Guangdong Medical Experimental Animal Center. All rats were kept at 20–25°C with humidity of 40–70% and a 12 h/12 h light-dark cycle. Rats had free access to drinking water and food.

Animal model

After regular feeding for 1 week, the rats were randomly divided into 5 groups with 16 rats in each group: the Bronchitis model group (BM), the Budesonide group (BG), the Astragaloside IV group (AG), the Astragaloside IV combined with Budesonide group (CG), and the blank control group (BC). The bronchitis model was established [14]. Briefly, after mixing ovalbumin (Sigma, USA) with aluminum hydroxide solution, inactivate Bordetella pertussis vaccine (Beijing Tiantan Biological Products Co., Ltd, China) was added to prepare 1 mL of sensitization solution containing 10 mg ovalbumin, 100 mg aluminum hydroxide, and 5×10^9 inactivated Bordetella pertussis. Rats in the Bronchitis model group, Budesonide group, Astragaloside IV group, and Astragaloside IV combined with Budesonide group were intraperitoneally injected with 1 ml of sensitization solution. After 14 days, the rats were placed in a glass box (20×30×40 cm) connected to the exhalation tube of an atomizing inhaler (BARI, MASTER, Germany). The rats were continuously exposed to aerosol inhalation of ovalbumin solution for 7 days, 1 time each day and for 20 mins each time. Blank control group rats were injected with saline and exposed to aerosol inhalation of saline. In the Budesonide group, after being injected with 0.64 ml/kg budesonide (AstraZeneca, Australia), the rats were exposed to 10 ml ovalbumin solution aerosol inhalation for 15 min. Astragaloside IV (Chia Tai Youth Po Pharmaceutical Co, China) group rats were injected with 2.5 ml astragaloside IV solution, then exposed to 10 ml ovalbumin solution aerosol inhalation for 15 min. Astragaloside IV combined with Budesonide group rats were injected with 2.5 ml astragaloside IV solution and 0.64 ml/kg budesonide and exposed to 10 ml ovalbumin solution aerosol inhalation for 15 min.

Measurement of airway hyperresponsiveness

At 24 h after the last challenge, rats were anesthetized with 25% urethane (Sigma, USA) 4.0 ml/kg intraperitoneally and intubated intratracheally. The rats were placed in a sealed, body-plethysmography box. A Medlab biological signal acquisition and processing system recorder (Nanjing Medese Science and Technology Co., Ltd, Nanjing, China) was used to record the airway velocity, trans-pulmonary pressure, and tidal volume before and after aerosol inhalation of Methacholine (MCh) (Sigma, USA) in anesthetized rats. The MCh inhaled was atomized by

an ultrasonic nebulizer (BARI, MASTER, Germany). Inhalation concentration were 0.4, 0.8, 1.6, 4.0, and 8.0 g/L^{-1}, and the rats inhaled each concentration for 20 s at intervals of 5–10 min. The change of airway velocity was recorded. Airway resistance was calculated as the ratio of trans-pulmonary pressure to respiratory airflow velocity and the unit is cm H$_2$O/ml/s. Lung compliance was calculated as the ratio of mean tidal volume to mean transpulmonary pressure in units of ml/(cm H$_2$O).

Hematoxylin-eosin (HE) staining of lung tissue

The rats were anesthetized and killed by exsanguination. The right lobe was dissected and fixed in 10% formalin solution (Sigma, USA). The sections (4 µm) (Leica Microsystems GmbH, Wetzlar, Germany) were baked and deparaffinized. Hematoxylin and eosin (HE) (Beijing Solarbio Science & Technology Co., Ltd., China) staining was performed for pathological examination.

Enzyme-linked immunosorbent assay (ELISA)

The blood was collected from the abdominal aorta and spun at 4000 rpm for 10 min at 4°C. The supernatant was aliquoted and frozen at –20°C. The activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) (Sigma, USA) content were detected using ELISA kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIZOL reagent (OmegaBio-tek, Georgia, USA) according to the instructions. A single-stranded cDNA was generated using a reverse-transcription kit (Promega, USA). The amplification primers were synthesized by Shanghai Shengong Biology Company. Products were amplified with the primers shown in Table 1. PCR products were run on 1.5% agarose gel (containing 0.5 µg/ml ethidium bromide) for electrophoresis. A Bio-Rad Imager (Bio-Rad, USA) was used to take photos and analyze data. Amplification of β-actin cDNA in the same samples was used as an internal control for all PCR amplification reactions.

Western blot

Briefly, the lung tissue was dissociated with lysis buffer to extract total protein. Protein concentration was measured by BCA method. We separated 100 µg total proteins by SDS-PAGE followed by transfer to a PVDF membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Then, the membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), and subsequently incubated overnight at 4°C with the primary antibodies rabbit anti-Nrf2, -Keap1, -Bach1, -Bcl-2, and -Bax (Santa Cruz, USA, 1: 200). After washing with TBS-T for 30 min at room temperature,

Table 1. Primers sequence list.

Primer	Sequence
Nrf2 forward (5'-3')	AACCAGTGGATCTGCCAACTACTC
Nrf2 reverse (5'-3')	CTGCGCCAAAAGCTGCAT
Bach1 forward (5'-3')	AAGCTGTTCAAGCAATTTGG
Bach1 reverse (5'-3')	CTGATTCAAGATTCTGTATACAGTCAA
Keap1 forxard (5'-3')	CCTTCAGCTACACCCTGGAG
Keap1 reverse (5'-3')	AACATGGCCTTGAAGACAGG
Bax forxard (5'-3')	GACACCTGAGCTGACCTTGG
Bax reverse (5'-3')	GAGGAAGTCCAGTGTCCACC
Bcl-2 forxard (5'-3')	ATCGCTCTGTGGATGACTGAGTAC
Bcl-2 reverse (5'-3')	AGAGACAGCCAGGAGAATCAAAC
β-actin forward (5'-3')	GTCCACCTTCCAGCAGATGTG
β-actin reverse (5'-3')	GCATTTGCGGTGGACGAT

the membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit IgG (Sigma, USA, 1: 5,000) for 1.5 h at 37°C. Finally, protein bands were visualized with Amersham ECL substrates. The relative abundances of target proteins were measured by image analysis. β-actin (Sigma, USA, 1: 1000) served as the internal reference.

Statistics

Results were analyzed using SPSS 19.0 statistical software. All data are shown as mean ± standard deviation (SD). One-way ANOVA followed by LSD post-test was used. $p<0.05$ was considered to be statistically significant.

Results

Airway resistance and pulmonary compliance changes

As shown in Figure 1, the MCh responsiveness of the bronchitis model group was significantly enhanced under MCh inhalation stimulation of 1.6, 4.0, and 8.0 g/l concentration, airway resistance significantly increased at the dose of 0.8 g/L, and lung compliance decreased significantly (p<0.05). Compared with the blank control group, the Budesonide group and Astragaloside IV group had significantly increased airway resistance and decreased pulmonary compliance at 4.0 and 8.0 g/L concentrations of MCh (p<0.05). Compared with the Bronchitis model and Astragaloside IV groups, the Astragaloside IV combined with Budesonide group had significantly decreased airway resistance and increase pulmonary compliance when MCh was inhaled (p<0.05).
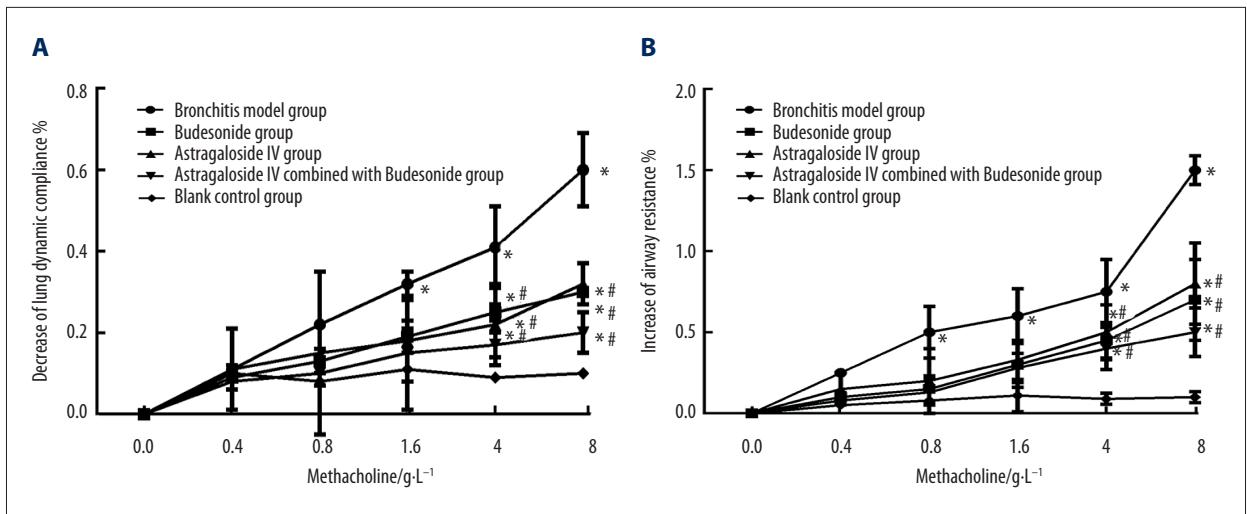
Figure 1. (**A–B**) Methacholine-induced increase of lung resistance (RL) and decrease of lung compliance (Cdyn) in groups. * p<0.05, compared with blank control group; # p<0.05, compared with Bronchitis model group.
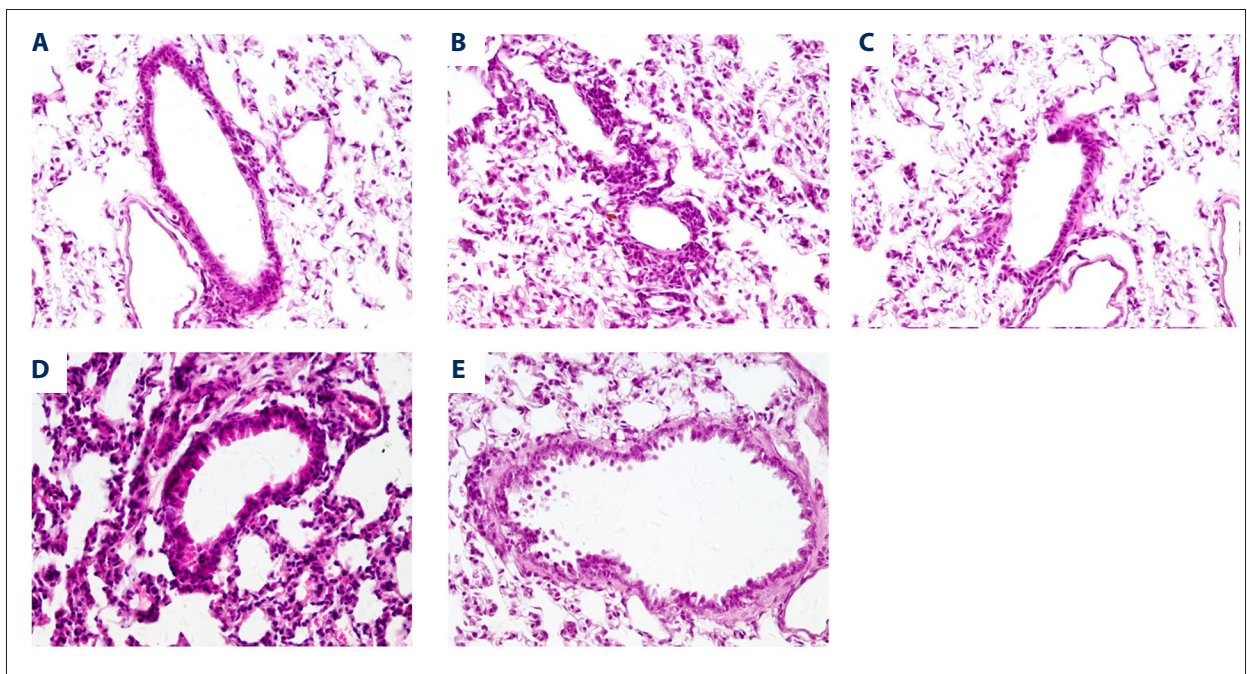
Figure 2. HE staining of airway tissues. (**A**) Blank control group. (**B**) Bronchitis group. (**C**) Budesonide group. (**D**) Astragaloside IV group. (**E**) Astragaloside IV combination with budesonide group (400×). The drug groups, especially the combination group, significantly improved the pathological structure of airway tissue.

Histological evaluations of airway tissue after treatment

We examined the airway wall tissues microscopically after H&E staining. As shown in Figure 2, the alveolar structure in the blank control group was clear and there was little collagen deposition in the airway wall (Figure 2A). In the bronchitis model group, we observed that the deposition was significantly increased, with an extensive distribution in the airway wall (Figure 2B). Collagen deposition in the rats treated with astragaloside or budesonide was found to be significantly decreased (Figure 2C, 2D). However, the pathological changes of airway tissue in the Astragaloside IV combined with budesonide group produced the best result (Figure 2E).

Effect of Astragaloside IV and budesonide treatment on oxidation factor

Since the anti-oxidative reaction is important after the combination of astragaloside IV and budesonide, the activity of CAT,
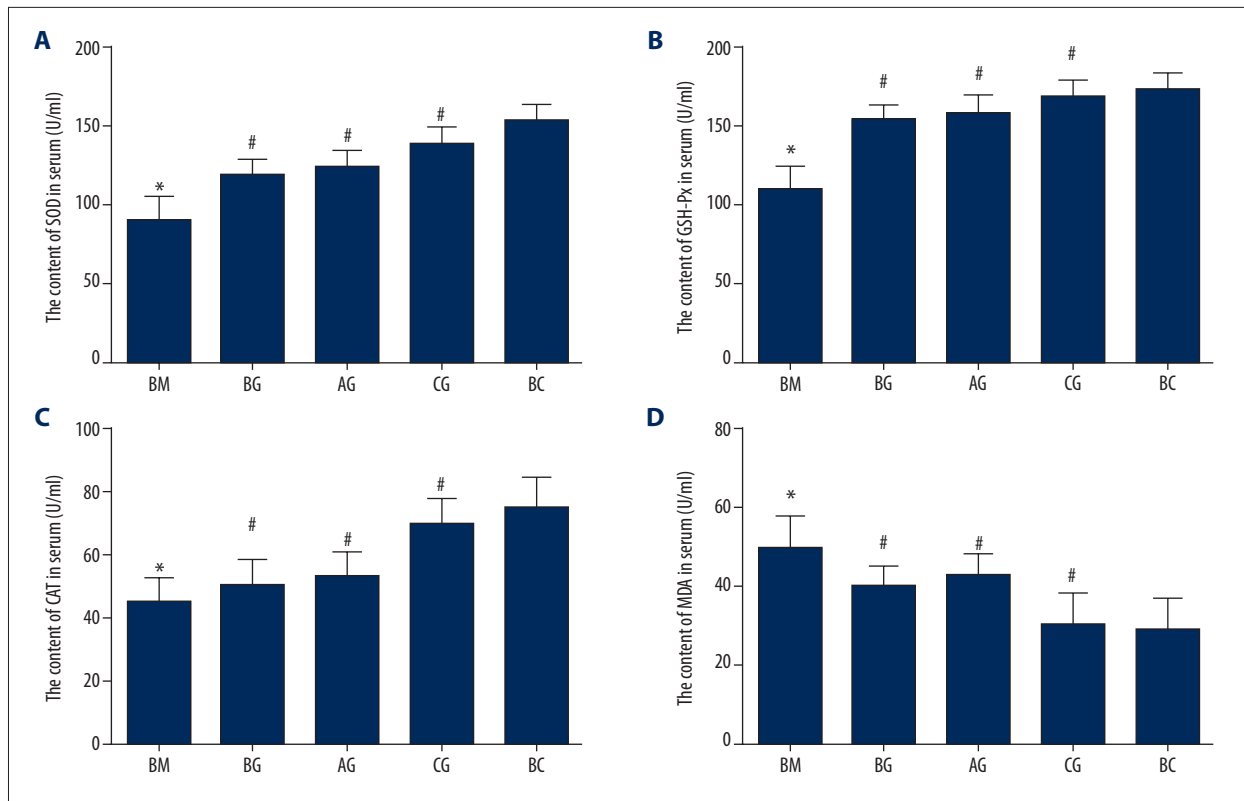
Figure 3. Comparison of (**A**): SOD, (**B**): GSH-Px, (**C**): CAT, (**D**): MDA in serum in Bronchitis model group (BM), Budesonide group (BG), Astragaloside IV group (AG), Astragaloside IV combined with Budesonide group (CG), and blank control group (BC). * p<0.05, compared with blank control group; # p<0.05, compared with Bronchitis model group.

SOD, GSH-Px, and the MDA content in lungs were measured using ELISA (Figure 3). Compared with the blank control group, SOD, GSH-Px, and CAT activity decreased in the Bronchitis model group, while the MDA content was increased (p<0.05). Compared with the Bronchitis model group, the activity of SOD, GSH-Px, and CAT increased, while MDA content was significantly decreased in the Budesonide group, Astragaloside IV group, and Astragaloside IV combined with Budesonide group, which displayed the same trend (p<0.05). The results suggest that both budesonide and astragaloside IV had the effects of increasing SOD, GSH-Px, and CAT activity and decreasing MDA content in blood. Compared with the Budesonide group and Astragaloside IV group, the activities of SOD, GSH-Px, and CAT in the Astragaloside IV combined with Budesonide group were increased and the content of MDA was decreased, which indicates that astragaloside IV combined with budesonide treatment can enhance anti-oxidation effects in rats.

The drug combination protects lung tissue by regulating the Nrf2/Keap1 pathway

The Nrf2/Keap1 signaling pathway is very important to the modulation of cellular defense mechanisms against oxidative stress; therefore, we detected the Nrf2/Keap1 pathway-related

factors Nrf2, Keap1, Bach1 mRNA, and protein expression. The results were shown in Figure 4. Compared with blank control group, Nrf2 and Keap1 mRNA were decreased while Bach1 was increased in the Bronchitis model group (p<0.05). After being treated with budesonide or/and astragaloside IV, the levels of Nrf2 and Keap1 mRNA were obviously increased, while the levels of Bach1 mRNA decreased (p<0.05). The changed trend of protein expression was consistent with the mRNA expression in the Budesonide group, Astragaloside IV group, and Astragaloside IV combined with Budesonide group (Figure 4). In the drug combination group, the change was the strongest. The results suggest that astragaloside IV combined with budesonide protects against lung injury by activating the Nrf2/Keap1 signaling pathway.

Astragaloside IV and Budesonide affected the apoptosis pathway in lung disease

To investigate the expression of apoptosis protein in rats, we examined the expression of the apoptosis-related factors Bcl-2 and Bax (Figure 5). Compared to control rats, the expression of Bax increased and Bcl2 decreased in the bronchitis rats (p<0.05). However, compared with the BM group, the expression level of Bax in the drug treatment groups declined relatively, especially
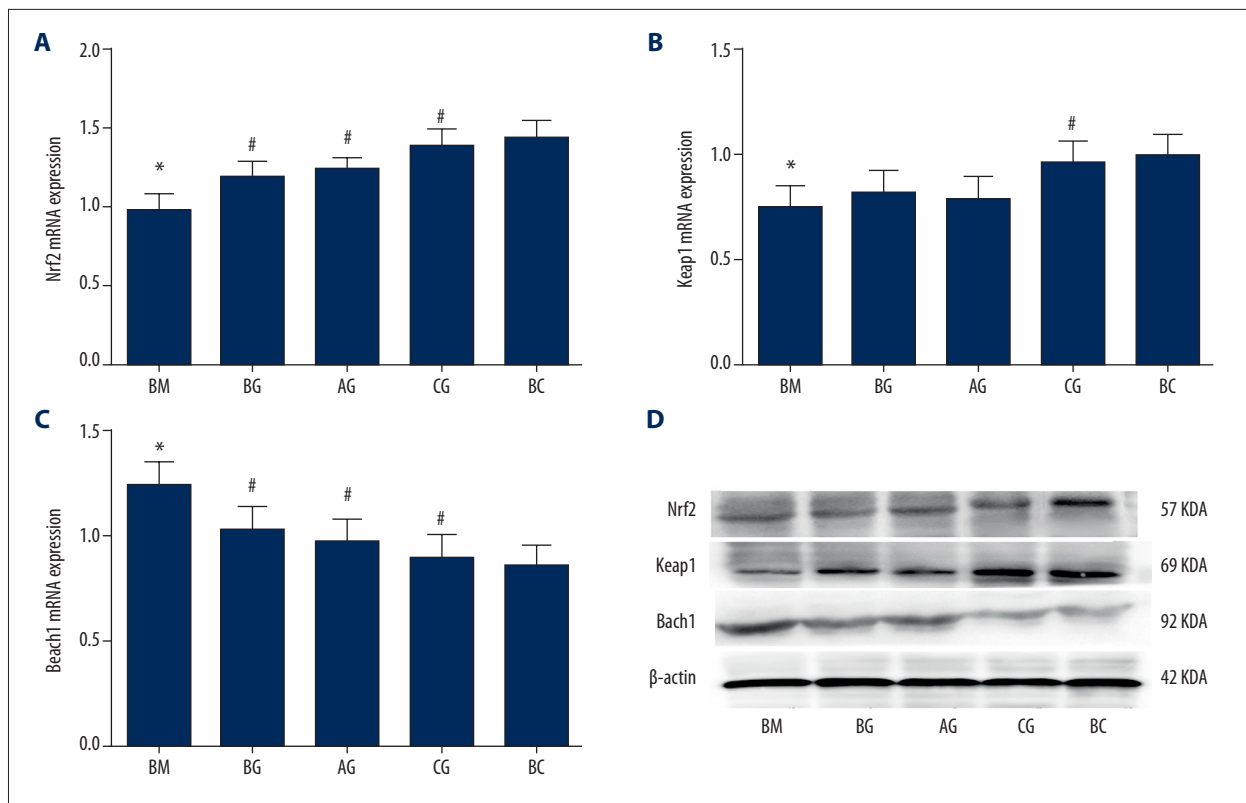
Figure 4. The expression of (**A**): Nrf2, (**B**): Keap1, and (**C**): Bach1 mRNA in the lung tissue, (**D**): The protein levels of Nrf2, Keap1, and Bach1 were detected by Western blot. Bronchitis model group (BM), Budesonide group (BG), Astragaloside IV group (AG), Astragaloside IV combined with Budesonide group (CG) and blank control group (BC) (6 rats in each group). Data are expressed as the mean ±SD. * $p < 0.05$, compared with blank control group; # $p < 0.05$, compared with Bronchitis model group.

in the CG group ($p < 0.05$), but the expression of Bcl-2 had the opposite trend in drug treatment groups.

Discussion

From the above investigation, we revealed that the efficacy of the combination of astragaloside IV with budesonide treatment was more effective than individual budesonide treatment in protecting lung functions from bronchitis. Specifically, the combination treatment rescued the lung structural damage, activated anti-oxidative gene expressions of SOD, GSH-Px, CAT, Nrf2, and Keap1, and inhibited the expression of apoptosis-related genes such as Bcl-2 and Bax.

Oxidative stress plays a key role in the pathogenesis of various diseases, which can cause an imbalance between free radical generation and anti-oxidant defenses, and it is related to the occurrence of many diseases by damaging lipids, proteins, and DNA and inactivating the anti-oxidant enzymes [15]. MDA is the end-product of the oxygen-derived free radicals and lipid oxidation, which reflects the damage caused by reactive oxygen species [16]. CAT, SOD, and GSH-Px are important endogenous

anti-oxidative enzymes that provide cellular protection against the apoptosis caused by oxygen-derived free radicals [17–19]. Anti-oxidants protect the cells and tissues from oxidative stress by scavenging free radicals and reactive oxygen species. These anti-oxidants may be endogenous or exogenous. Astragalus constituents of the dried roots of Astragalus (*Radix astragali*), provide significant protection against heart, brain, kidney, intestine, liver, and lung injury in various models of oxidative stress-related diseases [20,21]. Astragaloside IV is a natural saponin purified from *Astragalus membranaceus*. However, no studies have reported the effect of each ingredient of astragaloside IV, or combinations the various ingredients of astragaloside IV with budesonide treatment on lung disease or bronchitis, which deserves further investigation. In this study, the results suggested that the activities of SOD, GSH-Px, and CAT in the Astragaloside IV combined with Budesonide group are increased and the content of MDA is decreased, which indicates that astragaloside IV combined with budesonide treatment can enhance the anti-oxidation action in rats.

Numerous anti-oxidative pathways are involved in the redox balance of cells, of which the nuclear factor-E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) signaling
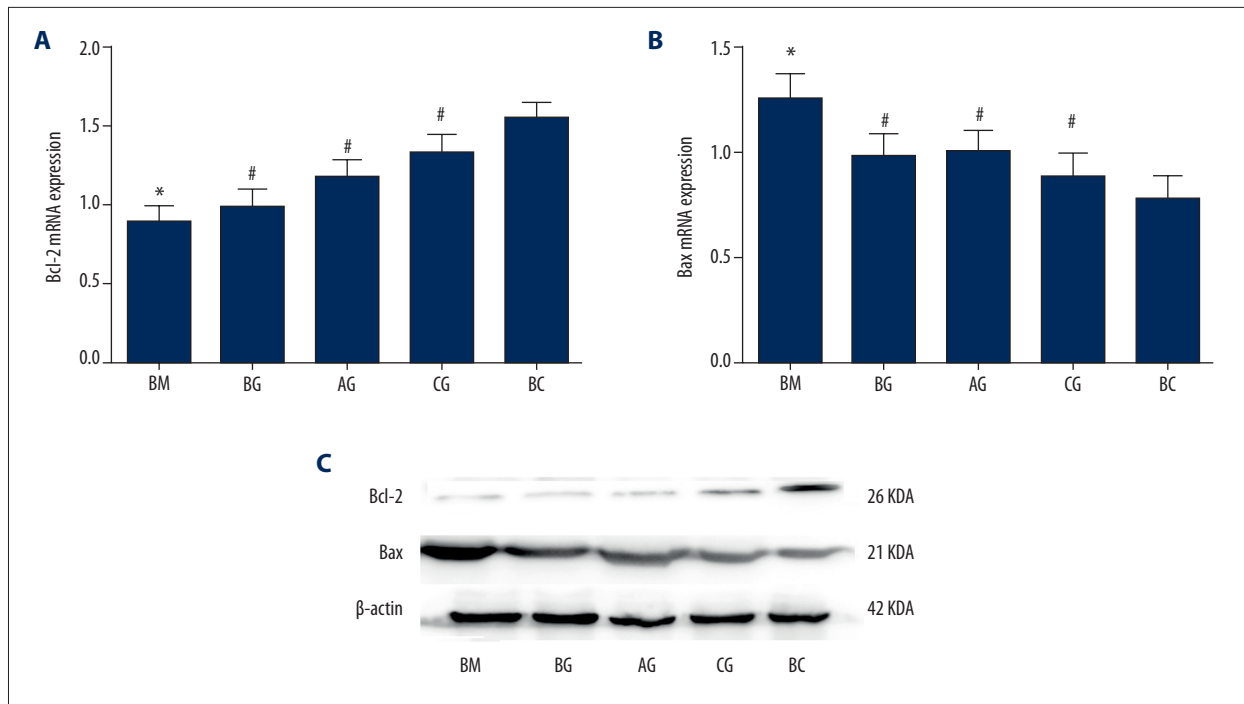
Figure 5. The expression of (**A**): Bcl-2, (**B**): Bax mRNA in the lung tissue, (**C**): The protein levels of Bcl-2 and Bax were detected by Western blot. Bronchitis model group (BM), Budesonide group (BG), Astragaloside IV group (AG), Astragaloside IV combined with Budesonide group (CG), and blank control group (BC). Data are expressed as the mean ±SD. * $p<0.05$, compared with blank control group; # $p<0.05$, compared with Bronchitis model group.

pathway may be the most prominent [22–24]. It has been shown that, in healthy cells, Keap1 binds Nrf2, and targets Nrf2 to be ubiquitinated and degraded by the proteasome [25,26]. Then, in response to increased oxidative stress, cysteine thiols on Keap1 are modified, allowing Nrf2 to be released from Keap1 and activating transcription of target genes [27]. In humans, the Keap1/Nrf2 pathway is frequently disrupted in lung cancer [28–30]. Our data showed that the Nrf2/Keap1 signaling pathway is involved in the bronchitis stress, and the combination of Astragaloside IV with budesonide treatment upregulated Nrf2-Keap1 expression and downregulated Bach1 expression, suggesting that the Nrf2/Keap1 axis modulates the oxidative reaction in bronchitis.

The Bcl-2 family has been discovered to play a key role in promoting or inhibiting intrinsic apoptotic pathways triggered by mitochondrial dysfunction [31,32]. Therefore, the balance between pro- and anti-apoptotic members of this family can determine the cellular fate. Bax promotes cell death through

permeabilization of mitochondrial outer membrane in response to different cellular stresses. In contrast, Bcl-2 prevents apoptosis by inhibiting the activity of Bax [33]. In our experiments, we observed that after drug treatment, the expression level of Bax decreased and bcl-2 expression increased, suggesting that astragaloside IV and budesonide inhibited cell apoptosis. In general, this study addressed the anti-oxidant and apoptosis effect of combination of astragaloside IV with budesonide treatment on bronchitis, which may help elucidate the underlying mechanism and may provide a basis for clinical therapy.

Conclusions

Our study showed that astragaloside IV combined with budesonide has a protective effect on the treatment of bronchitis in rats. The function of the drugs in combination may be have an anti-oxidant effect through activating the Nrf2/Keap1 pathway.

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