ORIGINAL ARTICLE

Anti-Inflammatory Effects of Reduning Injection (热毒宁注射液) on Lipopolysaccharide-Induced Acute Lung Injury of Rats[*]

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ABSTRACT **Objective:** To evaluate the protective effects of Reduning Injection (热毒宁注射液, RDN), a patent Chinese medicine, on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats and its underlying mechanisms of action. **Methods:** Sixty male Sprague-Dawley rats were randomly divided into 6 groups, including normal control, model, dexamethasone (DEX, 5 mg/kg), RDN-H (720 mg/kg), RDN-M (360 mg/kg) and RDN-L (180 mg/kg) groups, with 10 rats in each group. Rats were challenged with intravenous injection of LPS 1 h after intraperitoneal treatment with RDN or DEX. At 6 h after LPS challenge, lung tissues and bronchoalveolar lavage fluid (BALF) were collected, and the number of inflammatory cells was determined. The right lungs were collected for histopathologic examination, measurement of gene and protein expressions, superoxide dismutase (SOD) and myeloperoxidase (MPO) activities. **Results:** In vivo pretreatment of RDN (360, 720 mg/kg) significantly reduced the weight of wet to dry (W/D) ratio of lung, protein content in BALF, and led to remarkable attenuation of LPS-induced histopathological changes in the lungs. Meanwhile, RDN enormously decreased BALF total inflammatory cells, especially neutrophil and macrophage cell numbers. Moreover, RDN increased SOD activity, inhibited MPO activity, alleviated LPS-induced tumor neurosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) expression in lung tissues. Furthermore, RDN (720 mg/kg) efficiently weakened nuclear factor-kappa B (NF-κB) gene and protein expression. **Conclusion:** Anti-inflammatory effects of RDN was demonstrated to be preventing pulmonary neutrophil infiltration, lowering MPO activity, TNF-α and iNOS gene expression by inhibiting NF-κB activity in LPS-induced ALI.

KEYWORDS acute lung injury, lipopolysaccharide, neutrophils, chemokine, nuclear factor-kappa B, Reduning Injection, Chinese patent medicine

Acute lung injury (ALI) is a clinical complication mainly due to gram-negative bacterial infection and an important cause of morbidity and mortality in critically ill patients.[1] Lipopolysaccharide (LPS), major composition of the outermost membrane of gram-negative bacteria, is recognized as a key factor in the pathogenesis of ALI,[2] which even can cause acute respiratory distress syndrome (ARDS).[3] Intravenous administration of LPS has been widely used as an experimental animal model of ALI/ARDS.[4] LPS-induced ALI is characterized by the infiltration of neutrophils and macrophages into alveoli,[5] the release of chemokines and pro-inflammatory cytokines from activated macrophages and neutrophils,[6,7] as well as the increase of protein content in the bronchoalveolar lavage fluid (BALF).[8] Among these pro-inflammatory cytokines, tumor necrosis factor α (TNF-α), which participates in both early and later inflammatory responses, plays a critical role in the progression of ALI.[9,10] The excessive accumulation of inflammatory mediators, especially cytokines, chemokines, adhesion molecules, and bioactive lipid products[11,12] contribute to the increased pulmonary vascular permeability, interstitial edema, disruption of epithelial integrity, and lung parenchymal injury.[13,14] Numerous studies have suggested that nuclear factor-kappa B (NF-κB) is activated after LPS exposure to the experimental animal. Activated NF-κB plays a key role in the regulation of inflammatory mediator

transcription,[15] which contributes to the vigorous acquisition of neutrophils in the lung and is crucial to the pathogenesis of ALI.[16]

LPS challenge also activates neutrophils and macrophages within the lung vasculature, which exaggerates production of reactive oxygen species (ROS), including hydroxyl radical, hydrogen peroxide and superoxide anion radical.[17,18] They are believed to contribute to the severity of several diseases, including ALI and ARDS. The increased ROS will induce lipid peroxidation and alter antioxidant enzyme activity,[17,19] resulting in increased pulmonary vascular permeability and the release of inflammatory cell in BALF.[20] Thus, ROS is believed to be crucial in the induction of lung damage caused by pneumonia, while therapeutic agents that could effectively scavenge ROS may prevent or reduce the deleterious effects of pneumonia.

Reduning Injection (热毒宁注射液, RDN) is a patented Chinese medicine prepared by effective components from the extract of *Artemisia annua*, *Gardenia jasminoides Ellis*, and *Flos Lonicerae*. It has been widely used to treat common cold, cough, acute upper respiratory infection (AURI) and acute bronchitis in clinic. Clinical studies reported that RDN was safe and effective in curing pneumonia in the elderly population[21] and also had good therapeutic effect to AURI in children.[22] At the same time, it was reported to be used as a new drug for prophylactic treatment to *Staphylococcus aureus*, *Klebsiella pneumoniae* and influenza virus in clinic.[23] However, there is little experimental data regarding the effects of RDN on inflammation. The present study was undertaken in rats to evaluate the effects of RDN on ALI induced by bacterial LPS through examining cellular infiltration and histological changes in the lungs.

METHODS

Drugs and Reagents

RDN (Batch No. 100906) was provided by Jiangsu Kanion Pharmaceutical Co., Ltd. (Jiangsu, China). Its chemical pattern was obtained by high-performance liquid chromatography (HPLC) analysis. The injection was submitted to Dianon HP-20 Toyopearl HW40 (Mitsubishi, Japan) and preparative HPLC chromatographic columns (Silicycle Canada), yielding compounds of the main peaks in the HPLC fingerprint. The structures of these compounds were identified by spectrophotometer (Waters, USA), mass spectrometer (Bruker, USA), and nuclear magnetic resonance imaging (NMR) spectrometer (Bruker, USA). As illustrated in Figure 1, the compounds of the main peaks on the HPLC fingerprint were identified as neochlorogenic acid (peak 1, t_R 8.3 min), chlorogenic acid (peak 2, t_R 13.0 min), cryptochlorogenic acid (peak 3, t_R 14.4 min), secologanic acid (peak 4, t_R 15.8 min), caffeic acid (peak 5, t_R 17.1 min), geniposide (peak 6, t_R 18.6 min), secologanin (peak 7, t_R 26.1 min), isochlorogenic acid B (peak 8, t_R 29.2 min), isochlorogenic acid C (peak 9, t_R 29.0 min), and isochlorogenic acid A (peak 10, t_R 35.1 min).

LPS (*E. Coli* L6511) was purchased from Sigma Chemical (St. Louis, USA). The kits used to determine superoxide dismutase (SOD) and myeloperoxidase (MPO) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dexamethasone sodium phosphate injection (DEX) was purchased from Guangzhou Baiyunshan Tianxin Pharmaceutical Co., Ltd. (Guangdong, China).

Experimental Animals and Treatment

Sixty adult male Sprague-Dawley rats (specific pathogen-free grade, weighing 180–200 g) were purchased from the Center of Experimental Animals of Southern Medical University (Guangdong, China, certificate No. SCXK 2006-0015). The rats were kept in a room with temperature maintained at 25 ± 1 ℃ and an alternating 12 h light-dark cycle. Animals were stratified randomly according to body weight and divided into 6 groups, which were normal control, model, DEX (5 mg/kg), RDN-H (720 mg/kg), RDN-M (360 mg/kg) and RDN-L (180 mg/kg) groups, with 10 animals in each group. Animals were anesthetized with an inhalation of diethyl ether, and then LPS (5 mg/kg) or saline was injected intravenously via the tail vein to induce lung injury. LPS was dissolved in saline shortly before use. One hour before LPS challenge, saline, DEX and RDN were injected intraperitoneally to respective groups. Six hours after LPS treatment, the animals were sacrificed with intraperitoneal injection of 10% chloral hydrate. BALF and lung samples were immediately collected. All animal care and experimental procedures were approved by the Animal Care and Use Committee of Jinan University and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Determination of the Number of Inflammatory Cells (Macrophages and Neutrophils) and Protein Contents in BALF

Following euthanasia, left lungs were lavaged three times with a total volume of 5 mL sterilized phosphate balanced solution (PBS). The recovered BALF was centrifuged at 825×g for 10 min at 4 ℃ to collect cell pellets. The cell pellets were resuspended in 50 μL PBS to determine the number of inflammatory cells using a Z2 coulter particle count and size analyzer (Beckman, USA). The machine can classify the macrophages and neutrophils by diameter of the cells and record total cell number as well as single type of cell number. The amount of proteins in the supernatant of the BALF was assayed by Bradford assay. Protein concentration was expressed in milligram protein per milliliter BALF.

Measurement of Lung Wet/Dry Weight Ratio

The water content of lungs was determined by calculating the wet/dry (W/D) weight ratio. The left lung was slightly rinsed in PBS, dried, and then weighed to obtain the "wet" weight. The tissue was then dried in an oven at 80 ℃ for 72 h to obtain the "dry" weight. The W/D ratio was calculated to assess tissue edema.

Histopathologic Examination

The right lungs were fixed in 4% paraformaldehyde for more than 48 h. After routine processing and paraffin embedding, each sample was sliced into thin sections (4 μm) and stained with hematoxylin and eosin (HE). The severity of lung injury was evaluated by examining interstitial inflammation, inflammatory cell infiltration, and tissue edema using a Olympus microscope (Olympus, Japan).

Measurement of SOD and MPO Activities

Lung was homogenized in normal saline using a homogenizer. The activities of SOD and MPO in 10% lung homogenate were determined using assay kits. All procedures were performed according to the manufacturer's instructions. The activity of MPO in the homogenates was determined using o-dianisidine as peroxidase substrates. Its absorbance was read on a spectrophotometer at 460 nm. The data were presented as U/g lung tissue. Total SOD activity, including Cu-Zn SOD activity and Mn SOD activity, was determined by hydroxylamine assay developed from xanthine oxidase.
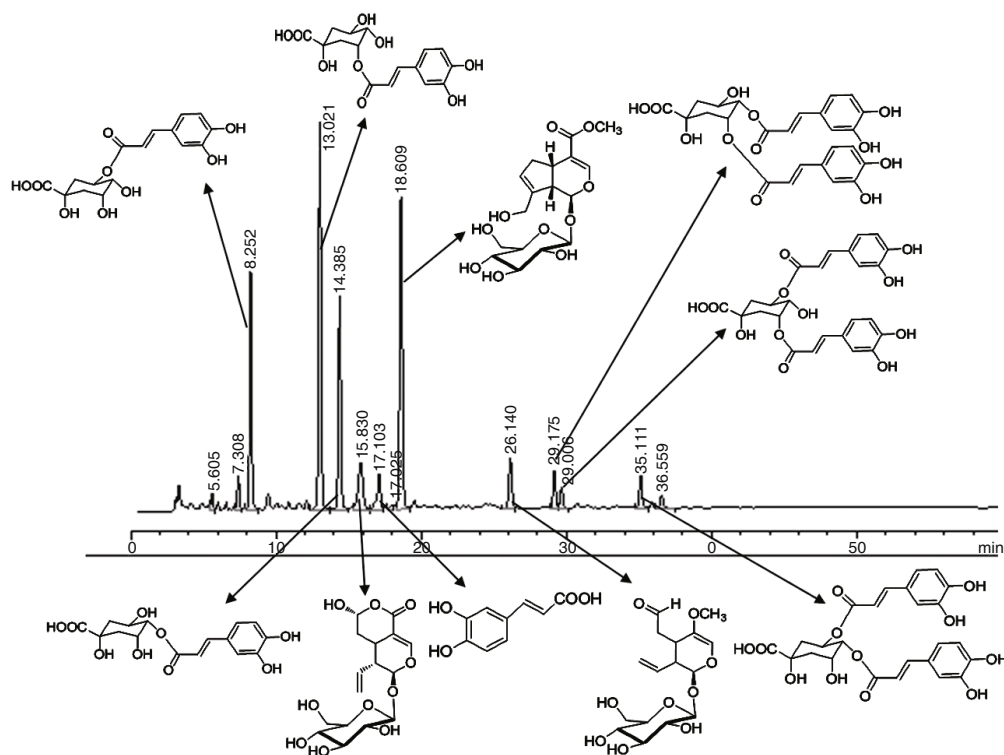

Figure 1. Chemical Profile of RDN Analyzed by HPLC
Notes: Several batches of RDN were analyzed by HPLC with UV detection at 225 nm, and similar profiles were obtained. The analysis was performed with a Welchrom TM XB-C$_{18}$ column (4.6 mm×250 mm, Welch Materials, USA) at 35 ℃. The compounds were eluted (eluent A, 0.1% phosphoric acid in water; eluent B, 0.1% phosphoric acid in methanol) at a flow rate of 0.8 mL/min using a gradient program (eluent B content linear gradient from 20% B to 60% B in 50 min, and 60% for 10 min)

Reaction mixture was allowed to stand at room temperature for 30 min before its absorbance being read on a spectrophotometer at 560 nm. The enzyme activity was expressed as U/mg protein.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was isolated from lung tissue by TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by optical density measurement at 260 nm on a spectrophotometer (Thermo, USA). RNA (3 μg) was reversely transcribed with M-MLV reverse transcriptase, and a master mix containing reaction buffer, dNTPs, Taq polymerase, and 1 μL cDNA underwent polymerase chain reaction (PCR) in 25 μL reaction system. The size of amplified PCR products was subjected to electrophoresis on a 1% agarose gel (Invitrogen, CA, USA) for about 30 min and were analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA) with 18S as a normalization control. PCR primers for TNF-α, inducible nitric oxide synthase (iNOS), NF-κB and 18S contained the following sequences: TNF-α sense (5'-TTATCGTCTACTCCTCAG-3') and antisense (5'-ACTACTTCAGCGTCTC-3'), NF-κB sense (5'-TGGAGCAAGCCATTAG-3') and antisense (5'-GGGCACGGTTATCAA-3'), iNOS sense (5'-TTTGTGGCGATGTGC-3') and antisense (5'-GAGTCTTGTGCCTTTGG-3'), 18S sense (5'-GGGAGAGCGGGTAAGAGA-3') and antisense (5'-ACAGGACTAGGCGGAACA-3').

Western Blot Analysis

The lungs were removed and proteins were extracted with lysis buffer (Beyotime, China) for 30 min on ice. The cytosol and nuclear extracts were harvested after 13,201 × g centrifugation at 4 ℃ for 10 min. Total protein concentrations of lungs were determined by Bradford assay. The extracts were placed in a boiling water bath for 5 min with loading buffer. Protein samples were subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h at room temperature with 3% balanced scoreinteriord approach (BSA) in tris buffered saline, with tween-20 (Tris-NaCl-Tween20, TBST) and then incubated with a 1:1000 dilution of primary antibody (NF-κB p65 mouse mAb) in blocking buffer overnight at 4 ℃. After three washes with TBST

for 10 min, a 1:1000 dilution of secondary antibody (goat anti-mouse IgG) was added and incubated for 1 h at room temperature. After three further washes, the blots were developed using enhanced chemiluminescence reagent and exposed to a film.

Statistical Analysis

The data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analyses were carried out using a nonparametric one-way ANOVA of the SPSS 13.0. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Effects of RDN on Lung W/D Ratio and BALF Protein Content in Rats with LPS-Induced ALI

To quantify the severity of pulmonary edema and lung microvascular permeability, the lung W/D ratio and protein content in BALF of ALI rat was evaluated. As shown in Figure 2, compared with the normal control group, the lung W/D ratio and the total protein content in the BALF were evidently increased in the model group ($P<0.01$). However, the LPS-induced increase of both lung W/D ratio and the total protein content were obviously inhibited by pretreatment of RDN (360, 720 mg/kg) and DEX ($P<0.01$ or $P<0.05$).
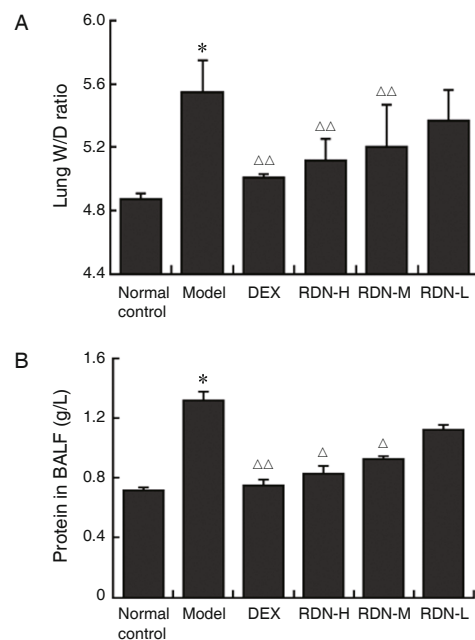

Figure 2.   Effect of RDN on the Lung W/D Ratio and Protein Concentration in the BALF of Rats with LPS-Induced ALI ($\bar{x} \pm s$)

Notes: The lung W/D ratio (A) and total protein concentration in the BALF (B) were determined at 6 h after LPS challenge. *$P<0.01$, compared with the normal control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with the model group

Effects of RDN on the Number of Inflammatory Cells in BALF of LPS-Induced ALI Rats

As shown in Figure 3, LPS induced a dramatic increase in the total number of inflammatory cells, as well as the number of neutrophils and macrophages in BALF when compared with the normal control group ($P<0.01$). However, pre-administration of RDN (360, 720 mg/kg) and DEX significantly decreased the number of neutrophils and macrophages, as well as the total number of inflammatory cells in BALF in rats treated with LPS ($P<0.05$ or $P<0.01$).

Effect of RDN on Histopathological Changes in Lungs of Rats with LPS-Induced ALI

The effect of RDN on the lungs of rats was determined by HE staining. As shown in Figure 4, alveolar structure was clear in the normal control group. However, lung tissues from rats of the model group showed marked inflammatory alterations as characterized by the presence of edema, inflammation, recruitment of neutrophils and leukocytes into the alveolar spaces. In contrast, histological damage was found relieved in all RDN-treated rats.

Effect of RDN on SOD and MPO Activities in the Lungs of Rats with LPS-Induced ALI

As shown in Figure 5, LPS challenge resulted in a significant decrease of SOD activity and an evident increase of MPO activity ($P<0.01$). However, pretreatment with RDN (360, 720 mg/kg) and DEX significantly elevated SOD activity and remarkably lowered MPO activity in the lungs of LPS-induced ALI rats ($P<0.05$ or $P<0.01$).
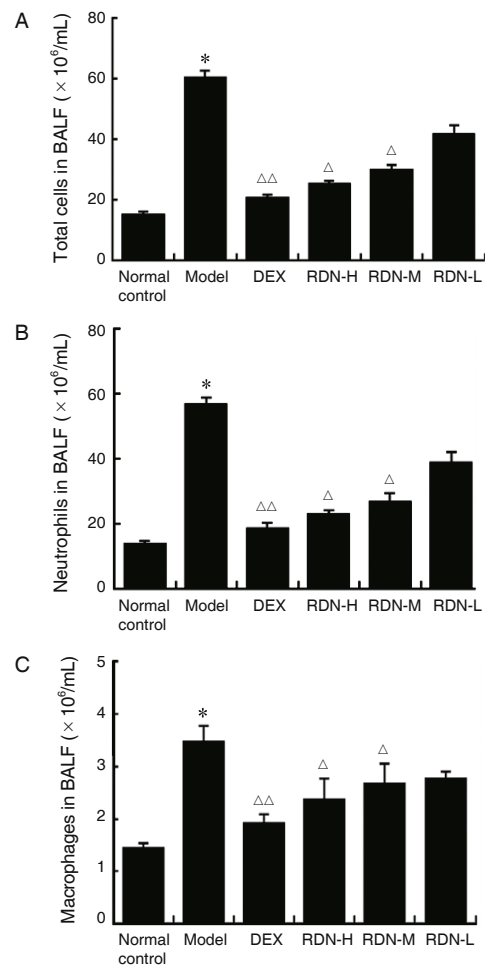

Figure 3.   Effect of RDN on Inflammatory Cell Accumulation in BALF of LPS-Induced ALI Rats ($\bar{x} \pm s$)

Notes: BALF was collected from rats 6 h after LPS challenge to measure the number of total cells (A), neutrophils (B), and macrophages (C). The results were expressed as the cell numbers for each population in 1 mL of BALF. *$P<0.01$, compared with the normal control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with the model group
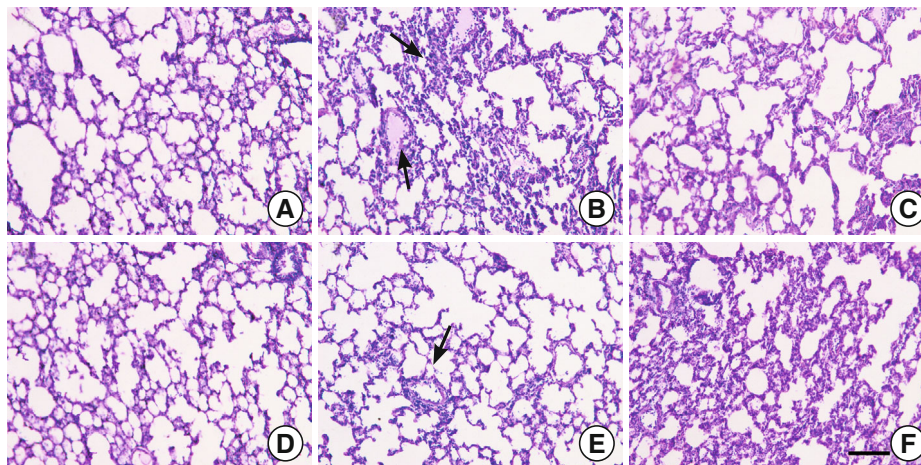

Figure 4.   Effect of RDN on Histopathological Changes in Lungs of LPS-Induced ALI Rats (HE staining, $\times 100$)

Notes: A: normal control group; B: model group; C: DEX group; D: RDN-H group; E: RDN-M group; F: RDN-L group. Arrows: inflammatory cells infiltration. Bar = 10 μm
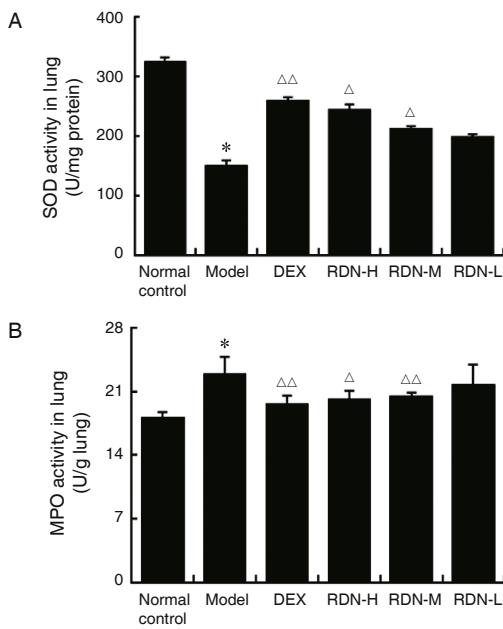
Figure 5. Effect of RDN on SOD and MPO Activities in the Lungs of Rats with LPS-Induced ALI ($\bar{x} \pm s$)

Notes: *P<0.01, compared with the normal control group; △P<0.05, △△P<0.01, compared with the model group

Effect of RDN on TNF-α, iNOS and NF-κB mRNA Expression in the Lungs of Rats with LPS-Induced ALI

As shown in Figure 6, LPS stimulation obviously elevated TNF-α, iNOS and NF-κB mRNA expressions (P<0.05 or P<0.01). RDN (360, 720 mg/kg) and DEX could significantly suppress the increase mRNA expressions of TNF-α, iNOS and NF-κB induced by LPS (P<0.05 or P<0.01).

Effect of RDN on Protein Expression of NF-κB in Lung Tissues of Rats with LPS-Induced ALI

The protein expression of NF-κB was evidently increased in the model group compared with that of the normal control group (P<0.05). RDN (720 mg/kg) and DEX significantly prevented the activation of NF-κB expression (P<0.05 or P<0.01, Figure 7).

DISCUSSION

ALI is commonly found in patients with severe illnesses and is often complicated by systemic inflammation. As a major stimulus for the release of cytokines, LPS challenge can induce inflammatory responses by activating numerous inflammatory cells, which are commonly found in ALI patients.[24] ALI is characterized by increased capillary permeability, interstitial and alveolar edema, and influx of circulating inflammatory cells.[25] In the present study, results
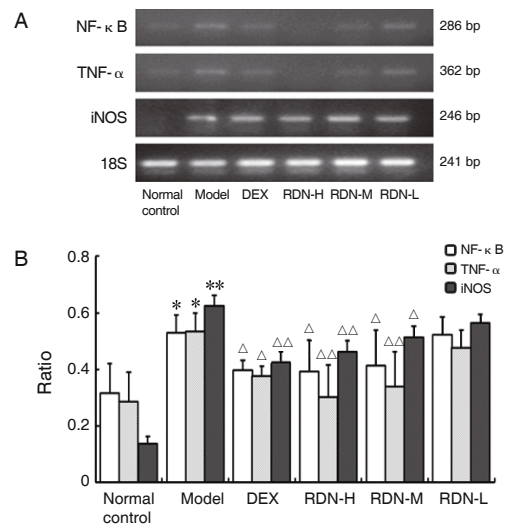

Figure 6. Effect of RDN on mRNA Expressions of TNF-α, iNOS and NF-κB in Rats with LPS-Induced ALI ($\bar{x} \pm s$)

Notes: Cytokines were measured in the lung tissues obtained 6 h after LPS challenge. *P<0.05, **P<0.01, compared with the normal control group; △P<0.05, △△P<0.01, compared with the model group

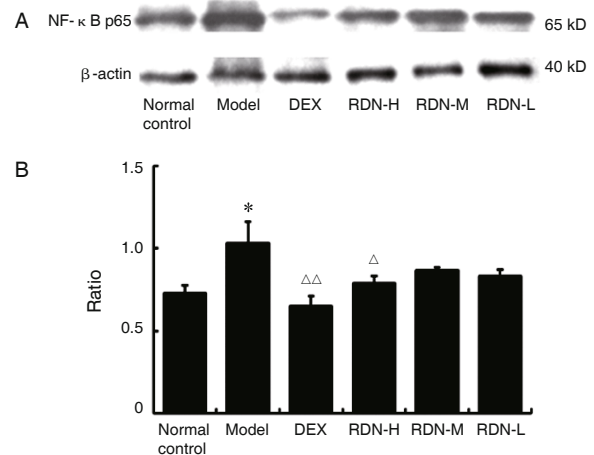**Figure 7. Effect of RDN on Protein Expression of NF-κB in Rats with LPS-Induced ALI ($\bar{x} \pm s$)**

Notes: *P<0.05, compared with the normal control group; △P<0.05, △△P<0.01, compared with the model group

showed that LPS challenge increased lung W/D ratio, inflammatory cells migration into the lung, and protein leakage in BALF. Histological examination also demonstrated that LPS exposure showed a thickening of the alveolocapillary membrane and massive extravagation of polymorphonuclear leukocytes into the alveolar spaces. The results above confirmed that LPS challenge elevated the capillary permeability and induced the pulmonary edema, which are the major characters of ALI.[26] Pretreatment with RDN greatly improved the alteration of pulmonary histology by LPS, decreased lung W/D ratio, prevented the

migration of inflammatory cells, and reduced protein content in BALF, demonstrating the alleviation effect on LPS-induced ALI.

RDN has been commonly used for relieving cough and acute bronchitis in clinic for years. HPLC analysis indicated that RDN mainly contained neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, secologanin and geniposide. High levels of chlorogenic acid and neochlorogenic acid can reduce *Monilinia laxa* infection.[27] Chlorogenic acid was also demonstrated to possess anti-inflammatory, analgesic and antipyretic activities and its isomers exhibited strong antioxidant activities.[28,29] Geniposide has been reported to protect mice against LPS-induced ALI,[30] inhibit the production of ROS and down-regulate LPS-elevated iNOS expression.[31] These active components in RDN could possibly contribute to the alleviation of lung inflammation on LPS-induced ALI. The sign of pulmonary inflammation in ALI is the presence of infiltrating leukocytes. Excessive infiltration of macrophages and neutrophils, which are predominant inflammatory cells and play key roles in the early development of ALI, is found in lung of rats challenged with LPS. When ALI occurs, neutrophils adhere to the injured capillary endothelium and migrate into the air spaces. The activated neutrophils will release oxidants, proteases, and other inflammatory mediators.[32] It is the adhesion and activation of neutrophils that causes lung injury.

MPO, an enzyme mainly located in the primary granules of neutrophils, is abundantly expressed in activated neutrophils.[33,34] The level of MPO activity can indirectly reflect the degree of inflammation. Meanwhile, macrophages are known to play important roles in LPS-induced ALI.[35] The increased microvascular permeability to endotoxin is abrogated if the animals are subjected to intravascular macrophage depletion.[36] As expected, increased protein content, massive recruitment of inflammatory cells including neutrophils and macrophages in BALF and elevated MPO activity were found in the lungs of ALI rats challenged with LPS. In contrast, pretreatment with RDN could decrease LPS-induced inflammatory cells migration into the lung, protein content and MPO activity. The results suggested that the protective effect of RDN on LPS-induced ALI was related to the attenuation of the sequestration and

migration of inflammatory cells in lung tissue.

Pro-inflammatory cytokines appear in the early phase of an inflammatory response and contribute to the severity of lung injury.[37] A network of pro-inflammatory cytokines including TNF-α and chemokines is critical for initiating, amplifying, and perpetuating the inflammatory response in ALI. LPS triggers monocytes and macrophages to produce several inflammatory cytokines and mediators, as well as biological mediators involved in the control of pathogens. TNF-α plays a key role in stimulating a variety of chemokine production and activation of neutrophils. Activated neutrophils will generate oxygen radicals,[38] which can induce lipid peroxidation and cause serious damage to cellular structure and functions.[39] Besides, the over-production of nitric oxide (NO) induced by iNOS may lead to both direct and indirect cytotoxic effects on endothelial cells via the formation of peroxynitrite, and lead to increased pulmonary microvascular permeability.[40,41] We observed that LPS challenge led to the elevation of TNF-α and iNOS gene expressions in lungs. Besides, we also found that LPS challenge decreased the activity of SOD in lungs of rats. However, pre-administration of RDN markedly reduced gene expressions of TNF-α, iNOS, and increased the activity of SOD in lungs of rats treated with LPS. These results suggested that the protective effect was due to the down-regulation of cytokine expressions in the lungs of rats.

On the other hand, NF-κB is a capital transcription factor for a number of inflammatory mediators that are involved in the pathogenesis of ALI.[42] It is normally located in the cytoplasm in an inactive form through being associated with an inhibitor of κB (IκB) protein.[43] Following activation, IκB protein breaks down and liberates NF-κB to enter the nucleus where it binds to promoter regions of target genes, and eventually induces the expressions of inflammatory cytokines and mediators.[44] In our study, the results showed that LPS induced an elevation of NF-κB gene expression and protein expression in lungs. RDN pretreatment could inhibit the activation of NF-κB induced by LPS in rat lung tissues. It was suggested that the inhibitory effects of RDN on pro-inflammatory cytokine expressions in the lung tissues of ALI rats were related to down-regulation of NF-κB activation.

In conclusion, the results of the present investigation showed that pretreatment of RDN significantly attenuated pulmonary inflammation in rats with LPS-induced ALI. The protective effect of RDN in ALI was mainly demonstrated by improvement of histological disorders in lung tissues, and decrease in microvascular leakage and inflammatory cell infiltration. The effect of RDN was believed to be the inhibition of pro-inflammatory cytokine expression through down-regulating the activation of NF-κB. On the basis of these results, further investigation is required to clarify the potential efficacy for clinical usefulness of RDN as an adjunctive therapy of ARDS.

Conflict of Interest

The authors declare no competing financial interest.

Author Contributions

Tang LP performed the experimental operations and wrote the article. Li YF modified the article. He RR and Kurihara H guided the design of experiment and article writing. Xiao W, Wang ZZ, Yao XS and Li HB offered the chemical profile of RDN analyzed by HPLC.

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