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Jinhua Qinggan granules attenuates acute lung injury by promotion of neutrophil apoptosis and inhibition of TLR4/MyD88/NF-κB pathway

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ABSTRACT
Ethnopharmacological relevance: Acute lung injury (ALI) is one of the fatal complications of respiratory virus infections such as influenza virus and coronavirus, which has high clinical morbidity and mortality. Jinhua Qinggan granules (JHQG) has been approved by China Food and Drug Administration in the treatment of H1N1 influenza and mild or moderate novel coronavirus disease 2019 (COVID-19), which is an herbal formula developed based on Maxingshigan decoction and Yinqiao powder that have been used to respiratory diseases in China for thousands of years. However, the underlying mechanism of JHQG in treating infectious diseases remains unclear.

Aim of the study: This study investigated the effects of JHQG on neutrophil apoptosis and key signaling pathways in lipopolysaccharide (LPS) -induced ALI mice in order to explore its mechanism of anti-inflammation.

Materials and methods: The effect of JHQG on survival rate was observed in septic mouse model by intraperitoneal injection of LPS (20 mg/kg). To better pharmacological evaluation, the mice received an intratracheal injection of 5 mg/kg LPS. Lung histopathological changes, wet-to-dry ratio of the lungs, and MPO activity in the lungs and total protein concentration, total cells number, TNF-α, IL-1β, IL-6, and MIP-2 levels in BALF were assessed. Neutrophil apoptosis rate was detected by Ly6G-APC/Annexin V-FITC staining. Key proteins associated with apoptosis including caspase 3/7 activity, Bcl-xL and Mcl-1 were measured by flow cytometry and confocal microscope, respectively. TLR4 receptor and its downstream signaling were analyzed by Western blot assay and immunofluorescence, respectively.

Results: JHQG treatment at either 6 or 12 g/kg/day resulted in 20% increase of survival in 20 mg/kg LPS-induced mice. In the model of 5 mg/kg LPS-induced mice, JHQG obviously decreased the total protein concentration in BALF, wet-to-dry ratio of the lungs, and lung histological damage. It also attenuated the MPO activity and the proportion of Ly6G staining positive neutrophils in the lungs, as well as the MIP-2 levels in BALF were assessed. Neutrophil apoptosis rate was detected by Ly6G-APC/Annexin V-FITC staining. Key proteins associated with apoptosis including caspase 3/7 activity, Bcl-xL and Mcl-1 were measured by flow cytometry and confocal microscope, respectively. TLR4 receptor and its downstream signaling were analyzed by Western blot assay and immunofluorescence, respectively.

Results: JHQG treatment at either 6 or 12 g/kg/day resulted in 20% increase of survival in 20 mg/kg LPS-induced mice. In the model of 5 mg/kg LPS-induced mice, JHQG obviously decreased the total protein concentration in BALF, wet-to-dry ratio of the lungs, and lung histological damage. It also attenuated the MPO activity and the proportion of Ly6G staining positive neutrophils in the lungs, as well as the MIP-2 levels in BALF were reduced. JHQG inhibited the expression of Mcl-1 and Bcl-xL and enhanced caspase-3/7 activity, indicating that JHQG partially acted in promoting neutrophil apoptosis via intrinsic mitochondrial apoptotic pathway. The levels of TNF-α, IL-1β, and IL-6 were significantly declined in LPS-induced mice treated with JHQG. Furthermore, JHQG reduced the protein expression of TLR4, Myd88, p-p65 and the proportion of nuclei p65, suggesting that JHQG treatment inhibited TLR4/MyD88/NF-κB pathway.

Conclusion: JHQG reduced pulmonary inflammation and protected mice from LPS-induced ALI by promoting neutrophil apoptosis and inhibition of TLR4/MyD88/NF-κB pathway, suggesting that JHQG may be a promising drug for treatment of ALI.

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1. Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) as a life-threatening respiratory disease with high morbidity and mortality is mostly associated with uncontrolled, overwhelming inflammatory lung injury (Li et al., 2021). It is characterized by non-cardiogenic pulmonary edema, hypoxic respiratory failure, and increased vascular permeability due to the presence of protein-rich exudates and neutrophil infiltrates in the alveolar spaces (Vichare and Janjic, 2022). Recently, severe influenza infection with pulmonary epithelial damage and endothelial dysfunction is widely believed to contribute to ALI, and there is no specific pharmacological treatment of ALI/ARDS in severe influenza patients (Rommel et al., 2020). Another global literature survey reported that the mortality rate in novel coronavirus disease 2019 (COVID-19)-associated ARDS is 45%, and the incidence of ARDS among non-survivors of COVID-19 surged to 90% (Tzotzos et al., 2020). ALI/ARDS is a frequent complication in various respiratory diseases, with the mortality rate as high as 35–46% (Bellani et al., 2016). Accordingly, developing new drugs for treatment of ALI/ARDS is in great demand. Neutrophils as primary effector cells are activated and migrate into pulmonary interstitial and alveolar cavities to release inflammatory mediators such as reactive oxygen species, proteolytic enzymes, and neutrophil extracellular traps (NETs), which can exacerbate inflammatory mediators (Vichare and Janjic, 2022). There is no specific pharmacological treatment of ALI/ARDS for treating ALI/ARDS. In present study, we hypothesized that JHQG alleviated inflammatory response in LPS-induced ALI mice model by promoting neutrophil apoptosis in intrinsic mitochondrial apoptosis pathway and inhibiting of TLR4/MyD88/NF-κB pathway in ALI.

Jinhua Qinggan granules (JHQG), an innovative Chinese Patent Medicine, is developed based on Maxingshigan decoction and Yingqiao powder. Both classic formulae are used to treat respiratory infections recorded in Treateize on Exogenous Febrile Disease (about 210 AD) and Systematic Differentiation of Warm Diseases (1978 AD), respectively (Shi et al., 2021; Wu et al., 2021). JHQG, composed of 12 Chinese herbs including Lonicer japonica Thunb. (Jin Yin Hua), Gypsum Fibrosum (Shi Gao), honey-fried Ephedra sinica Stapf (Mi Ma Huang), stir-baked Prunus sibirica L. (Chao Ku Xing Ren), Scutellaria baicalensis Georgi. (Huang Qin), Forsythia suspensa (Thunb.) Vahl (Lian Qiao), Pfitzaria thunbergii Miq (Zhe Bei Mu), Anemorrhena asphodeloids Bunge (Zhi Mu), Arctium lappa L. (Niu Bang Zi), Artemisia annua L. (Qing Hao), Mentha haplocalyx Briq. (Bo He), and Glycyrrhiza glabra L. (Gan Cao), has been approved by China Food and Drug Administration to treat H1N1 influenza virus infection and mild or moderate COVID-19 (General Office of National Health Commission of the People’s Republic of China and Office of National Administration of Traditional Chinese Medicine, 2020). In clinical practice, Wang et al. showed that maxingshigan-yinqiaoan and oseltamivir, alone and in combination, reduced time to fever resolution in patients with H1N1 influenza virus infection (Wang et al., 2011). Liu et al. found that JHQG could effectively shorten the duration of nucleic acid detection and promote the absorption of pneumonia inflammatory exudate without obvious adverse reactions in patients with COVID-19 (Liu et al., 2020). An et al. also indicated that in suspected and confirmed COVID-19 patients, JHQG combined with antiviral drugs or antiviral drugs alone both could relieve symptoms such as fever, fatigue, and diarrhea, whereas JHQG treatment was superior in relieving fever and poor appetite (An et al., 2021). In addition, several studies reported the main active components, potential targets and mechanism of JHQG against the severe acute respiratory syndrome, the Middle East respiratory syndrome, and COVID-19 using network pharmacology and molecular docking (Niu et al., 2021; Huang et al., 2021). However, there are relatively few experimental designs on its anti-inflammatory mechanism. We speculate that the potential mechanism of JHQG in the treatment of influenza and COVID-19 may be associated with anti-inflammatory activity.

In previous studies, we have identified chemical composition of JHQG. A total of 184 components of JHQG were identified by ultra-high performance liquid chromatography-quadrupole/Orbitrap high resolution mass spectrometry, including 20 alkaloids, 78 flavonoids, 2 phenylpropanoid, 23 terpenoids, 2 quinones, 7 steroids, 16 phenylethanoid glycosides, 17 lignans, 11 organic acids, 4 coumarins, and 16 other components (Supplementary Fig. 1 and Tab. 1). In addition, we established an high performance liquid chromatography method for simultaneous determination of 14 components in JHQG (Supplementary Fig. 2), including neochlorogenic acid, loganic acid, chlorogenic acid, caffeic acid, swertiside, forsythoside A, apigenin-7-O-β-D-glucopyranoside, baikalin, forsythin, arctiin, wogonoside, baikalene, chrysophanol, and oxoroyalin A, and their contents in 10 batches were 1.19–1.42, 0.20–0.37, 6.51–7.72, 0.20–0.25, 2.12–2.80, 1.75–2.46, 0.89–1.14, 14.41–16.58, 0.41–0.64, 15.85–20.01, 2.80–3.44, 0.38–0.53, 0.02–0.03, 0.07–0.11 mg/g, respectively (Zhu et al., 2021). Baicalin and arctin may be used marker compounds in JHQG, because their contents in JHQG were higher than other compounds. In present study, we hypothesized that JHQG alleviated inflammatory response in LPS-induced ALI mice model by promoting neutrophil apoptosis in intrinsic mitochondrial apoptosis pathway and inhibiting of TLR4/MyD88/NF-κB pathway.

2. Materials and methods

2.1. Reagents

JHQG (Lot, 20200106) was purchased from Juxiechang (Beijing) Pharmaceutical Co., Ltd. (Beijing, China). Its quality conforms to the national medication standard YBZ00392016, established by CFDA. Dexamethasone (Lot, 20022002) was purchased from Sinopharm Rongsheng Pharmaceutical Co., Ltd. (Jiaozuo, China). LPS (Escherichia coli 055:B5, L2880) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against TLR4 (66350-1-Ig), Bcl-xl (10783-1-AP), McI-1 (16225-1-AP), CoraLite594-conjugated Goat Anti-Rabbit IgG (H + L) (SA00001-2) were purchased from ProteinTech Group (Chicago, IL, USA). Antibodies against MyD88 (4283s), NF-κB (3033s), interleukin-1 (16225-1-AP) (NF-B/p65 (p-p65) (3033s) were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (556547) was purchased from BD Pharmingen (San Jose, CA, USA). FITC anti-mouse Ly6G antibody (127605) was purchased from BioLegend (San Diego, CA, USA). APC conjugated Ly6g monoclonal antibody (17-9668-80) and CellEvent™ Caspase-3/7 Green Detection Reagent (C10723) were purchased from Invitrogen (Carlsbad, CA, USA). Quanticyto® mouse TNF-α (EMC012a.96), IL-1β (EMC001b.96), IL-6 (EMC004.96), and MIP-2 (EMC122.96) enzyme-linked immunosorbant assays (ELISA) kits were purchased from NeoBioscience Technology Co., Ltd. (Shenzhen, China). Radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B), protease inhibitors (ST506), phosphatase inhibitors (P0825), bicinecinonic acid.
(BCA) protein assay kit (P0010), and BeyoECL Star chemiluminescence detection kit (P0018AS) were purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). MPO activity kit (A0441-1) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Mice and experimental design

Specific pathogen-free male Bagg albino (BALB)/c mice (21 ± 2 g) were purchased from Beijing Vital River Laboratory Animal Technology company (approval ID: SCXK, 2016-0006). All animal experiments were conducted in accordance with the guidelines of Animal Welfare and Ethics of the Institutional Animal Care and Use Committee. The protocols described in the present study were approved by the Animal Care and Use Committee of Henan University of Chinese Medicine (approval ID: DWLLGZR2022022024). All mice were housed in cages under a 12 h light/dark cycle and with free access to food and water (25 °C, 50–60% humidity). The mice were randomly divided into the following six groups: 1) control (CON), 2) LPS (LPS), 3) LPS + dexamethasone (5 mg/kg/day) (DEX), 4) LPS + JHQG (3 g/kg/day) (JL), 5) LPS + JHQG (6 g/kg/day) (JM), and 6) LPS + JHQG (12 g/kg/day) (JH). DEX group was intraperitoneally injected with DEX at 5 mg/kg for 1 h before injection of LPS.

For survival experiment (n = 10/group), the mice were given LPS 20 mg/kg (i.p.), except CON received the same volume of phosphate buffered saline (PBS) (Su et al., 2016). JL, JM, and JH groups were administered JHQG by oral gavage at 2 h after LPS treatment and then administered every 24 h for 3 days, and LPS group was administered the same volume of saline. Survival of the mice was daily monitored for 96 h.

For pharmacological evaluation experiments, the mice (n = 6/group) were anesthetized with 1% pentobarbital sodium (i.p.) and then received intratracheal injection of 5 mg/kg LPS or PBS (Su et al., 2019). JL, JM, and JH groups were given JHQG by oral gavage at 2 h post treatment. Twenty-four hours after intratracheal injection of LPS, the mice were humanely killed by overdose of anesthesia, and lung tissues and bronchoalveolar lavage fluid (BALF) were collected. The left lobes of lung tissues were fixed in 4% (v/v) paraformaldehyde for 24 h then submitted for hematoxylin-eosin staining (HE), and immunofluorescence staining. The right lobes were divided into three parts and used for the measurement of wet-to-dry (W/D) ratio, myeloperoxidase (MPO) activity assay, and Western blot assay, respectively.

2.3. Measurement of wet-to-dry ratio of the lungs

At 24 h after LPS stimulation, the parts of right lung tissues were removed and the wet weight was determined. The lungs were then incubated at 60 °C for 72 h to obtain the dry weight and the lung W/D ratio was calculated (Ju et al., 2018).

2.4. MPO activity assay

100 mg right lung tissue was homogenized and the supernatant was analyzed with MPO Detection Kit according to the manufacturer’s instruction. The enzymatic activity was determined by measuring the absorbance at 460 nm on a 96-well microplate reader (Chen et al., 2015).

2.5. Lung histopathological analysis

The left lobes of lung tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sliced into 5 μm-thick section. Next, the sections were stained with HE, and observed under a light microscope at a magnification of 200×. Pulmonary histopathological changes were blindly evaluated by a pathologist according to previous assessment method (Cho et al., 2020). Briefly, four types of histopathological changes were scored independently (alveolar capillary congestion, hemorrhage, infiltration of neutrophils, alveolar wall thickness and hyaline membrane formation) and were determine histopathological scores based on a scale ranging from 0 to 4. The lung injury score is the sum of all scores for each item.

2.6. Lung immunofluorescence

The p65 nuclear translocation in lung tissues was assessed by immunofluorescence as previously described (Ju et al., 2018). In brief, the lung sections (5 μm thickness) were used for immunofluorescence analysis of neutrophil infiltration and p65 expression in cytoplasm and nucleus, respectively. The sections were deparaffinized and rehydrated, then blocked with 5% bovine serum albumin (BSA) for 30 min. The sections were next incubated with anti-Ly6G antibody (1:300) or anti-p65 antibody (1:500) overnight at 4 °C and Cy3-labeled secondary antibody for 1 h at 37 °C, followed by nuclear staining with DAPI for 10 min at room temperature. The staining results were observed with laser scanning confocal microscope (Nikon Eclipse C1, Japan). The populations of neutrophils stained for Ly6G or nuclei p65-positive cells were counted in six fields of view per section.

2.7. BALF collection and analysis

Briefly, the lungs were lavaged through the tracheal cannula using instillation of 2 × 1 mL PBS. The BALF samples were then centrifuged at 4 °C (10 min, 2000 rpm), and the supernatants were collected and stored in −80 °C for total protein concentration and cytokine measurements. Total protein concentration in BALF was determined with a BCA protein assay (Ju et al., 2018). TNF-α, IL-1β, IL-6, and MIP-2 levels were measured using ELISA assay according to the kit’s protocols. The cell pellet was re-suspended with 1 mL PBS. Total cell number was counted with Handheld Automated Cell Counter (Millipore, Billerica, MA, USA), then cell suspension was analyzed by flow cytometry and confocal microscopy, respectively.

2.8. Confocal microscopy

The cell pellet from BALF was re-suspended with 1 mL PBS, then 10 μl cell suspension was subjected to smear, fixation, permeability, blocking, and staining with antibody against Bcl-xL (1:100) or Mcl-1 (1:500) overnight at 4 °C and the corresponding CoraLite594-conjugated Goat Anti-Rabbit antibody (1:500) and FITC anti-mouse Ly6G antibody (1:200) for 1 h at 37 °C. Stain cells were counterstained with DAPI for 10 min at room temperature and analyzed with a Zeiss LSM700 confocal microscope. The positive number of neutrophils stained for Bcl-xL or Mcl-1 was counted in six fields per slide (Su et al., 2019).

2.9. Flow cytometry

BALF was prepared for neutrophil apoptosis analysis as previously described (Su et al., 2019). Briefly, the 950 μl cell suspension of BALF was divided into 2 portions. One portion was measured neutrophil apoptosis with FITC-Annexin V/APC-Ly6G. The cells were stained with APC-Ly6G (2 μL) for 30 min at 4 °C and washed with cold PBS. Then, samples were re-suspended with 100 μL 1 × Binding Buffer and incubated with FITC-Annexin V (5 μL) for 10 min without light at room temperature. After washing three times, samples were re-suspended with 500 μL PBS and measured by flow cytometry. The other portion was detected the activation of caspase-3/7. The cells were stained with CellEvent™ Caspase-3/7 Green Detection Reagent (0.2 μL) and APC-Ly6G (2 μL) for 30 min at 4 °C and washed for three times. The samples were re-suspended with 500 μL PBS and measured by An Accuri C6 Plus flow cytometer (BD Bioscience).
2.10. Western blot analysis

The right-lung lobes of mice were collected and lysed in a RIPA buffer containing protease inhibitors (1:100) and phosphatase inhibitors (1:50) according to the manufacturer’s instructions and Western blot analyses were performed as previously described (Zhu et al., 2019). A BCA protein assay kit was used to determine the protein concentration. All of the supernatants were dissolved in a 5× loading buffer and boiled for 10 min. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes, blocked with 5% skin milk or 5% BSA in Tris buffer saline containing 0.1% Tween 20 at room temperature for 2 h. The membranes were incubated at 4°C overnight with a primary antibody against TLR4 (1:1000), MyD88 (1:1000), p65 (1:1000), p-p65 (1:1000) or β-actin (1:5000). After washing three times, the membranes were incubated with HRP-conjugated second antibody (1:5000) at room temperature for 2 h. Then the blots were visualized using an ECL kit, imaged and analyzed using Tanon 4600SF Luminescent Imaging Workstation (Tanon Sciences Technology Co., Ltd., Shanghai, China).

2.11. Statistical analysis

All of the graphs were performed with GraphPad Prism 5.0 software. Kaplan-Meier survival curves were compared using the log-rank test. Data were shown as means ± standard deviation (SD) of three independent experiments. Differences were analyzed using one-way analysis of variance (ANOVA) with the Tukey’s test, and p values < 0.05 were considered statistically significant.

3. Results

3.1. JHQG protects mice from LPS-induced ALI

A systemic infection mouse model was established by intraperitoneal injection of LPS (20 mg/kg) to evaluate effect of JHQG in vivo. As shown in Fig. 1A, only a 20% survival was observed in LPS group, whereas JHQG treatment at either 6 or 12 g/kg/day elevated the survival up to 40%, similar to that of DEX treatment. To better pharmacological evaluation of JHQG, intratracheal administration of LPS (5 mg/kg) for 24 h was used. The pulmonary inflammation was evaluated by BALF total protein concentration, W/D ratio of the lungs, and histopathological analysis. As shown in Fig. 1B, LPS group augmented a 2-fold increase in the total protein concentration of BALF compared to CON group, whereas treatment with different doses of JHQG or DEX significantly reduced the total protein concentration. Additionally, LPS challenge significantly increased the lung W/D ratio compared with that of CON group. However, the lung W/D ratio was markedly decreased in the mice treated with 6 or 12 g/kg JHQG, suggesting that JHQG could alleviate lung edema induced by LPS (Fig. 1C). Lung injury scores for CON, LPS, JHQG, and DEX groups were consistent with these results (Fig. 1D). The histopathological scores of JM and JH groups significantly reduced compared with that of LPS group. As shown in Fig. 1E, LPS group induced inflammatory response characterized by congestion, prolifer exude, and neutrophil infiltration. The presence of DEX or JHQG effectively inhibited LPS-induced histological damage, particularly treatment with JHQG at either 6 or 12 g/kg obviously decreased development of lung injuries. These results indicated that JHQG improved the survival of LPS-induced mice and protected mice from LPS-induced ALI through suppressing pulmonary inflammation.

3.2. JHQG attenuates neutrophil accumulation in lung tissues

Twenty-four hours after LPS administration, neutrophil accumulation in lung tissues was evaluated by Ly6G staining of neutrophil, MPO activity, total cells and MIP-2 level in BALF of the mice with and without DEX or JHQG treatment, respectively. As shown in Fig. 2A and B, the signal for Ly6G staining was significantly increased in LPS group compared to CON group. After JHQG treatment, a significant decrease in Ly6G staining was observed, indicating that JHQG may prevent LPS induced neutrophil infiltration. Moreover, MPO, as a heme-containing peroxidase expressed mainly in neutrophils, is a local mediator of tissue damage resulting in inflammation (Aratani, 2018). The results showed that MPO activity significantly increased after LPS challenge. However, JHQG treatment (6 or 12 g/kg) weakened the MPO activity (Fig. 2C). Additionally, MIP-2, an intermediary chemoattractant for neutrophils, is known as a marker of neutrophil recruitment (Lee et al., 2019). LPS induced a 3-fold increase of MIP-2 level in BALF. Whereas, MIP-2 levels significantly decreased following administration of DEX or different doses of JHQG compared with that of LPS group (Fig. 2D). To evaluate the migration and filtration of pulmonary cells, the number of total cells in BALF were measured. The results showed that there was a...
significant increase in the number of total cells in mice exposed to LPS. Compared to LPS group, both DEX and JHQG treatment decreased the number of total cells, and the inhibition effects of JHQG at 6 and 12 mg/kg were similar to that of DEX (Fig. 2E). Together, these results suggested that neutrophil accumulation was attenuated by JHQG in a dose-dependent manner.

3.3. JHQG promotes apoptosis of pulmonary neutrophils

To evaluate neutrophil apoptosis, BALF were stained with FITC-Annexin V/APC-Ly6G or caspase-3/7 Green/APC-Ly6G and analyzed by flow cytometry. As shown in Fig. 3A and C, the ratio of apoptotic neutrophils in CON mice was 7.3%, whereas that in LPS mice significantly decreased to 5.0%. Compared with LPS mice, the ratio of apoptotic neutrophils in the mice treated with 3, 6 or 12 g/kg JHQG were higher to 5.3%, 10.9% or 17.7%, respectively. The effect of JHQG group on inhibiting neutrophil apoptosis was close to that DEX group. To further investigate the pro-apoptosis mechanism of JHQG, the activation of caspase 3/7 was measured. As shown in Fig. 3B and D, the proportion of activated caspase 3/7 was significantly increased from 2.4% (LPS group) to 5.5% (JL group), 11.7% (JM group), and 15.0% (JH group), and the activation of caspase 3/7 in JH group was obviously higher compared with DEX group (13.1%). Furthermore, Bcl-2 family proteins such as Bcl-xL and Mcl-1 could inhibit the apoptosis caused by caspase-3. As shown in Fig. 3E, F and G, immunofluorescence staining signal for Mcl-1 and Bcl-xL was stronger in LPS group compared with CON group. In contrast, the immunofluorescence level of Mcl-1 and Bcl-xL was weaker in LPS group treated with different doses of JHQG. These results indicated that JHQG could promote neutrophil apoptosis in intrinsic mitochondrial apoptosis pathway by inhibiting Mcl-1 and Bcl-xL expression and activating caspase 3/7.

3.4. JHQG inhibits TLR4/MyD88/NF-κB pathway

To investigate the effect of JHQG on TLR4/MyD88/NF-κB pathway, the expression of TLR4, MyD88, and p65, and p-p65 in lung tissues was detected by Western blot, and the expression of p65 in cytoplasm and nucleus was detected by immunofluorescence. As shown in Fig. 4A and B, LPS promoted the expression of TLR4, MyD88, and p-p65 and inhibited the expression of p65. In contrast, JHQG treatment markedly decreased the expression of TLR4, MyD88, and p-p65 and significantly increased the expression of p65. Consistently, immunofluorescence staining showed that the lung tissue sections in LPS group exhibited stronger staining of p65 in nucleus, whereas different doses of JHQG treatment prevented the transfer of p65 from the cytoplasm to nucleus (Fig. 4C and D). These results indicated that JHQG suppressed the activation of TLR4/MyD88/NF-κB pathway.

3.5. JHQG reduces the release of pro-inflammatory cytokines

To assess the effect of JHQG on inflammatory responses, the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in BALF were measured. As shown in Fig. 5A–C, LPS significantly increased the release of TNF-α, IL-1β, and IL-6 compared with CON group. However, JHQG treatment dose-dependently reduced TNF-α, IL-1β, and IL-6 over-production, indicating that JHQG inhibited inflammation in LPS-induced ALI mice. These results highlighted that the protective effect of JHQG on LPS-induced ALI was associated with the attenuation of inflammatory responses by promoting neutrophil apoptosis and inhibiting TLR4/MyD88/NF-κB activation (Fig. 6).
4. Discussion

In this study, the beneficial effects of JHQG against LPS-induced ALI in mice were identified. We demonstrated that (i) neutrophil infiltration in the lungs were alleviated; (ii) anti-apoptotic proteins Bcl-xL and Mcl-1 expression were suppressed and apoptotic executioners caspase 3/7 activity was enhanced, resulting in the increase of neutrophil apoptotic rate; (iii) the expression of TLR4, MyD88, and p-p65 were markedly decreased, whereas p65 expression was significantly increased, and the translocation of p65 from the cytoplasm to nucleus in lung tissues was prevented, lead to suppress the activation of TLR4/MyD88/NF-κB pathway; (iv) the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were reduced. Thus, we preliminarily revealed that the molecular mechanism of JHQG in the prevention and treatment of ALI was associated with promoting neutrophil apoptosis and inhibition of TLR4/MyD88/NF-κB pathway.

Recent studies have reported that various agents (e.g., hydrochloric acid, LPS, bacteria, viruses) which are delivered intratracheally or intranasally can induce direct ALI. In comparison, intraperitoneal or intravenous injection of LPS, cecal ligation and puncture, and hemorrhagic shock are used to induce indirect ALI (D’Alessio, 2018). Intratracheal injection of LPS is highly reproducible, causes robust neutrophilic alveolitis, and is used to evaluate both the early and resolution phases of ALI (Chen et al., 2010). In this study, we first evaluated the effect of JHQG on survival rate in septic mouse model by intraperitoneal injection of LPS (20 mg/kg). JHQG treatment at 6 and 12 mg/kg/day reduced the mice mortality rate, resulting in 20% increase of survival.

Next, the protective effects of JHQG against LPS-induced lung damage were investigated in a mouse model by intratracheal injection of LPS (20 mg/kg). JHQG treatment at 6 and 12 mg/kg/day reduced the mice mortality rate, resulting in 20% increase of survival.
treatment decreased the expression of TLR4, MyD88, and p-p65, and prevented activated p65 translocation into the nucleus, leading to a decline of the levels of TNF-α, IL-1β, and IL-6. These results indicated JHQG treatment inhibited the activation of TLR4/MyD88/NF-κB pathway.

Neutrophil accumulation in the lung is a characteristic pathological feature of ALI (Balamayooran et al., 2010). Our results showed that different doses of JHQG treatment significantly decreased the fluorescent signal for Ly6G and MPO activity in lung tissues and MIP-2 levels in BALF, indicating that JHQG treatment reduced neutrophil infiltration.

Abrogating disproportionate neutrophilic inflammation through promoting apoptosis is a therapeutic approach. Neutrophil apoptosis is a heavily regulated mechanism that is mediated by extrinsic death receptor pathway and intrinsic mitochondrial apoptotic pathway in which NF-κB and p38MAPK controlled proteins such as Bcl-2 family members and caspases are involved. Under normal condition, neutrophils express high levels of the pro-apoptotic proteins (Bax, Bak, and Bid) and low levels of the anti-apoptotic proteins (Bcl-2, Mcl-1, A1, and Bcl-xL). The cellular levels of pro-apoptotic proteins change very little during exposure to agents that accelerate or delay apoptosis. However, anti-
apoptotic proteins are highly and transiently expressed when neutrophils are exposed to the inflammatory mediators (IL-8, LPS, etc.) (Dyugovskaya and Polyakov, 2010). Notably, Mcl-1 and Bcl-2 can inhibit caspase 3-mediated apoptosis (Zhang et al., 2021). However, human neutrophils express the proteins Mcl-1, A1, and to a much lesser extent Bcl-xL, but not Bcl-2 (Milot and Filep, 2011). In addition, Su and colleagues reported that mesenchymal stem cell-conditioned medium (MSC-CM) therapy attenuated LPS-induced ALI by enhancing the apoptosis of BALF neutrophils through reducing the expression of Bcl-xL and Mcl-1 (Su et al., 2019). Therefore, we evaluated the effect of JHQG on neutrophil apoptosis by detecting the expression of the anti-apoptotic proteins Mcl-1 and Bcl-xL and the activation of apoptotic effector proteins caspase 3/7. The results showed that the ratio of apoptotic neutrophils in BALF markedly increased by JHQG treatment in a dose-dependent manner. The effect of JHQG on promoting neutrophil apoptosis was associated with inhibition of anti-apoptotic proteins Bcl-xL and Mcl-1 expression and increasing caspase-3/7 activity. Consistent with these results, Kageyama et al. reported that JHQG rapidly induced a significant decrease in the neutrophil/lymphocyte ratio and plasma level of IL-6 in healthy volunteers (Kageyama et al., 2022). Together, these results suggested JHQG treatment could reduce systemic inflammation via promoting neutrophil apoptosis and inhibiting TLR4/MyD88/NF-κB pathway.

In addition to neutrophil apoptosis, NETosis is another novel death process of neutrophils, in which excessive formation of NETs results in exacerbating tissue injury and immunothrombosis (Hamam and Palaniyar, 2019). Importantly, delayed neutrophil apoptosis is closely related to the formation of NETs in ARDS. Song et al. reported that promoting neutrophil apoptosis in ARDS could reduce NETs formation and inhibit inflammation, thereby alleviating ARDS (Song et al., 2022). Although the present study identified that JHQG promoted neutrophil apoptosis in LPS-induced ALI mice, it could reduce NETs formation remains to be elucidated. Moreover, the active ingredients in JHQG that play the anti-inflammatory activity should to be determined in future studies.

5. Conclusions

In summary, the present study demonstrated that JHQG could improve survival of septic mice and attenuate LPS-induced lung inflammation by promoting neutrophil apoptosis and inhibition of TLR4/MyD88/NF-κB pathway. These findings suggested that JHQG may be a promising drug for treatment of ALI induced by various factors.

CRediT authorship contribution statement

Yanhsui Zhu: Conceptualization, Methodology, Investigation, Writing – original draft. Qianqian Han: Conceptualization, Methodology, Investigation. Lei Wang: Conceptualization, Methodology. Baiyan Wang: Methodology, Investigation. Jianshuang Chen: Investigation. Bangrong Cai: Methodology, Writing – review & editing. Can Wu: Methodology. Xiali Zhu: Methodology. Fugang Liu: Formal analysis, Data curation. Deen Han: Software, Formal analysis. Haoran Dong: Resources, Formal analysis. Yongyan Jia: Conceptualization, Methodology. Yalin Liu: Conceptualization, Writing – review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled “Jinhua Qinggan granules attenuates acute lung injury by promotion of neutrophil apoptosis and inhibition of TLR4/MyD88/NF-κB pathway”. No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115763.
Glossary

| Abbreviation | Definition |
|--------------|------------|
| ALI          | acute lung injury |
| ARDS         | acute respiratory distress syndrome |
| BALF         | bronchoalveolar lavage fluid |
| BCA          | bicinchoninic acid |
| Bcl-xL        | Bcl-2-related protein long form of Bcl-x |
| COVID-19     | novel coronavirus disease 2019 |
| ELISA        | enzyme-linked immunosorbant assays |
| FITC         | fluorescein isothiocyanate |
| HE           | hemotoxylin-eosin staining |
| IL-1/IL-6    | interleukin-1β/interleukin-6 |
| LPS          | lipopolysaccharide |
| MAC           | myeloperoxidase |
| MPO           | myeloperoxidase |
| MyD88         | myeloid differentiation factor 88 |
| NETs          | neutrophil extracellular traps |
| NF-κB         | nuclear factor kappa-B |
| PBS           | phosphate buffered saline |
| RIPA          | Radioimmunoprecipitation assay |
| TLR4          | Toll-like receptor 4 |
| TNF-α         | tumor necrosis factor alpha |

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