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Lianqinjiedu decoction attenuates LPS-induced inflammation and acute lung injury in rats via TLR4/NF-κB pathway

Guiming Deng a, Hai He a, Zheng Chen a,*, Linqi OuYang a, Xiaojin Xiao b, Jinwen Ge b, Biao Xiang a, Sichen Jiang a, Shaowu Cheng b,⁎

a The First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, Hunan 410007, China
b The Key Laboratory of Hunan Province for Integrated Traditional Chinese and Western Medicine on Prevention and Treatment of Cardio-Cerebral Diseases, Hunan University of Chinese Medicine, Changsha, Hunan 410208, China

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ABSTRACT
Acute lung injury (ALI) and its severe form acute respiratory distress syndrome remain the leading cause of morbidity and mortality. LianQinJieDu (LQJD), which is a traditional Chinese medicine, has been clinically used for antiviral drug. The present study investigated whether Lianqinjiedu(LQJD) ameliorates lipopolysaccharide (LPS)-induced acute lung injury in rats and aimed to determine the anti-inflammatory effects of LQJD. Rat model with ALI induced by intraperitoneal injection of LPS was treated by oral administration of LQJD. The recruitment of body temperature and the histopathology of lung tissue from all groups were evaluated to grade the severity of the inflammation. The inflammatory cytokines levels, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), were examined by ELISA assay, and the TLR4 and NF-κB pathway were evaluated by Real time-PCR and western blotting. It was observed that LQJD reduced the LPS-induced body temperature, inflammatory cytokines levels, and lung injuries, and blocked the activation of TLR4/NF-κB signaling in lung tissue. This study demonstrates that LQJD has a protective effect on LPS-induced inflammatory rats through the signaling pathway of TLR4 and NF-κB65.

1. Introduction

Inflammation is a self-protection of microcirculation against harmful stimuli, including physical, chemical and biological stimuli. However, excessive and uncontrolled inflammation may cause fever, severe tissue damages and secondary inflammatory injuries [1–3]. Lipopolysaccharide (LPS), a component of the cell envelope of Gram-negative bacteria, is often associated with inflammation disease processes. It can cause damages to the host, including generation of inflammation, deleterious effects on organs (Lung Injury) [4].

Previous researches have confirmed that LPS induces inflammatory response mainly by activating Toll-like receptor 4 (TLR4) signaling pathway. Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated and damage-associated molecular patterns. TLRs can activate nuclear factor-κB (NF-κB) and induce the expression of inflammatory cytokines to modulate the inflammatory reaction. It has been established that this process is accomplished via myeloid differentiation protein (MyD88)-dependent and MyD88-independent pathways [5]. This process leads to high expression of endogenous pyroxenes such as TNF-α and IL-1 and leads to heat and lung injury through MyD88-dependent and MyD88-independent pathways [6,7].

LianQinJieDu (LQJD), which is an ancient Chinese traditional medicine, has been clinically used for respiratory tract infections for more than 30 years in The First Affiliated Hospital of Hunan University of Chinese Medicine. The main drug ingredients for this decoction are berberine, astragalus, and scutellaria. Prescription has been clinically used for the treatment of respiratory tract infections. However, its detail mechanisms of anti-inflammatory and antipyretic are still unclear.

In this study, Acute Lung Injury (ALI) inflammatory model in rats was induced by lipopolysaccharide (LPS). We evaluated the effects of LQJD on the expression of TLR4/NF-κB and its downstream signaling molecules and elucidated the mechanisms of its anti-inflammatory and antipyretic.
2. Materials and methods

2.1. Animal model

Sprague-Dawley (SD) rats (n = 48) weighing 180–220 g were obtained from Hunan Slack JingDa Experimental Animal Co, Ltd (Changsha, China). All animals were housed at a constant room temperature with a 12:12 h light-dark cycle and fed with a standard rodent diet and water. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine.

48 SD Rats were randomly divided into the following six groups: control, LPS(0.005 g/kg), LPS + LQJD(0.61, 1.22, 2.44 g/kg) and LPS + Aspirin(0.1 g/kg). Rats were injected with LPS (0.005 g/kg) (Escherichia coli055:B5L2880, Sigma, USA) to establish the rat Acute Lung Injury model based on a previous study [8,9].

2.2. Drugs

LQJD was extracted with 10 times and 8 times 60% ethanol for 1.5 h and 1 h, respectively, and concentrated in vacuum and dried under vacuum at 60 °C (pressure −0.08 MPa) to obtain dry extract. The drug dosage setting is based on the LQJD daily dosage of the adult 60 kg weight, which is 3.832 g extractum LQJD liquidum per day. The adult daily dose was converted into the low dosage of rat, which is 0.61 g/kg. Based on this dosage, the medium and higher dosage was set. Aspirin was bought from Tianjin Pharmaceutical Jiaozuo Co (Tianjin, China) [10]. Aspirin tablets were dissolved in saline, and then given orally as solution depending on the weight of the rat in this experiment. LQJD (0.61, 1.22, 2.44 g/kg) and Aspirin (0.1 g/kg) was administered orally 1 h before the LPS i.p. injection. [11,12].

2.3. Temperature detection

After the animal model was established, the rectal temperature (Ti) was measured at 1 h intervals from 1 h to 6 h after the i.p. injection of lipopolysaccharide, and the difference between the body temperature and the basal body temperature (ΔT, °C) was calculated.

2.4. Enzyme-linked immunosorbent assay

After six hours, the rats were sacrificed by exsanguinations under anesthetic (40 mg/kg pentobarbital intraperitoneally). The blood samples were obtained and immediately separated by centrifugation at 3000 rpm for 15 min at 4 °C. The cytokines in the Blood samples of rats were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Rat TNF-α,IL-6 ELISA kits are from R & D Systems, Co.).

2.5. Lung histology

The rats were sacrificed by exsanguinations under anesthetic and lungs were excised. The right upper lobes and the middle lobes of the right rat lungs were stored with 4% Paraformaldehyde. Subsequently, the middle lobes of the right rat lungs were embedded in paraffin blocks and sectioned (5 μm) for conventional hematoxylin and eosin staining, and examined by a pathologist blinded to the protocol and experimental groups [13,9].

2.6. Western blot analysis

Protein extracts were obtained from the lung tissues and protein concentrations were quantified by BCA method (ASPEN Institute of Biotechnology). 40 μg of protein samples were dissolved with equal volume of loading buffer, separated on 10% SDS-PAGE and then electrotransferred to PVDF membrane. Membranes were blocked in TBST containing 5% non-fat dried milk for 1 h at room temperature followed by incubation with primary antibodies at 4 °C overnight. The primary antibodies used were rabbit anti-NF-κBp65 (1:2000; Bioworld Technology, Inc, China), anti-TLR4 (1:1000; Bioworld Technology, Inc, China), GAPDH (1:2000; Bioworld Technology, Inc, China). Membranes were washed extensively in TBST and incubated with secondary antibodies for 1 h at room temperature. The signal was detected by an enhanced chemiluminescence method and quantified by Image J software.

2.7. Real time PCR analysis

The Total RNA of the lung tissue was extracted using Trizol reagent (TIANGEN, Inc). The cDNA was generated by using Transcriptor First Strand cDNA Synthesis Kit (K106-02, TIANGEN, Inc). Real-time PCR Light Cycler Fast Start DNA Master SYBR Green I kit (TIANGEN, Inc). TLR4 gene (Forward: 5'-TCTAACTCACAG ACCTCACA-3' and Reverse: 5'-GGCTTATGCCCTCTCCCTTAG-3'), NF-κBp65 gene (Forward: 5'-GGGAGTCTTGGACGC-3' and Reverse: 5'-GTGCGTCTTCTCGAG-3') and β-actin gene (Forward: 5'-CGAGATATGCACCC-3' and Reverse: 5'-GGCTTATGCCCTCTCCCTTAG-3') (TIANGEN, Inc). Real-time PCR was performed by CFX96 Real-Time System (Bio-Rad, CA, USA). Reactions were performed in triplicate using the following protocol: reverse transcription at 95 °C for 3 min and 95 °C for 20 s, followed by 36 cycles at 64.5 °C for 30 s, 65 °C for 5 s and 95 °C for 5 min.

2.8. Statistical analysis

SPSS17.0 statistical software was used for statistical analysis. All data were repeated at least 3 times. The results were expressed as mean ± standard deviation. Differences between multiple groups were compared with a one-way analysis of variance. In all tests, P < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of LQJD on LPS induced rat body temperature

To investigate the antipyretic effect of LQJD in LPS-induced acute lung injury, as depicted in Fig. 1, a dose of LPS (0.005 g/kg) induced a febrile response with peaks at 1 h and 6 h and the body temperature rise from 0.5 h post-injection. The value of body temperature (ST) was

![Fig. 1. Antipyretic Effect of LQJD on LPS induced rats](image)
significantly higher than those in saline group at corresponding time points. LQJD (0.61, 1.22, 2.44 g/kg) were remarkably suppressed the elevation of body temperature ($\Delta T$) in 6 h ($P < 0.01$). The results showed that LQJD significantly inhibited LPS induced hyperthermia in rats.

### 3.2. Effects of LQJD on LPS induced rat lung damage

As shown in Fig. 2, the normal lesions structures of lung could be seen in blank control group (Fig. 2). In LPS group, the inflammatory cells infiltration in the lung tissue of the model group was serious, and the cell stroma was thickened. The alveolar structure was disorder and the alveolar wall was not complete (Fig. 2B). In LPS + LQJD group, the infiltration of inflammatory cells in the lungs of LQJD group and the positive group were decreased. The interstitial cells, the continuity of alveolar structure and the incomplete of alveolar wall were improved in different dose of LQJD (Fig. 2(C–F)). These results suggest that LQJD has protective effects on LPS-induced lung injury in inflammatory rats.

### 3.3. Effects of LQJD on LPS-induced cytokine production in inflammatory rat blood

The release of LPS can cause fever and inflammatory damage by stimulating peripheral monocytes and macrophages to synthesize and release various cytokines. Of which, TNF-$\alpha$ and IL-6 are of great importance. The results showed that in vitro stimulation with LPS dramatically enhanced the production of TNF-$\alpha$ and IL-6 in rat blood. LQJD (0.61, 1.22, 2.44 g/kg) treatment produced a marked decrease in the increasing levels of TNF-$\alpha$ and IL-6 (Fig. 3).

### 3.4. Effects of LQJD on LPS-induced TLR4 and NF-$\kappa$Bp65 mRNA levels in the rat lung tissue

The mRNA levels of related genes in lung assessed by RT-PCR were shown in Fig. 4A and B. Compared with blank control group, the mRNA levels in LPS group involving TLR4, NF-$\kappa$B p65 mRNA in lung were significantly up-regulated. Compared with the model group, the expression of TLR4, NF-$\kappa$Bp65 mRNA in lung were significantly down regulated by LQJD (0.61, 1.22, 2.44 g/kg) (Fig. 4). These findings demonstrated that LQJD inhibited the production of TLR4, NF-$\kappa$Bp65 mRNA in lung induced by LPS.

### 3.5. Effects of LQJD on LPS-induced TLR4 and NF-$\kappa$Bp65 protein levels in the rat lung tissue

The western blotting analysis of the TLR4, NF-$\kappa$Bp65 protein levels were performed in lung. As shown in Fig. 5, we found that the protein levels of TLR4 and NF-$\kappa$Bp65 in LQJD (0.61, 1.22, 2.44 g/kg) group were lower than those in model group. These findings demonstrated that LQJD inhibited the production of TLR4 and NF-$\kappa$Bp65 protein in the rat lung tissue induced by LPS.

### 4. Discussion

Toll-like receptor-4 (TLR4) signaling has been implicated in innate immunity and acute inflammation following acute lung injury (ALI). The studies have indicated that TLR4 are the main regulators involved in the LPS induce inflammation response, of which TLR4 plays a major role as an LPS receptor [14]. LPS, as a potent cytotoxic inducer of inflammation, could cause lots of damages to organs, including heart, liver, spleen, and lung [15,16]. In this study, the results demonstrate
that pretreatment with LQJD inhibited LPS induced expression inflammatory cytokines (TNF-α, IL-6) in blood, improved the lung injury induced by LPS-induced acute lung injury and inhibited body temperature rise in rat. Moreover, LQJD significantly inhibited protein expression of TLR4 and NF-κBp65 activation. These observations demonstrate that the protective effect of acute lung injury inflammation the response to LPS with LQJD is partial dependent on TLR4/NF-κB. These findings are consistent with previous animal studies, which indicated that TLR4 signaling pathway is closely related to anti-inflammatory LPS-induced.

The main drug ingredients for LQJD decoction are berberine, astragalus, scutellaria. In vitro and in vivo studies have shown that berberine has anti-inflammatory and antibacterial effects and TLR4/NF-κB pathway regulation of inflammatory factors are closely related [17,18].

Baicalin flavonoid is the main active ingredient of Radix Scutellariae, which possesses significant antipyretic effects and is an important component of a variety of antipyretic and detoxifying prescription drugs [19,20]. In vivo studies have shown that the antipyretic effects of baicalin are associated with its ability to lower the levels of TNF-α, IL-1β, IL-6 and other pyrogenic cytokines in the serum, hypothalamus, and cerebrospinal fluid [21]. However, the previous studies have predominantly focused on downstream inflammatory factors and a paucity of information exists with regard to the upstream signaling pathways related to its antipyretic effects.

Astragalus mongholicus can relieve inflammatory fever of rats by down-regulating LPS-induced expression of inflammatory factors (IL-1β, IL-6) [22]. Astragalus polysaccharide can regulate LPS-induced inflammation of rats by TLR4/NF-κB signal transduction [23,24]. But whether the only through the regulation of TLR4/NF-κB pathway to inflammatory injury, inflammatory factor expression to be further studied.

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