Indian Journal of Experimental Biology
Vol. 60, July 2022, pp. 557-562

Protective effect of breviscapine on acute lung injury in rats with infectious shock

Bo Zhou[1†], Yicong Huang[2†], Yuxiang Chen[2†], Fei Yu[3], Quanzhen Wang[1], Jian Zhang[1], Zhegang Zhou[1], Fanbin Meng[1] & Yanbin Peng[1]*

[1]Department of Hand & Microsurgery, Peking University Shenzhen Hospital, Shenzhen 518036, China

[2]Department of Orthopedic Surgery, Affiliated Dongguan People's Hospital, Dongguan 523000, China

[3]Department of Bone & Joint, Peking University Shenzhen Hospital, Shenzhen 518036, China

Received 19 January 2022; *revised 10 June 2022*

Acute lung injury (ALI), during the progression of infectious shock, leads to systemic inflammatory response syndrome with increased pulmonary capillary membrane permeability due to pulmonary inflammation and uncontrolled inflammatory responses. It may cause fatality in patients. Here, we evaluated the protective effect of breviscapine on ALI in rats with infectious shock. Sprague-Dawley (SD) rats were assigned into Sham, model [lipopolysaccharide (LPS) group], and breviscapine treatment groups (LPS + breviscapine group) and weighed. The lung coefficient, and the wet-to-dry weight ratio (W/D) and moisture content of lung tissues were calculated. The pathological changes of the lung tissues were detected using hematoxylin-eosin (HE) staining, and the protein expressions of interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) were determined by enzyme-linked immunosorbent assay. Western blotting was conducted to measure the protein expressions of toll-like receptor-9 (TLR-9) and nuclear factor-kappa B (NF-κB) (p65). Compared with LPS group, breviscapine significantly lowered the lung coefficient and the W/D and moisture content of lung tissues, relieved the pathological changes of lung tissues, reduced the protein expression levels of IL-1β, IL-6, and TNF-α, weakened the activation of NF-κB (p65) in lung tissues, and repressed the protein expressions of TLR-9 and NF-κB (p65).

Keywords: Interleukin, Ischemia, Sepsis syndrome, Tumor necrosis factor-alpha (TNF-α)

Infectious shock, also named septic shock, refers to the sepsis syndrome with shock caused by such products as microorganisms and their toxins[1]. The microorganisms and their toxins, as well as cell wall products in foci of infection invade the blood circulation and activate all kinds of cellular and humoral systems in the host, producing cytokines and endogenous mediators that act on multiple organs and systems and affect the blood perfusion. The mortality rate of infectious shock reaches more than 50%[2]. Acute lung injury (ALI) tends to develop during the progression of infectious shock, especially systemic inflammatory response syndrome, and it is mainly characterized by the increased pulmonary capillary membrane permeability due to pulmonary inflammation and uncontrolled inflammatory responses, which is also one of major causes of death in patients with infectious shock[3]. However, the specific pathogenesis of ALI in infectious shock remains elusive, and there have been no effective treatments until now.

*Correspondence:
E-Mail: p1y1b1@163.com
†Contributed equally

Breviscapines, which are extracted from *Erigeron breviscapus* (Vant.) Hand-Mazz., possess active ingredient of general flavone, and mainly contain scutellarins and a few apigenin-7-O-glucronides. They can expand blood vessels, reduce blood viscosity, improve microcirculation, and prevent thrombosis and have been applied in the treatment of cardiovascular and cerebrovascular diseases since many years[4]. Further, its protective effect on ALI caused by infectious shock has also been reported recently[5]. In this study, we assessed the protective effect of breviscapine on ALI in rats with infectious shock using LPS-induced model.

Materials and Methods

Reagents and apparatus

Breviscapine injection (specifications: 20 mg/5 mL, purity >95%) was sourced from Shineway Pharmaceutical Group Ltd., and rabbit antibody against nuclear factor-kappa B (NF-κB) was provided by Cell Signaling Technology. Cy3-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) was purchased from TM® Jackson. Reverse transcription kit and Hieff™ quantitative polymerase chain reaction (qPCR) SYBR

Green Master Mix (Low Rox Plus) were bought from Yeasen. Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) were offered by Jiangsu Kete Biotechnology Co., Ltd. An inverted fluorescent biological microscope (FM-500) was provided by Shanghai Puda Optical Instruments Co., Ltd., and an ultraviolet-visible spectrophotometer (UV9100) and a microplate reader (DNM-9602A) were purchased from Xingtai Runlian. A qPCR instrument was bought from Bio-Rad.

Establishment of rat model with infectious shock and grouping

Specific pathogen-free male Sprague-Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. with a certificate No. of SCXK (Beijing) 2016-0011. The rats were raised at 3 per cage and had free access to food and water in a 12 h light/dark cycle at a room temperature of (20±2)°C, and a relative humidity of 55-65%. After adaptive feeding for 3 days, they were randomly assigned into three groups: Sham group, model group [lipopoly-saccharide (LPS) group], and treatment group (LPS + breviscapine group). The rats in Sham group were intravenously injected with normal saline as controls. In LPS group, endotoxin was intravenously injected at 15 mg/kg, and 2 min later, normal saline was continuously injected at 1 mL/kg/h. The rats in LPS + breviscapine group were intravenously injected with endotoxin at 15 mg/kg, and breviscapine was injected incessantly at 5 μg/kg/h.

Measurement of lung tissue coefficient, W/D and moisture content

The rats were sacrificed after being treated with drug for 6 h, and thoracotomy was performed to rapidly take out the lungs. They were then placed in pre-cooled normal saline, and the bloodiness was rinsed. The right lung was excised, and additional tissues were removed carefully. After that, the surface moister was sucked up using filter paper, and the right lung was quickly weighed. The lung coefficient (W/D×100%) was calculated. Finally, the right lung was dried using a 60°C oven until a constant weight, and W/D and moisture content [(1-W/D)×100%] were calculated.

Hematoxylin-eosin (HE) staining

The upper lobe tissues of the left lung were cut, fixed in 4% paraformaldehyde, dehydrated, transparentized, and embedded in paraffin, followed by sectioning at a thickness of about 4 μm. Then the sections were taken at an interval of 10 pieces and baked in a 60°C thermostatic oven for 20 min prior to use. The paraffin-embedded sections in each group were deparaffinized using fresh xylene twice, hydrated in 100, 95, 85 and 75% ethanol solution in turn, and soaked in distilled water for 5 min. Then they were stained with Harris hematoxylin dye at 60°C for 5 min and rinsed with running water for 5-10 s to remove the hematoxylin solution. After that, the sections were differentiated for 1-3 s using 1% hydrochloric acid-ethanol mixtures, and rinsed by running water for 5-10 s, by 1% ammonia water for 5-10 s and by running water again for 15-30 s. They were then stained with eosin dye for 30-60 s, and the colour changes were observed under a microscope. The resulting sections were washed using running water for 5-10 s, dehydrated using gradient ethanol, namely 80% ethanol for 1-2 s, 95% ethanol for 1-2 s, and absolute ethanol for 1-2 s, transparentized with xylene for 2-3 s, dried in the air, and mounted in neutral resin. A total of 6 section specimens were taken from each group, and the histological changes were observed under a light microscope.

Detection of levels of IL-1β, IL-6, and TNF-α in lung tissue homogenates by ELISA

About 100 mg of lower lobe tissues of the left lung were placed in an in-cut electric homogenizer, added with phosphate-buffered saline in five-fold volume, and homogenized in ice bath. The resulting homogenate was centrifuged at 4°C and 12,000 r/min for 20 min, and then the supernatant was taken to determine the levels of IL-1β, IL-6, and TNF-α in the tissue homogenate in each group using ELISA according to the operation instructions of the kit. The blank well was used for zeroing, and the absorbance value was measured using the microplate reader at a wavelength of 450 nm. The concentrations of IL-1β, IL-6, and TNF-α in the sample were calculated on the basis of the standard curve.

Determination of toll-like receptor-9 (TLR-9) and NF-κB (p65) levels in lung tissues using Western blotting

The lung tissues were taken from all groups of rats, added separately with lysis buffer, protease inhibitor, phosphatase inhibitor, and phenylmethylsulfonyl fluoride, homogenized on ice for 30 min, and centrifuged at 4°C and 12,000 r/min for 15 min, and the supernatant was stored in a 1.5 mL centrifugal tube for later use. After the concentrations of the proteins were determined by the bicinchoninic acid method, the proteins of the same weight were taken from each group, subjected to sodium dodecyl sulfate-

polyacrylamide gel electrophoresis, transferred onto membranes, and sealed in 5% skimmed milk powder sealant for 1 h. Later, the membranes were washed using tris-buffered saline-Tween 20 (TBST) for 5 min thrice, and the TLR-9, NF-κB (p65) and glyceraldehyde-3-phosphate dehydrogenase antibody diluents were then added at 1:1,000, and the resulting membranes were washed using TBST again for 5 min thrice. Subsequently, horseradish peroxidase-labeled secondary antibodies were added at 1:1,000 for 1 h of incubation at room temperature. An enhanced chemiluminescent kit was used to detect light emission, the gel imaging system was adopted for imaging, and the gray value was calculated using Image J software. Finally, the ratio of the optical density of the target protein band to that of the internal reference protein band was used for statistical analysis.

Statistical analysis

GraphPad Prism 5 software was employed for calculation and analysis. All data were expressed as ($\bar{\chi} \pm s$), and one-way analysis of variance was performed among groups. P <0.05 suggested that difference was statistically significant.

Results

Breviscapine lowered lung coefficient, W/D and moisture content

The lung coefficient, W/D, and moisture content were (0.81±0.11)%, (4.12±0.32)%, and (75.14±3.25)%, respectively, in Sham group, and (1.14±0.12)%, (5.62±0.31)%, and (80.72±5.13)%, respectively, in LPS group, showing statistically significantly differences between LPS group and Sham group (P <0.05). After treatment with breviscapine, the lung coefficient, W/D, and moisture content were (0.82±0.05)%, (4.83±0.34)%, and (79.34±2.52)%, respectively, and they were statistically significantly different from those in LPS group (P <0.05). These results suggest that breviscapine lowered the lung coefficient, W/D, and moisture content in ALI in rats with infectious shock.

Breviscapine reversed pathological changes of lung tissues

Severe damage to alveolar wall, thickening of the alveolar septum, and severe edema, exudation and hemorrhage of the lung interstitium and alveolar cavity, as well as infiltration and hyaline membrane formation of large numbers of granulocytes, were microscopically observed in LPS group. After treatment with breviscapine, the microscopic observation revealed that most of the alveolar walls were intact, and the alveolar septum was significantly narrower than that in LPS group. Besides, only small amounts of bleeding, exudation and inflammatory cell infiltration were seen in the cavity (Fig. 1). These findings showed that breviscapine reversed the pathological changes in the ALI tissues in rats with infectious shock.

Breviscapine decreased levels of IL-1β, IL-6, and TNF-α in lung tissues

The rats with infectious shock had significantly higher levels of IL-1β, IL-6, and TNF-α in the lung tissues than those in Sham group, with statistically significant differences (P <0.01). After being treated with breviscapine, the rats with infectious shock had significantly decreased levels of the pro-inflammatory factors IL-1β, IL-6 and TNF-α in the lung tissues, with statistically significant differences (P <0.01), suggesting that breviscapine can repress the inflammatory responses in the lung tissues in rats with infectious shock (Fig. 2).

Breviscapine inhibited the TLR-9/NF-κB signaling pathway in lung tissues

Compared with Sham group, the protein expression levels of TLR-9 and NF-κB (p65) rose significantly in LPS group (P <0.01), indicating that LPS can activate the TLR-9/NF-κB signaling pathway. The protein

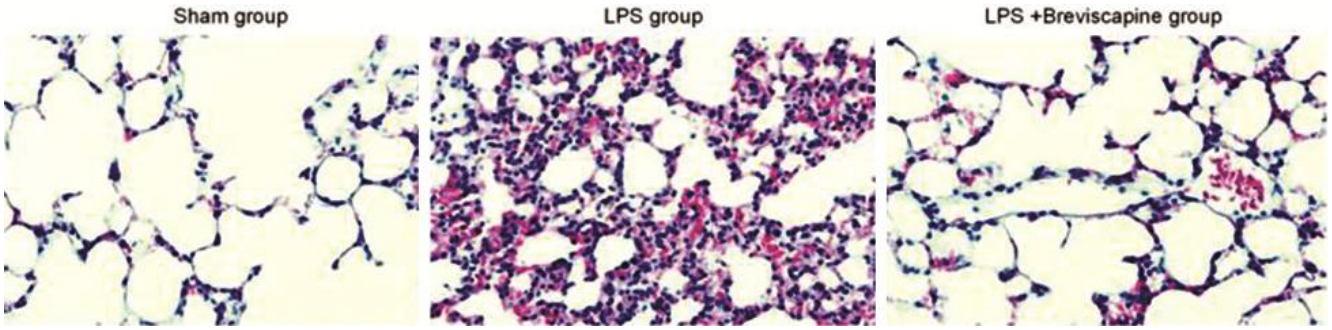

Fig. 1 — Pathological changes of lung tissues observed by HE staining.

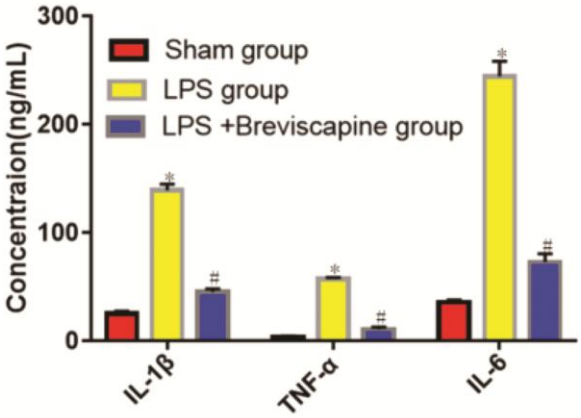

Fig. 2 — Levels of IL-1β, IL-6, and TNF-α in lung tissues. **P* <0.05 *vs.* Sham group, and #*P* <0.05 *vs.* LPS group.

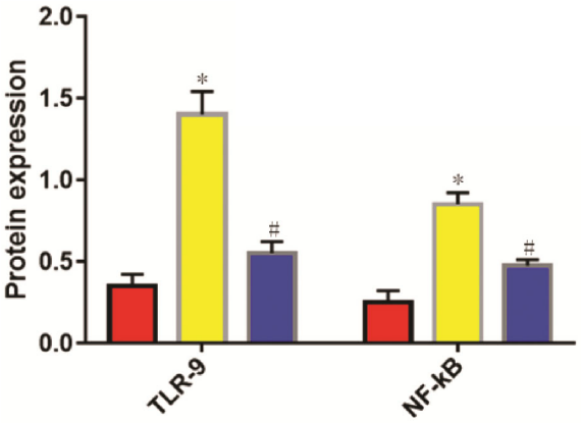

Fig. 3 — Protein expression levels of TLR-9 and NF-κB (p65) determined by Western blotting. **P* <0.05 *vs.* Sham group, and #*P* <0.05 *vs.* LPS group.

expression levels of TLR-9 and NF-κB (p65) in the lung tissues in LPS + breviscapine group were significantly lower than those in LPS group, showing statistically significant differences (*P* <0.05), suggesting that breviscapine has a protective effect on the lung in rats with infectious shock by inhibiting the TLR-9/NF-κB signaling pathway (Fig. 3).

Discussion

Severe infections, especially those from gram-negative bacteria, can often cause infectious shock. During the course of the disease, the toxic products such as LPS, the wall teichoic acid and peptidoglycan of G bacteria, mold yeast polysaccharide, and *Staphylococcus aureus* toxin will be released, by which the humoral and cell-mediated response systems are activated, thereby producing various inflammatory mediators and cytokines, such as TNF-α, IL-6, IL-1, IL-19, histamine, prostaglandins, prostacyclin and thromboxane A2[19]. These bioactive substances can severely damage blood vessels, affect vascular tension, and promote the increase in capillary permeability and platelet aggregation. Once initiated, such a reaction can continue to be augmented, resulting in circulatory disorders, without depending on the original trigger factors[6,7]. This strong self-destructive systemic inflammatory response is the main pathophysiological process of infectious shock. ALI, which mainly features increased pulmonary capillary membrane permeability, is caused by the generation and release of massive inflammatory mediators and cytokines when the cellular and humoral immune systems are activated during the progression of infectious shock, and it is one of the leading causes of death in patients with infectious shock[8,9]. In the present experiment, the rats with infectious shock were intravenously injected with LPS to establish the model of ALI and received intervention treatment with breviscapine, and its efficacy was evaluated. According to the results, breviscapine can lower the lung coefficient, W/D, and moisture content in rats with infectious shock and reverse the pathological changes caused by lung injury, thereby exerting a protective effect on lung injury.

Despite the fact that hundreds of inflammatory factors are associated with the onset of infectious shock, the major players therein include TNF-α, IL-1β, and IL-6. Various inflammatory factors interact with each other and form complex network systems, and they together with some kind of synergy among multiple inflammatory mediators mediate and enhance inflammatory cascade effects[10,11]. It was found that the expressions of the inflammatory factors IL-1β, IL-6, and TNF-α were significantly stronger in the lung tissues of rats with infectious shock in LPS group than those in Sham group, and the increases in the expressions of IL-1β, IL-6, and TNF-α were reversed after treatment with breviscapine. These results fully unravel the inhibitory effect of breviscapine on the inflammatory responses and further suggest that it can protect the lung from injuries through reducing the expressions of the inflammatory factors in the lung tissues of rats with infectious shock.

Located on human chromosome 3p21.3, TLR9 can mediate bacterial CpG and activate multiple inflammatory mediators. All these suggest that TLR9 plays a certain part in the pathogenesis of infectious

shock[12,13]. It has been revealed that TLRs recognize their ligands and activate NF-κB through the canonical MyD88-dependent signaling pathway in the TLRs/NF-κB signaling pathway, a TLR-mediated signal transduction pathway. NF-κB is a ubiquitous important transcription factor in inflammatory and immune responses, and it is activated by various signals through degrading IκBs. The activated NF-κB binds to the specific sequence on the deoxyribonucleic acid (DNA) chain, and it initiates and adjusts the gene transcription and regulation of multiple inflammatory and immune cytokines and inflammatory mediators, especially the expression regulation of the immediate-early genes related to the body defense[14,15]. The activated NF-κB initially enters the cell nucleus to bind to DNA. IκBs are firstly catalyzed by the kinase (IKK), composed of one regulatory subunit IKK-γ (also known as NEMO) and two catalytic subunits IKK-α and IKK-β, to phosphorylate their two conservative serine residues. Then IκBs are degraded by proteases due to the polyubiquitination under the catalysis of Skp1-cullin 1-F-box E3 ubiquitin ligase complex. The activated NF-κB translocates to the nucleus and binds to the relevant DNA motif to induce the transcription of the target gene. This pathway is activated by multiple signals including the compositions of various pathogens like LPS, proinflammatory cytokines TNF and IL-1, and mitogens[16-18]. In the present experiment, the expression of NF-κB (p65) was augmented in the lung of rats in LPS group, suggesting that NF-κB (p65) is activated, consistent with the above description. Besides, the expression of NF-κB (p65) declined after treatment with breviscapine, illustrating that breviscapine reduces the expression of NF-κB (p65) in the lung tissues of rats with infectious shock.

Conclusion

The study proved that breviscapine can alleviate the LPS-caused ALI in rats with infectious shock, and it is confirmed to play a therapeutic role through mitigating inflammatory responses by inhibiting the release of inflammatory factors in the lung tissues of rats with infectious shock possibly by the mechanism that the TLR9/NF-κB signaling pathway is repressed.

Acknowledgement

This study was financially supported by the Science and Technology Planning Project of Shenzhen City (No. JCYJ20180228175315535).

Conflict of interest

Authors declare no competing interests.

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