Am J Transl Res 2021;13(11):12786-12796
www.ajtr.org /ISSN:1943-8141/AJTR0137543

Original Article

Anti-inflammatory effects of brucea javanica oil via inhibition of NF-κB activation

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Received July 20, 2021; Accepted October 29, 2021; Epub November 15, 2021; Published November 30, 2021

Abstract: Objective: As a traditional herbal medicine extracted from the seeds of *Brucea javanica*, *Brucea javanica* oil (BJO) has been clinically used to treat wart, chronic gastroenteritis and a variety of malignant tumors, including gastrointestinal cancer and lung cancer. We have recently reported the anti-tumor role and possible molecular mechanisms of BJO in treatment of lung cancer. However, it remains elusive whether BJO also has an anti-inflammatory effect. Methods: The pneumonia-related inflammatory factors of macrophages under LPS treatment were investigated by real-time PCR and ELISA assays. LPS-induced acute pneumonia rat model was established. Hematoxylin and eosin (HE) examination was performed to detect histopathological changes in the lung tissues. Real-time PCR and ELISA assays were also used to detect the pneumonia-related inflammatory factors in lung tissues. Results: LPS-induced expression and secretion of pneumonia-related inflammatory factors (TNF-α, IL-1β, IL-6 and IL-8) were significantly suppressed by BJO in a concentration-dependent manner in RAW264.7 cells. However, BJO did not affect cell proliferation and survival rate. Further mechanistic studies revealed that BJO down-regulated the phosphorylation of IκB and p65, thereby inhibiting NF-κB pathway of macrophages and exerting its anti-inflammatory function. Western blot analysis showed that the phosphorylation levels of IκB and p65 were significantly up-regulated while the protein level of IκB was inhibited upon LPS stimulation in RAW264.7 cells and in lung tissue. Notably, LPS stimulation levels of IκB and p65 were effectively reversed under BJO co-treatment. The expression level of p65 was not influenced by LPS and BJO treatment. HE staining results showed that BJO can reduce the infiltration of inflammatory cells in lung. Conclusion: BJO can reduce the level of inflammatory factors in lung tissue, which provides a theoretical basis for BJO emulsion as an adjuvant therapy for pneumonia.

Keywords: Anti-inflammatory, *Brucea javanica* oil, inflammatory cytokines, NF-κB

Introduction

Brucea javanica oil (BJO) is a traditional herbal medicine extracted from the seeds of *Brucea javanica* [1], containing oleic acid, linoleic acid, palmitic acid, arachidonic acid, stearic acid, and other anticancer active ingredients [2]. It is mainly produced in the coastal tropical and subtropical regions of China, such as provinces of Hainan, Guangxi, Guangdong, and Yunnan [3, 4]. Previous studies have revealed that the anti-tumor mechanism of BJO may be attributed to its significant inhibition of cancer cell proliferation by inhibiting the synthesis of deoxyribonucleic acid (DNA) and blocking the division cycle of tumor cells in G2/M during cancer cell proliferation [5, 6]. Current studies have shown that BJO can be used to treat chronic gastritis, chronic colitis, condyloma acuminatum, and a variety of malignant tumors including lung, gastric and breast cancer [7-10]. In addition, we have recently demonstrated that BJO can regulate the expression of cyclin D1 and p53, and then induce G_0/G_1 phase arrest in non-small cell lung cancer A549 cells and small cell lung cancer H446 cells, and thus, inhibit the proliferation of these two cell lines [11]. BJO also elevated the generation of cellular reactive oxygen species (ROS), which subsequently induced apoptosis of A549 and H446 cells via mitochondrial/caspase-mediated signaling pathway [11]. Detailed analysis of BJO on non-small cell

lung cancer and small cell lung cancer will be conducive to the further clinical application of BJO.

In addition to the anti-tumor effect, brusatol and related quassinoids extracted from *Brucea javanica* were reported to exert an anti-inflammatory effect as early as 1983 [12]. Recent research has also found the anti-inflammatory role of BJO in ulcerative colonic mouse model induced by dextran sodium sulfate (DSS) [13]. However, it remains unclear whether BJO has an anti-inflammatory effect. Therefore, in this study, we investigated the anti-inflammatory effect of BJO and the related mechanisms, with the purpose of providing a theoretical basis for BJO emulsion as an adjuvant treatment drug for pneumonia.

Material and methods

Cell culture and treatment

RAW264.7 mouse macrophages were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China were cultured in DMEM medium containing 10% FBS, 1% glutamine (Gibco, Grand Island, NY, USA), 100 U of penicillin, and 100 μg/ml of streptomycin at 37°C with 5% CO_2.

RAW264.7 macrophages were treated with LPS (1 μg/mL) with or without BJO (5 or 10 μg/mL as indicated) for 12 h. LPS and BJO were dissolved or diluted in 0.9% NaCl solution, which was used as the vehicle control. Prior to treatment with agents, the medium was exchanged with serum-free Eagle's MEM containing sodium pyruvate and vitamins.

Experimental animals and grouping

Thirty SPF BALB/c male mice (26-30 g) were purchased from B&K Universal Group Limited, and kept in clean cages at 20-28°C. Then mouse models of acute pneumonia were established by intraperitoneally injecting the mice with lipopolysaccharide (LPS, L2880, Sigma, Saint Louis, MO) of Escherichia coli. The mice were randomly divided into three groups (each n=10), namely, normal control group, LPS group, and LPS+BJO-L group. Mice in the LPS group were intraperitoneally injected with LPS (5 mg/kg, sigma); those in the normal control group were intraperitoneally injected with 0.9% sodium chloride solution at the same amount with that of LPS; those in the LPS+BJO group were given BJO emulsion (Specification: 250 ml emulsion, Approval number: Chinese medicine approval word Z21020639, Shenyang Yaoda Leiyunshang Pharmaceutical Co., Ltd.) for gavage (1 g/kg, once daily) 3 hours after LPS modeling. Mice in the LPS group and the normal control group were gavaged with the same amount of saline. After 7 days of continuous treatment, the mice were sacrificed by cervical dislocation after anesthesia with intraperitoneal injection of pentobarbital sodium (40 mg/kg), and then the lung tissue was obtained. The drug doses were determined according to the effective and safe dose range commonly used in clinic and the calculation method of body surface area of human and animals [14]. All animal experiments were approved by the Ethics Committee. During the experiments, the animals were treated in strict accordance with the relevant health guidelines for the care and use of laboratory animals in international code of ethics and national health guidelines.

HE staining

The lung tissue was rinsed with 0.9% sodium chloride solution, dried with a filter paper, immobilized with 4% paraformaldehyde for 48 hours, and then dehydrated, embedded in paraffin, and sliced. The pathological slices with thickness of 4 μg? was placed in an oven at 65°C for 2 h, and then xylene was added for 10 min, followed by 2 min each of absolute ethanol, 95% ethanol, 90% ethanol, 85% ethanol, 75% ethanol, and 50% ethanol. The slices were stained with hematoxylin and eosin, sealed with neutral resins, and finally evaluated under a microscope.

Reagents and antibodies

BJO was purchased from Shenyang Yaoda Pharmaceutical Co., Ltd., Shenyang, China, with a primary concentration of 100 mg/mL (>99% purity). LPS (cat. #L2880) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Mouse anti-phospho-IκBα (Ser32/36) (cat. #9246), mouse anti-IκBα (cat. #4814), rabbit anti-Phospho-NF-κB p65 (Ser536) (cat. #3033) and rabbit anti-NF-κB p65 (cat. #8242), mouse anti-β-Actin (cat. #3700) antibodies were pur-

Table 1. Primer sequences

Subject	Primer sequences
TNF-α	Forward: 5'-AAAAGCAAGCAGCCAACC-3'
	Reverse: 5'-TGCCACAAGCAGGAATGA-3'
IL-1β	Forward: 5'-CTTCAAATCTCACAGCAGCATC-3'
	Reverse: 5'-GCTGTCTAATGGGAACATCACA-3'
IL-6	Forward: 5'-GCCTTCTTGGGACTGATGCT-3'
	Reverse: 5'-TGCCATTGCACAACTCTTTTC-3'
IL-8	Forward: 5'-CAAGGCTGGTCCATGCTCC-3'
	Reverse: 5'-TGCTATCACTTCCTTTCTGTTGC-3'
GAPDH	Forward: 5'-TGGCAAAGTGGAGATTGTTGCC-3'
	Reverse: 5'-AAGATGGTGATGGGCTTCCCG-3'

chased from Cell Signaling Technology, Boston, MA, USA.

Real-time PCR

Total RNA was isolated from cells and tissues using a Trizol RNA simple Total RNA Kit (Beyotime Biotechnology, Nanjing, China), and then reversely transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using THUNDERBIRD™ SYBR®qPCR Mix (Toyobo, Japan) with the specific primers listed below. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used as normalization controls. All primer sequences are shown in **Table 1**.

Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-1β, IL-6 and IL-8 in RAW264.7 cell supernatants and lung tissue was determined three times using ELISA kits (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) according to the manufacturer's instructions.

MTT assay

RAW264.7 macrophages were seeded in 96 well culture plates at a density of 1×10^5 per well. After adherence, they were treated with LPS (1 μg/mL) with or without BJO (5 or 10 μg/mL as indicated) for 12 h (4 wells per condition). MTT (Sigma-Aldrich) was then added at a concentration of 50 μg per well. Three hours later, DMSO (150 μL) was added to dissolve the formazan crystals for 15 min. Finally, the absorbance of each well optical density (OD value) was measured at 570 nm by a spectrophotometer. Cell viability rate was then calculated as average OD value of wells with administered drug/average OD value of the control wells × 100%.

Western blot analysis

Cells and tissues were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing 1 mM PMSF (Sigma-Aldrich) and a mixture of protease inhibitors (Roche), and then centrifuged at 14,000 g and 4°C for 30 min to collect the supernatant. Its protein concentration was determined using the BCA kit (Bio-Rad, Hercules, CA, USA). Equal amount of proteins were boiled for 7 min, then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted to a nitrocellulose membrane. After immersion in 5% fat-free milk, proteins were incubated with primary antibodies overnight and horseradish peroxidase conjugated secondary antibodies for 1 h. The immunoreactive products were detected using a horseradish peroxidase (HRP)-based system (Millipore, USA).

Statistical analysis

All statistical analyses were performed using SPSS 17.0. All data are expressed as the means ± S.E.M from three independent experiments. Statistical significance between two groups was determined by the student's *t*-test. Statistical analysis between groups was carried out using the one-way analysis of variance (ANOVA), followed by the Dunnett's test. $P < 0.05$ is defined as statistically significant.

Results

BJO has an anti-inflammatory effect in RAW264.7 cells

It has been found that inflammatory factors including TNF-α, IL-1β, IL-6, and IL-8 play important roles in the immune and pathological processes of pneumonia [14]. The level of IL-1β reflects the severity of infection [15]; IL-6 is related to the response of organism to stress [16]; IL-8 is primarily responsible for recruiting neutrophils to the inflamed sites and participating in the pathogenic process of pneumonia [17]. The role of TNF-α in the lung depends on its concentration. At a low concentration, it

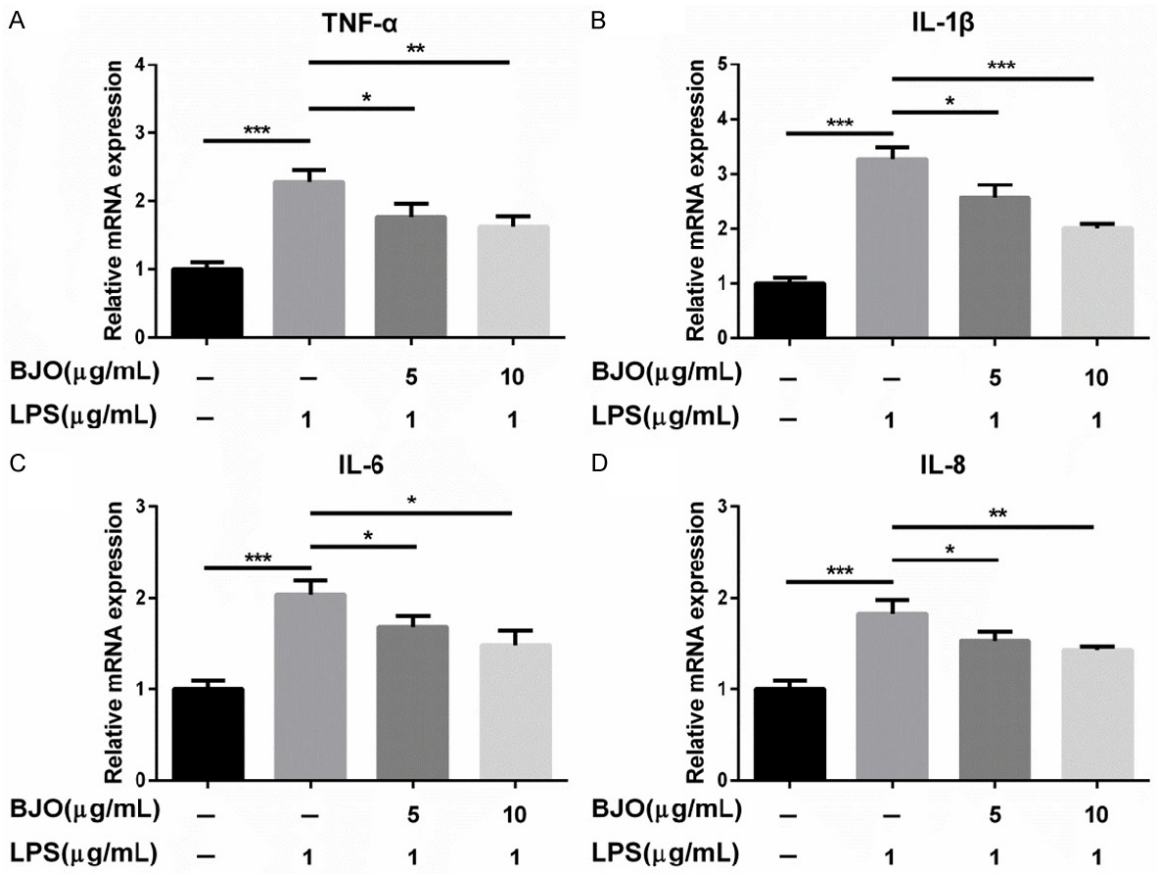

Figure 1. BJO inhibited LPS-induced expression of pneumonia-related inflammatory factors in macrophages. RAW264.7 macrophages were treated with LPS (1 μg/mL) with or without BJO (5 or 10 μg/mL as indicated) for 12 h. After that, total RNA of macrophages was extracted, and the mRNA expression levels were determined by real-time PCR. A: TNF-α; B: IL-1β; C: IL-6; D: IL-8. BJO: *Brucea javanica* oil. Student's t-test between two groups as the horizontal line indicated, statistical symbol: *P<0.05; **P<0.01; ***P<0.001.

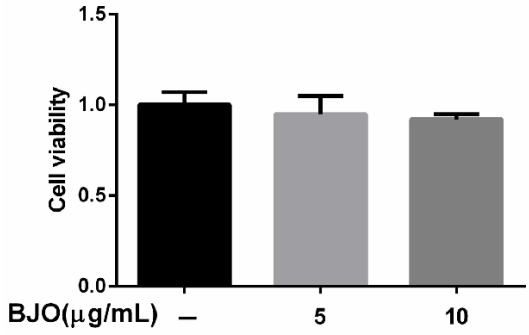

Figure 2. Effects of BJO on the survival rate of macrophages. RAW264.7 macrophages were treated with BJO (5 or 10 μg/mL as indicated) for 12 h, and then cell survival rate was detected by MTT assay. BJO: *Brucea javanica* oil.

helps to resist pathogenic invasion, while at a high concentration, it can cause damage to the body [18]. In order to investigate the anti-inflammatory effect of BJO, we stimulated RAW-264.7 macrophages with LPS to produce inflammatory factors, and then analyzed the effect of BJO inhibition on the expression and secretion of the mentioned pneumonia-related inflammatory factors induced by LPS.

Total RNA was extracted from RAW264.7 cells, and the expression levels of inflammatory factors were analyzed by real-time PCR. As a result, LPS induced the expression of these pneumonia-related inflammatory factors in RAW264.7 cells, which was significantly inhibited by BJO in a concentration-dependent manner (**Figure 1**). At these concentrations, BJO had no effect on the survival rate of RAW264.7 cells (**Figure 2**). The concentration of pneumonia-related inflammatory factors in the culture supernatant of RAW264.7 cells with or without LPS and BJO treatment was detected by ELISA to determine the secretion of these inflammatory factors.

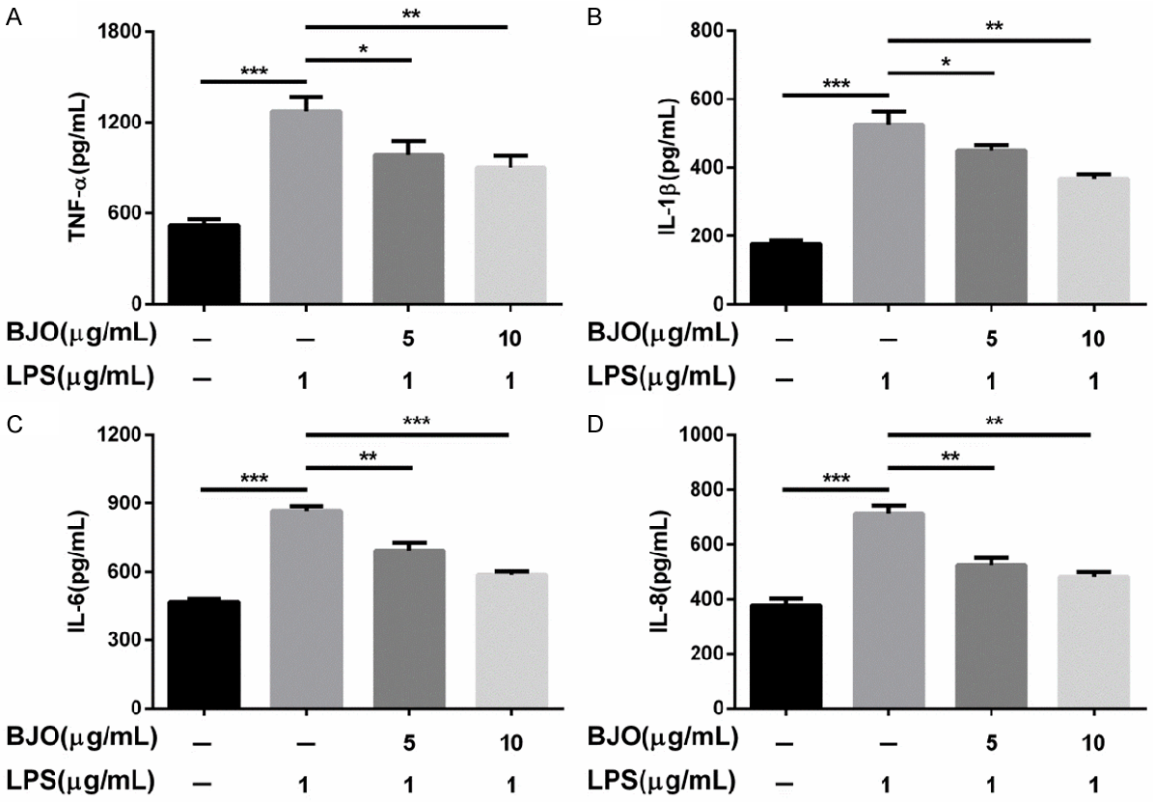

Figure 3. Inhibitory effect of BJO on LPS-induced pneumonia-related inflammatory factors secretion in macrophages. RAW264.7 macrophages were treated with LPS (1 μg/mL) with or without BJO (5 or 10 μg/mL as indicated) for 12 h. Culture supernatant was then collected, and the concentration of pneumonia-related inflammatory factors was detected by ELISA. A: TNF-α; B: IL-1β; C: IL-6; D: IL-8. BJO: *Brucea javanica* oil. Student's t-test between two groups as the horizontal line indicated, statistical symbol: $^{*}P<0.05$; $^{**}P<0.01$.

The results also showed that LPS could stimulate the secretion of pneumonia-related inflammatory factors including TNF-α, IL-1β, IL-6 and IL-8 in RAW264.7 cell culture supernatant. When cells were co-treated with BJO, the induction of LPS on the exocrine level of these inflammatory factors was significantly suppressed in a concentration-dependent manner (**Figure 3**). These results suggested an anti-inflammatory effect of BJO.

Molecular mechanisms underlying the inhibitory effect of BJO on the production of pneumonia-related inflammatory factors in RAW264.7 cells

We further explored the molecular mechanism of BJO inhibition on the production of pneumonia-related inflammatory factors in macrophages. Multiple inflammatory factors were known to be under transcriptional regulation of the transcription factor NF-κB, including TNF-α, IL-1β, IL-2, IL-6, IL-8, and IL-12. Therefore, we tested whether NF-κB was involved in the regulation of BJO on the expression and secretion of inflammatory factors by detecting the phosphorylation levels of IκB and p65, which were extensively used as markers for the activation of NF-κB pathway [19]. Western blot analysis showed that the phosphorylation levels of IκB and p65 were significantly up-regulated, while the protein level of IκB was inhibited upon LPS stimulation in RAW264.7 cells, which was effectively reversed under BJO co-treatment. However, the expression level of p65 was not influenced by LPS and BJO treatment (**Figure 4**). These results suggested that BJO might inhibit the expression and secretion of pneumonia-related inflammatory factors by down-regulating NF-κB pathway of macrophages, thus exerting its anti-inflammatory effect.

BJO has anti-inflammatory effect in LPS-induced acute pneumonia animal models

The pathological morphology of the lung tissue from mice was evaluated by HE staining. According to the results, under the light micro-

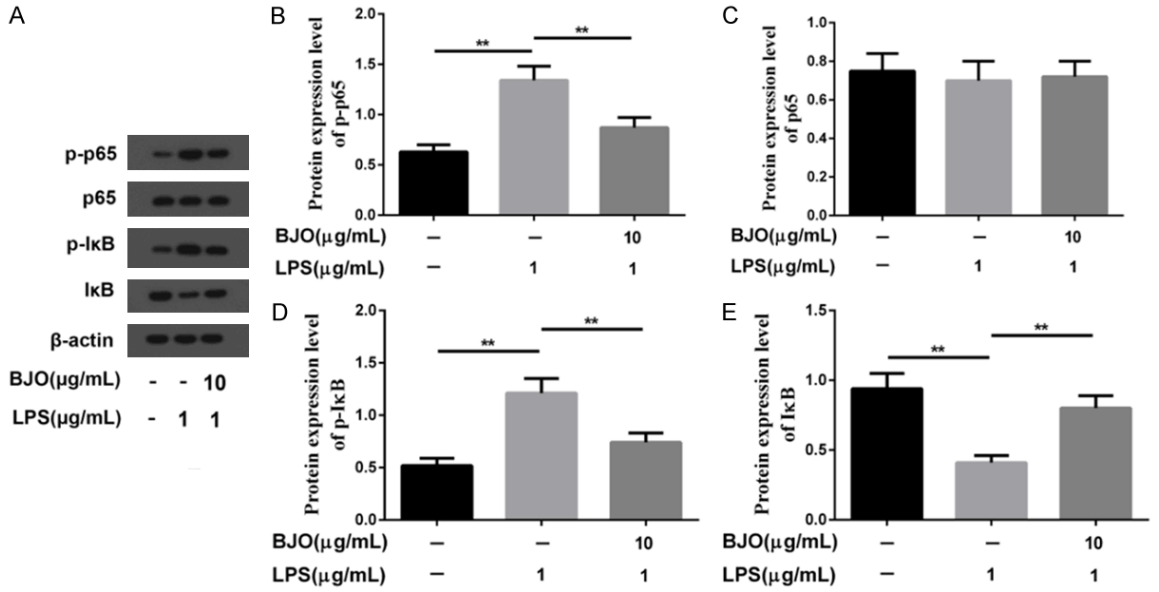

Figure 4. BJO suppressed phosphorylation of IκB and p65 induced by LPS RAW264.7 macrophages were treated with LPS (1 μg/mL) with or without BJO (10 μg/mL) for 12 h. After that, total protein of macrophages was extracted, and the phosphorylation and expression levels of IκB and p65 were determined by western blot. A: Bar graph of Western blot; B: Protein expression levelofp-p65; C: Protein expression level of p65; D: Protein expression level of p-IκB; E: Protein expression level of IκB; BJO: *Brucea javanica* oil. Student's t-test between two groups as the horizontal line indicated, statistical symbol: $^{**}P<0.01$.

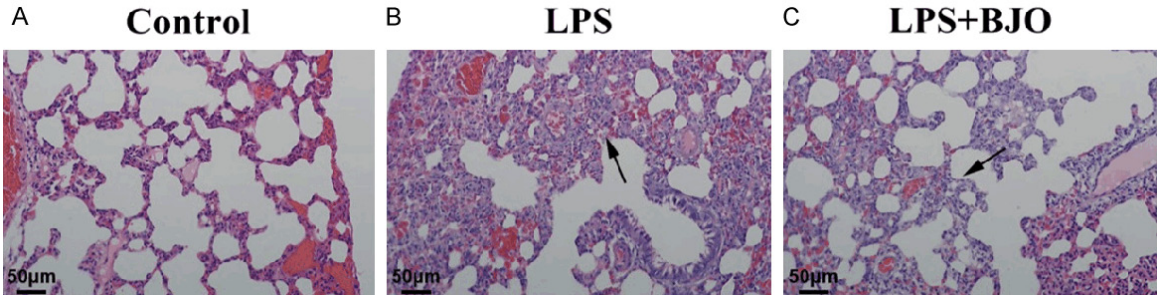

Figure 5. Pathological morphology of mouse lung tissue. Pathological changes of the lung tissue from mice were observed by HE staining (200 ×). A: Normal control group; B: LPS group; C: LPS+BJO group; BJO: Brucea javanica oil. The arrow indicated the infiltration of inflammatory cells.

scope, the normal control group showed complete lung tissue structure, clear alveolar cavity, and no inflammatory cell infiltration in the interstitial lung (**Figure 5A**); the LPS group showed destroyed alveolar structure, diffusely thickened alveolar wall, and obvious inflammatory cell infiltration (**Figure 5B**); Compared with the LPS group, the LPS+BJO group showed significantly reduced pathological changes and milder infiltration of inflammatory cells (**Figure 5C**). These results indicated that BJO could alleviate LPS-induced acute lung injury in mice.

Total RNA was extracted from the lung tissue of mice in each group, and the expression level of inflammatory factors was analyzed by real-time PCR. The results showed that the expression levels of TNF-α, IL-1β, IL-6 and IL-8 in the LSP group were significantly higher than those in the normal control group, while the expression levels of them in the LSP group were significantly lower than those in the normal control group ($P<0.05$, **Figure 6**). ELISA was also used to detect the concentration of pneumonia-related inflammatory factors in the supernatant of lung tissue suspension of mice in each

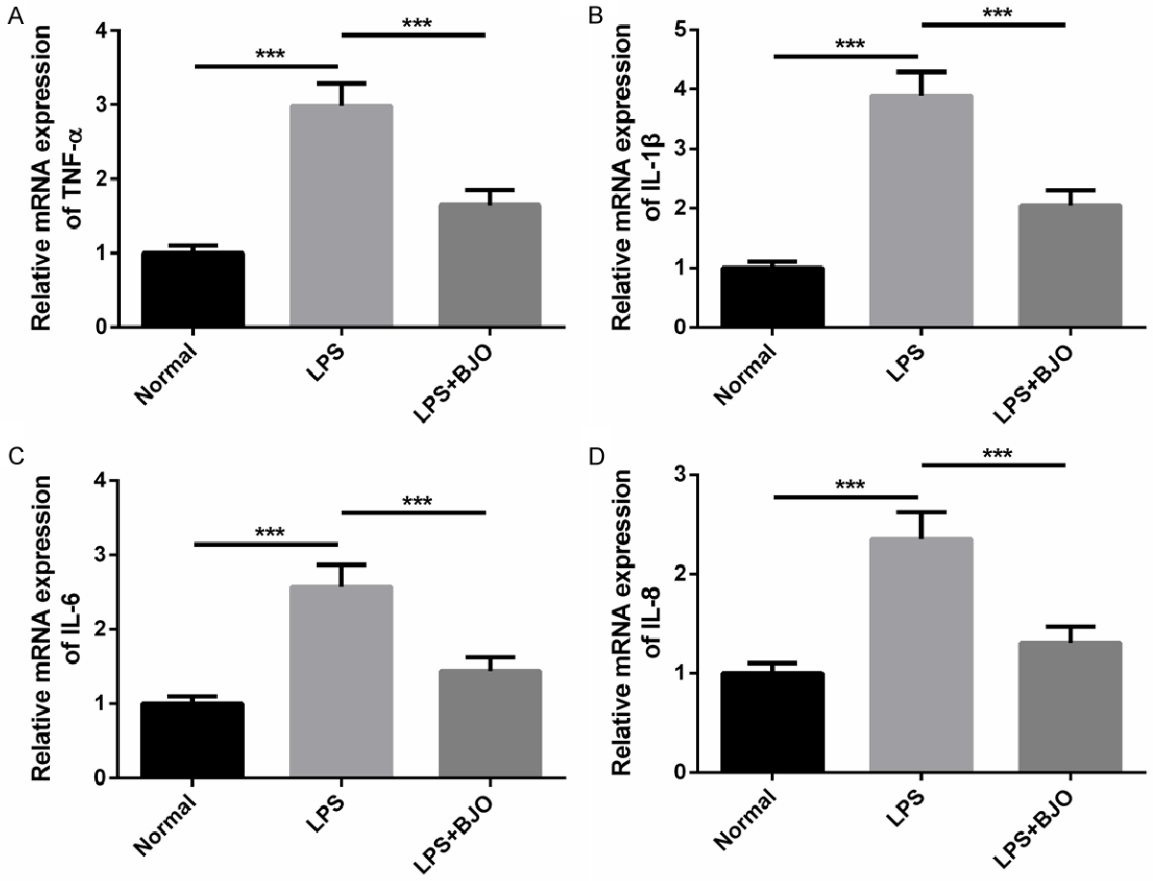

Figure 6. BJO inhibited LPS-induced expression of pneumonia-related inflammatory factors in LPS-induced acute pneumonia animal models. Total RNA was extracted from of the lung tissue of mice, and the mRNA expression levels were determined by real-time PCR. A: TNF-α; B: IL-1β; C: IL-6; D: IL-8. BJO: *Brucea javanica* oil. * means P<0.05; ** means P<0.01; *** means P<0.001. Student's t-test between two groups as the horizontal line indicated, statistical symbol: *P<0.05; **P<0.01; ***P<0.001.

group. The results showed that LPS treated with BJO significantly reduced the concentrations of TNF-α, IL-1β, IL-6 and IL-8 in the lung tissue of mice, which indicated that BJO had an anti-inflammatory effect (**Figure 7**).

The molecular mechanism underlying the inhibition of BJO in the production of pneumonia-related inflammatory factors in acute pneumonia animal models

We also detected the phosphorylation levels of IκB and p65 in acute pneumonia mouse models. The results of Western blot showed that compared with the normal control group, the LPS group showed significantly higher phosphorylation levels of IκB and p65 and significantly lower expression of IκB protein (both P< 0.05), but showed no significant difference in the expression of p65 protein (P>0.05) (**Figure 8**).

After BJO treatment, the phosphorylation levels of IκB and p65 in the lung tissue of the LPS+BJO group decreased significantly, while the expression of IκB protein increased significantly (P< 0.05) (**Figure 8**). The expression of p65 protein was not affected by LPS and BJO treatment.

Discussion

Inflammatory response is the basis of diseases in various tissue and organ systems, such as inflammatory, cancer and autoimmune diseases [20]. Macrophages play a crucial role in the process of inflammatory response by secreting cytokines that not only activate inflammatory cells and promote their phagocytosis and killing functions, but also cause cascaded expansion of inflammatory effects leading to tissue damage [21]. Therefore, the decrease in the levels of inflammatory factors, such as TNF-α and

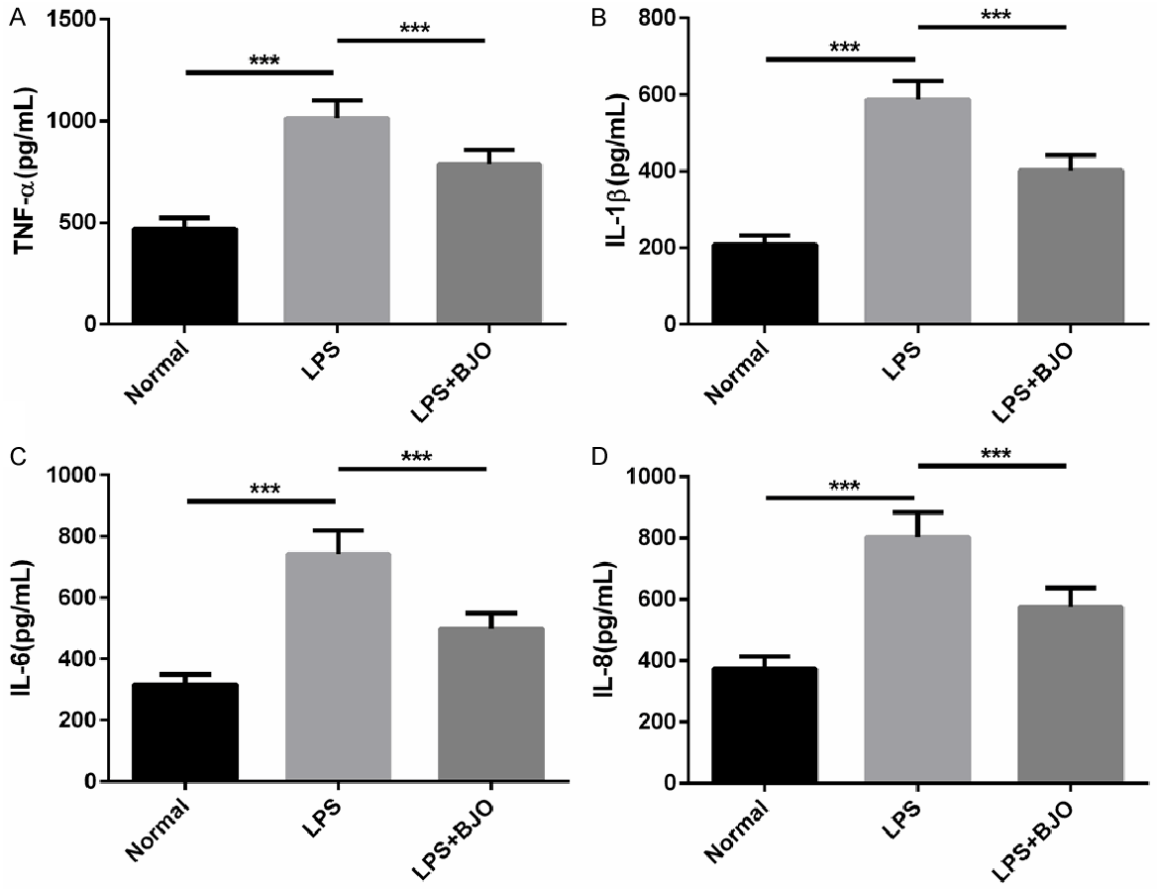

Figure 7. Inhibitory effect of BJO on the secretion of LPS-induced pneumonia-related inflammatory factors in LPS-induced acute pneumonia animal models. The concentration of pneumonia-related inflammatory factors was detected by ELISA. A: TNF-α; B: IL-1β; C: IL-6; D: IL-8. BJO: *Brucea javanica* oil. Student's t-test between two groups as the horizontal line indicated, statistical symbol: *P<0.05; **P<0.01; ***P<0.001.

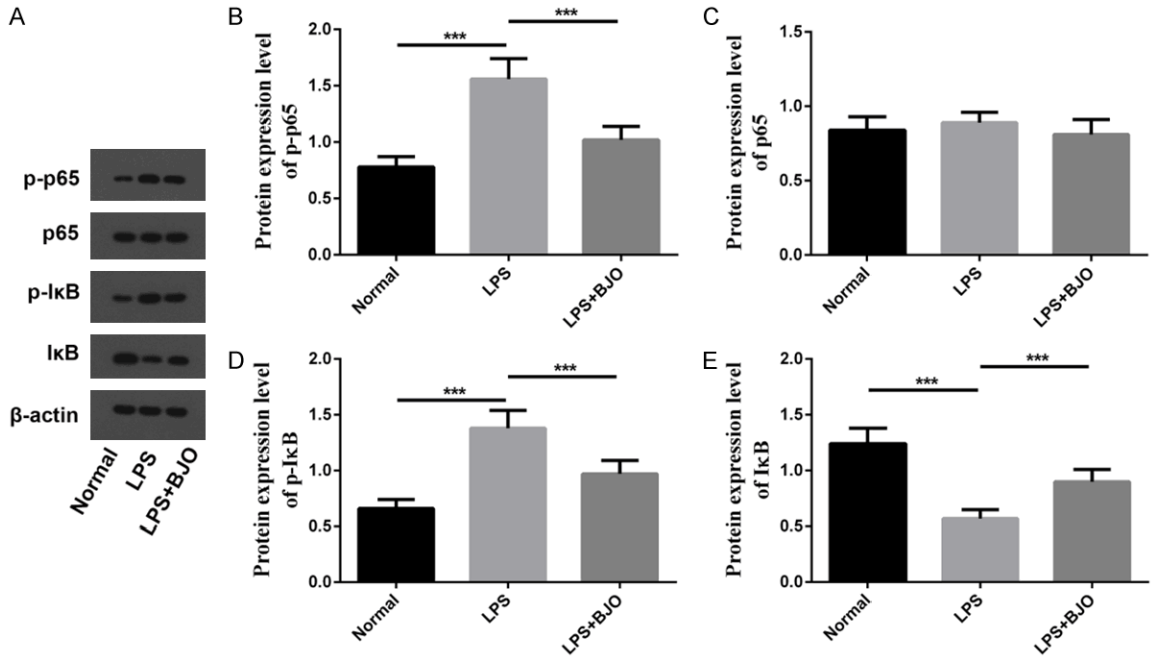

Figure 8. BJO suppressed phosphorylation of IκB and p65 induced by LPS. Total protein was extracted from the lung tissue of mice, and the phosphorylation and expression levels of IκB and p65 were determined by western blot. A: Bar graph of Western blot; B: Protein expression level of p-p65; C: Protein expression level of p65; D: Protein expression level of p-IκB; E: Protein expression level of IκB; BJO: *Brucea javanica* oil. Student's t-test between two groups as the horizontal line indicated, statistical symbol: ***P<0.001.

IL-6, can be used as detection indexes for the anti-inflammatory effect of drugs. It has been reported that mycoplasma pneumoniae induced RAW264.7 cells to secrete inflammatory factors TNF-α, IL-1β, IL-6 and IL-8, which can be intervened by drugs [16-18]. In the present study, macrophages were treated with bacterial lipopolysaccharide to induce inflammation models, and the effect of BJO on the levels of these pneumonia-related cytokines and the related mechanism were further studied.

BJO is a traditional Chinese medicine extracted from the seeds or nuts of Brucea javanica with petroleum ether [22], which is used not only to treat lung cancer, with anti-gastritis and colitis effects [23, 24]. BJO also has the potential to treat pneumonia. In our study, the expression and secretion of TNF-α, IL-1β, IL-6 and IL-8 were negatively correlated with the dosage of BJO, indicating that BJO prevented macrophages from producing and releasing pneumonia-related inflammatory factors and therefore exerted an anti-inflammatory effect. TNF-α and IL-1β are two of the main inflammatory factors secreted in the early stage of inflammation, which promote endothelial cells to produce chemokines and adhesion factors that further recruit neutrophils, and thus play key roles in secondary inflammatory reaction [25]. These early inflammatory factors cause the release of the secondary inflammatory factors, such as IL-6 and IL-8 [26]. Therefore, the present study revealed that BJO not only inhibited the production of inflammatory factors TNF-α and IL-1β in the early stage of pneumonia, but also suppressed the secondary inflammatory response of pneumonia by blocking the release of the secondary inflammatory factors IL-6 and IL-8.

Numerous studies have reported NF-κB as an important transcription factor in inflammation [27, 28]. NF-κB in an inactive state is detained by IκB in the cytoplasm. Upon stimulus such as LPS, IκB is phosphorylated and undergoes degradation, leading to the release and phosphorylation of NF-κB/p65, which subsequently translocates to the nucleus and promotes the transcription of its downstream target genes, including multiple inflammatory factors [29]. In this study, we have found that BJO can suppress the activity of NF-κB pathway by inhibiting the phosphorylation of IκB and NF-κB/p65 and thus alleviate inflammatory response.

In conclusion, BJO exhibits an anti-inflammatory effect by inhibiting NF-κB activation, which provides a possible theoretical basis for BJO emulsion as an adjuvant treatment drug for pneumonia. However, there are still some limitations of this study, as the effect of BJO is not limited to anti-inflammatory, some other functions such as anti-oxidant effects need further investigation. Besides, there might be other pathway that is activated during anti-inflammatory process. Hence, more experiments are needed to further understand the mechanism of anti-inflammatory in BJO treatment of MI.

Acknowledgements

This paper is supported by a Startup Fund for scientific research from Fujian Medical University, China (Grant number: 2017XQ1129), and a fund for young and middle-aged backbone from Fujian Provincial Health Science and Technology Project, China (Grant number: 2020GGB002).

Disclosure of conflict of interest

None.

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