Benzoylpaeoniflorin activates anti-inflammatory mechanisms to mitigate sepsis in cell-culture and mouse sepsis models

Chaeyeong Kim
Kyungpook National University
Hyunchae Sim
Kyungpook National University
Nayeon Kim
Kyungpook National University
Jong-Sup Bae (baejs@knu.ac.kr)
Kyungpook National University https://orcid.org/0000-0002-5756-9367

Research Article

Keywords: Benzoylpaeoniflorin, Lipopolysaccharide, Inflammation, Endothelium, CLP, Sepsis

Posted Date: April 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1548378/v1

Abstract

Background: *Xuebijing* injection (XBJI) (comprising of five herbs) is a widely used traditional Chinese medicine for sepsis treatment. However, the bioactive components of XBJI and the mechanisms responsible for its sepsis-mitigating action have not been experimentally determined. One of the main bioactive compounds in XBJI- benzoylpaeoniflorin (BPF) - inhibits the expressions of key mediators of inflammation such as nuclear factor kappa B (NF-κB), cyclooxygenase-1 (COX-1) and COX-2. However, its effects on sepsis are not determined yet. Therefore, here, we investigated the immunomodulatory effect of BPF on severely inflamed endothelial cells, THP-1 macrophages, peritoneal macrophages, and mice.

Methods: Human umbilical vein endothelial cells (HUVECs) and THP-1-macrophages were activated using lipopolysaccharide (LPS) after pretreatment with BPF. Subsequently, changes in expression profiles of pro-inflammatory cytokines including inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 were determined using quantitative real-time polymerase chain reaction (qPCR) and western blot analysis. Further, we monitored the phosphorylation of NF-kB and mitogen-activated protein kinases (MAPKs) to determine their activation levels. Using LPS-induced mouse model of sepsis we studied the effects of BPF on inflammatory cytokine production, pulmonary histopathology, and survival rates. Finally, we evaluated if BPF protects against cecal ligation and puncture (CLP)-induced sepsis, as it closely mimics human sepsis.

Results: BPF pretreatment inhibited LPS-induced increase in mRNA and protein levels of iNOS, TNF-α, and IL-6 in HUVECs and THP-1-macrophages. It also suppressed LPS-mediated phosphorylation p65, p38, JNK, and ERK. Mice with LPS-induced-sepsis who were treated with BPF had lower serum levels of IL-6, TNF-α, IL-1β, CXCL1, and CXCL2 than the control mice treated with BPF. Histopathology revealed that BPF treatment alleviated LPS-induced lung damage. In addition, in mice given a lethal dose of LPS, BPF treatment showed a dose-dependent improvement in survival rates. BPF treatment dose-dependently inhibited the LPS-induced IL-6, TNF-α, and CXCL1 production in peritoneal macrophages. BPF treatment also dose-dependently improved the survival rates in mice with CLP-induced sepsis.

Conclusion: These results show that BPF alleviates LPS-stimulated septic conditions and protects mice from CLP-induced sepsis. Our research marks BPF as a potential drug in the treatment of sepsis and various inflammatory diseases.

Introduction

Sepsis is the main cause of death in intensive care units. It is characterized by whole-body inflammation known as “systemic inflammatory response syndrome” or SIRS. It is increasingly responsible for common illnesses and deaths, especially in older adults, immunocompromised, and severely ill patients [1-3]. Pattern recognition receptors (PRRs) are typical innate immune sensors that recognize a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [1-3]. Overproduction of PAMP and DAMP sensors during sepsis is implicated in multiple organ failure

(MOF) [1-3]. Toll-like receptor 4 (TLR4) is a PRR that recognizes lipopolysaccharide (LPS) [3]. Upon binding to LPS, TLR4 recruits adaptor proteins such as myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) which activates inflammatory signaling cascade. This inflammatory signaling cascade further activates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), ultimately leading to expression of inflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS) [3]. Although regulated activation of the innate immune response plays a pivotal role in host defense against microbial infection and cellular homeostasis, excessive immune activation can lead to a variety of inflammation-related diseases (such as autoimmune diseases and cytokine release syndrome). Therefore, it is important to medically control the immune overdrive [4]. However, the clinical methods for treatment of septic injury are limited with no approved drugs currently available for sepsis management. Therefore, finding a safe, non-toxic yet effective treatment is vital to manage sepsis.

Traditional Chinese medicine prescriptions (TCMP) have historically been used to prevent and treat various diseases. *Xuebijing* injection (XBJI) is a clinical TCMP. XBJI is an aqueous extract of five traditional medicinal herbs - viz. *Flos Carthami*, *Radix Paeoniae Rubra*, *Radix Salviae Miltiorrhizae*, *Rhizoma Chuanxiong,* and *Radix Angelicae Sinensis* [5, 6]. XBJI was approved by China Food and Drug Administration (CFDA) in 2004 for clinical treatment of SIRS, MOF, and sepsis [7]. It is effective in suppressing the cytokine storm, reducing inflammation, activating blood circulation, detoxifying, and reducing organ damage [8]. The four active compounds of XBJI are - safflor yellow A, oxypaeoniflorin, benzoylpaeoniflorin (BPF, Fig. 1A), and ferulic acid. Among them BPF inhibits NF-κB activity and cyclooxygenase-1 (COX-1) and COX-2 expressions [9, 10]. However, the in vivo immunomodulatory and immunoregulatory effects (and mechanisms) of BPF alone have not been studied yet. Therefore, here, we tried to determine the immunomodulatory effects of BPF and its molecular mechanisms in endothelial cell culture, THP-1-macrophages, peritoneal macrophages and murine sepsis models.

Methods

Reagents and antibodies

BPF (purity > 98%) was purchased from Selleck Chemicals (Houston, TX, USA). LPS derived from bacteria (serotype: 0111:B4, L5293) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-iNOS and anti-β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as previously described [11]. Cells were cultured in EBM-2 basal media (Cambrex Bio Science) at 37°C under 5% CO_2. THP-1 cells, derived from a human monocyte line, were purchased from the ATCC (Manassas, VA, USA) and cultured as previously described [12].

Differentiation of THP-1 cells into macrophages was induced by incubating them in complete medium containing PMA (50 μM) for 48 h followed by incubation in complete medium without PMA for 48 h.

Animal experiments

Male C57BL/6 mice (8-week-old, 27 ± 0.5 g) were purchased from Orient Bio Co. (Sungnam, Republic of Korea). All animal experiment protocols were approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2020-107). Each animal sepsis model was established as below-

LPS-induced septic shock model: LPS (2.5 mg/kg) was administered to mice by intraperitoneal injection. Simultaneously, BPF was administered intravenously. For cytokine analysis, the mice were euthanized 4 h later. The blood and ascitic fluid were collected and centrifuged at 9500 *g* at 4 ° C for 10 min. The supernatant was used for cytokine analysis using ELISA. For lung histological analysis, the septic shock was extended to 24 h.

LPS-induced lethal septic shock model: Lethal dose of LPS (25 mg/kg) was administered to mice by intraperitoneal injection. Simultaneously, BPF was administered intravenously. Mouse survival was observed every 4 h for 36 h.

Cecal Ligation and Puncture (CLP) sepsis model: Mice were prepared as described previously [13]. Sham operated animals underwent laparotomy without ligation and puncture of the cecum. BPF was injected intravenously and the mice were observed every 12 h for 96 h.

Murine peritoneal macrophage isolation

Mice were administered 3 mL of 3% thioglycolate by intraperitoneal injection. Three days later mice were euthanized. Peritoneal macrophages were collected and washed with PBS. Subsequently, they were seeded in 48-well plates at a density of 5 x 10^5 cells/mL.

Methyl-thiazolyl-tetrazolium (MTT) cell viability assay

The cultured HUVECs and THP-1-macrophages were incubated with BPF at the indicated concentrations in serum-free medium. After 48 h, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to estimate cell viability [14, 15].

Enzyme-linked immunosorbent assay (ELISA)

The activities of pp65, pp38, pJNK, and pERK cellular proteins were determined by using commercially available ELISA kits (Cell Signaling Technology). The levels of IL-6, TNF-α, CXCL1, and CXCL2 in the cell culture supernatants were determined by using ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR)

RNA was purified using TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) extraction. The purified RNA was reverse transcribed with a PX2 Thermal Cycler (Thermo Fisher Scientific) using 0.5 mg/ml of the oligo (dT)-adapter primer (Thermo Fisher Scientific) and M-MLV reverse transcriptase (Thermo Fisher Scientific) in a 20 μL reaction mixture. The primer sequences were as follows: IL-6; forward, 5'-GCA CTG GCA GAA AAC AAC CT-3' and reverse, 5'-TCA AAC TCC AAA AGA CCA GTG A-3'. TNF-α; forward, 5'-CCC AGG GAC CTC TCT CTA ATC-3' and reverse, 5'-ATG GGC TAC AGG CTT GTC ACT-3'. iNOS; 5'-TCC AGG AGG ACA TGC AGC AC3' and reverse, 5'-CGC CCT TCC GCA GTT CT-3'. β-actin; forward, 5'-CCT GGC ACC CAG CAC AAT-3' and reverse, 5'-GCC GAT CCA CAC GGA GTA CT-3'. Quantitative gene expression levels are normalized to the expression levels of β-actin.

Immunoblotting

Lysis buffer containing Nonidet P-40, complete protease inhibitor cocktail (Roche, Mannheim, Germany), 2 mM dithiothreitol, and phosphatase inhibitor cocktail 2 (Sigma-Aldrich) was used to lyse the cells. The proteins in the treated cell lysate were separated using SDS-PAGE and transferred to nitrocellulose membranes. Transferred proteins were incubated with primary antibodies corresponding to each target protein and then with the relevant secondary antibodies.

Measurement of nitrite levels in cell culture supernatants

Conditioned cell culture supernatants were harvested and nitric oxide concentrations were determined using the Griess reaction assay.

Hematoxylin and eosin (H&E) staining

Five mice were used for histopathological analysis. Mice from LPS-induced septic shock model were euthanized and the left lobes of lungs were fixed in 10% neutral formalin for 24 h, followed by tissue processing and paraffin embedding. The paraffin blocks were sectioned into 2-μm thick sections and stained with H&E. Histopathological scoring was done by three experimental veterinary medicine experts using a random score index based on the degree of inflammatory cell infiltration and the extent of the injury area (0, normal; 1, mild; 3, moderate; 5, severe) with slight modifications from previous studies [16, 17].

Statistical analysis

Results are expressed as mean ± standard deviation (SD) of three independent experiments. Results were analyzed using ANOVA (one-way) followed by Tukey's post-hoc tests and differences at $p < 0.05$ were considered significant. The Kaplan–Meier method was used to compare differences in survival outcomes following LPS- or CLP-induced sepsis experiments.

Results

BPF is non-cytotoxic and reduces LPS-induced inflammatory cytokine production by HUVECs and THP-1-macrophages

Endothelial cells elicit a strong immunological response and secrete a variety of inflammatory cytokines in response to LPS stimulation [18]. To investigate whether BPF regulates this inflammatory response, HUVECs pretreated with BPF were stimulated with LPS. The IL-6 and TNF-α expression levels were measured. BPF pretreatment significantly suppressed the mRNA expression of IL-6 and TNF-α by LPS in a time- (Fig. 1B, C) and dose-dependent (Fig. 1D, F) manner. BPF pretreatment also reduced the LPS-induced increase in IL-6 and TNF-α protein levels in a dose-dependent manner (Fig. 1F, G). Consistently, in THP-1-macrophages, BPF pretreatment significantly inhibited LPS-induced IL-6 and TNF-α mRNA expression in a time- (Fig. 2A, B) and dose-dependent manner (Fig. 2C, D), and effectively suppressed the LPS-induced IL-6 and TNF-α production (Fig. 2E, F). Moreover, MTT assay revealed that 48 h exposure to BPF (<50 μM) was non-cytotoxic to both cell types (Fig. 2G).

BPF inhibits LPS-induced expression of inducible nitric oxide synthase (iNOS) and LPS+IFN-γ-induced production of nitric oxide (NO) by HUVECs

We evaluated whether BPF regulates the expressions of iNOS (mainly responsible for NO synthesis, in the endothelium) and NO. BPF pretreatment significantly inhibited LPS-induced iNOS mRNA levels in a time- (Fig. 3A) and iNOS protein levels in a dose- (Fig. 3B) dependent manner in HUVECs. We found that LPS stimulation was not enough for NO production by HUVECs; interferon-γ (IFN-γ) had to be co-applied with LPS to stimulate NO production. BPF markedly inhibited LPS+IFN-γ-induced NO production in a dose-dependent manner in HUVECs (Fig. 3C), (consistent with the iNOS expression results).

BPF reduces LPS-induced phosphorylation of NF-κB (p65) and MAPK (p38, JNK, and ERK), in HUVECS and THP-1-macrophages

Since MAPK-activated transcription factor NF-kB and activator protein 1 (AP-1) mediate the expression of IL-6, TNF-α, and iNOS during PRR activation, we next determined the effect of BPF on the phosphorylation of NF-κB (p65) and MAPKs (such as p38, JNK, ERK) in LPS-stimulated HUVECs and THP-1-macrophages. BPF pretreatment considerably reduced the LPS-induced activation of p65, p38, JNK, and ERK (Figs. 4A-4D) in HUVECs and in THP-1-macrophages (Figs. 4E, 4F) in time- and dose-dependent manner. Thus, BPF suppresses LPS-induced inflammation by inhibiting activation of inflammation-related transcription factors in human cells.

BPF reduces cytokine production, pulmonary damage, and mortality in LPS-induced mouse septic shock model

We next verified if these in vitro results could be replicated in vivo. We used the LPS-induced mouse septic shock model. We found that BPF protected against LPS-induced sepsis. BPF (i.v. 0.22 or 0.44 mg/kg) treatment significantly reduced the cytokine levels in serum and peritoneal fluid (Figs. 5A-5E), and lung tissue damage (Figs. 5F, 5G) in LPS-stimulated mice (LPS sublethal dose, 2.5 mg/kg). Furthermore, BPF

protected against lethal dose of LPS. Effect of BPF treatment on mice injected with LPS (lethal 25 mg/kg) was studied by monitoring every 4 h for 36 h. While only 2 out of 20 control (lethal LPF + no BPF treatment) mice survived, 8 out of 20 and 12 of 20 of mice administered lethal LPS + 0.22 mg/kg BPF and lethal LPS + 0.44 mg/kg BPF, survived, respectively (Fig. 5H). Therefore, administration of BPF significantly reduced LPS-induced mortality in mice.

BPF inhibits the CLP-induced cytokines production by peritoneal macrophages and improves survival rate of mice with CLP-induced sepsis

Sepsis peritoneal macrophages modulate SIRS by producing anti-inflammatory cytokines [19]. CLP-induced sepsis model is the golden standard for animal sepsis model mimicking human sepsis [20]. Therefore, we checked if BPF inhibits the CLP-induced production of inflammatory cytokines by peritoneal macrophages. BPF treatment, indeed, inhibited the CLP-induced production of IL-6, TNF-α, and CXCL1 by sepsis-induced peritoneal macrophages (Figs. 6A-6C) (consistent with the results in HUVECs and THP-1-macrophages). To determine whether BPF affects survival of mice with CLP-induced sepsis, we administered BPF intravenously to CLP-operated mice and monitored their survival for 96 h. While all control mice (treated with DMSO) died 48 h after CLP surgery, 9 of 20 BPF (0.22 mg/kg)-injected mice and 13 of 20 BPF (0.44 mg/kg)-injected mice survived for 96 h post CLP surgery (Fig. 6D).

Discussion

Over the years, many traditional Chinese herbal medicines have been adopted for medical use owing to their verified clinical effects; they are widely studied and have well-known safety profiles with low toxicity. Natural chemicals extracted from these medicinal herbal plants have been recognized as pharmacologic supplements in various inflammatory disorders [21, 22]. These chemicals inhibit inflammatory mediators (NF-κB, MAP Kinases), immune-related transcription factors and expressions of inflammatory cytokines (TNF-α or IL-series) [23, 24]. XBJI treatment reduces mortality, anal temperature, and the expression of inflammatory factors such as TNF-α, IL-1β, and IL-6 when administered to mouse sepsis models [7, 8]. Since NF-κB inhibition is the key action of XBJI in alleviating sepsis, six ingredients with NF-κB inhibitory properties —senkyunolide I, paeoniflorin, danshensu, safflor yellow A, oxypaeoniflorin and BPF — were identified in XBJI using UPLC-Q/TOF based protocols [9]. We focused on BPF as a sepsis drug candidate because among the six NF-κB inhibitors in XBJI, BPF is (1) the most abundant component, (2) the most effective inhibitor of NF-κB activity, and (3) an inhibitor of COX-1 and COX-2 activities. Thus, we aimed to clarify novel in vitro and in vivo sepsis-mitigating and anti-inflammatory effects of BPF. Here, we show that BPF suppresses the LPS-induced inflammatory cytokines release by HUVECs and TPH-1-macrophages. It also exerts anti-inflammatory effects on LPS- and CLP- induced sepsis . Thus, it protects mice from sepsis.

Long-term dysregulation of inflammatory genes including inflammatory cytokines, iNOS, and COX leads to chronic inflammation that contributes to a variety of inflammatory diseases [25]. Further, MAPKs (such as p38, JNK, and ERK) respond to various stimuli by inducing expression of pro-inflammatory mediators.

The amplified inflammatory responses can thus be seen as interplay of NF-κB and MAPKs pathways. Logically, drugs for inflammatory diseases must target MAPKs activities [25]. Thus, agents which modulate anti-inflammatory mediators emerge as good candidates for developing therapeutics for a variety of inflammatory diseases. We found, here, that BPF exerts several anti-inflammatory effects by suppressing LPS-induced : 1) iNOS and NO generation; 2) TNF-α and IL-6 expressions; and 3) JNK, ERK, and p38 MAPK activation. BPF also suppresses the PRR activation-induced strong expression of COX-1 and -2 [10]. Overall, BPF suppresses the increase in iNOS and COX-2 levels by inhibiting the LPS-stimulated activation of JNK and p38 MAPK. Thus, BPF protects against the effects of the inflammatory immune response via the blocking of NF-κB-JNK/P38K pathway, which in turn inhibits expression of pro-inflammatory genes.

Severity and prognosis of inflammatory diseases are closely associated with exaggerated inflammatory responses [26]. In particular, sepsis develops when appropriate host responses that are amplified initially, to tackle an infection, are combined with poor control of subsequent infections, leading to an imbalance between inflammatory and anti-inflammatory responses [26, 27]. Expression and regulation of inflammatory and anti-inflammatory cytokines are significantly related to the prognosis of patients with sepsis [27]. For example, the excessive production of pro-inflammatory cytokines launched by the host for effective immune response can lead to cytokine storm and subsequent septic shock, MOF, and death [27]. This kicks in overproduction of anti-inflammatory cytokines (such as IL-10 and IL-1 receptor antagonists) to inhibit excessive inflammatory responses and maintain host homeostasis which leads to the inhibition of the immune system. Thus, promoting bacterial burden and increasing host mortality [26, 27]. Therefore, treatment of patients with sepsis entails mitigating the overactivation of pro- and anti-inflammatory responses to alleviate septic shock and bacterial burden.

Based on the results of anti-inflammatory effects of BPF on THP-1-macrophages, we hypothesized that BPF would effectively inhibit the release of pro-inflammatory cytokines in sepsis. We verified this using LPS-induced mouse septic shock model. As expected, BPF significantly suppressed LPS-induced IL-6, TNF-α, CXCL1 and CXCL2 production and mortality in the mice. Moreover, our data showed that BPF significantly inhibits the LPS-induced production of inflammatory cytokines by peritoneal macrophages, which play an important role in SIRS [28]. Even in CLP-induced mouse sepsis model, administration of BPF inhibited the production of pro-inflammatory cytokines by peritoneal macrophages and improved the survival rate.

Xigris, used to treat sepsis, was withdrawn from market in 2011 due to issues with side effects and lack of efficacy [29] (licensed by Food and Drug Administration (FDA) in 2001 and by European Medical Agency (EMA) in 2002) [30]. Since then there has been no approved treatment for sepsis. Therefore, development of alternative drugs for sepsis has becomes imperative. Although studies on side effects and toxicity of BPF are lacking (and need to be conducted in future), results of our study indicate that BPF will be a good candidate for treatment of sepsis and various inflammatory diseases.

Conclusion

In conclusion, BPF is protective as it significantly inhibits LPS-induced inflammatory cytokine production in endothelial cells, ameliorates lung tissue damage, and reduces mortality in septic mice. Thus, BPF may be a potential drug in treating various vasculitis-related diseases.

Abbreviations

XBJI, Xuebijing injection; BPF, benzoylpaeoniflorin; NF-κB, nuclear factor kappa B; COX-1, cyclooxygenase-1; HUVECs, Human umbilical vein endothelial cells; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha; IL-6, interleukin (IL)-6; qPCR, quantitative real-time polymerase chain reaction; MAPKs, mitogen-activated protein kinases; CLP, cecal ligation and puncture; SIRS, systemic inflammatory response syndrome; PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; MOF, multiple organ failure; TLR4, Toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β; TCMP, Traditional Chinese medicine prescriptions; CFDA, China Food and Drug Administration; PMA, phorbol 12-myristate 13-acetate; MTT, Methyl-thiazolyl-tetrazolium; AP-1, activator protein 1; FDA, Food and Drug Administration; EMA, European Medical Agency

Declarations

Acknowledgments; Not applicable.

Authors' contributions; Nayeon Kim and Hyunchae Sim, Conceptualization, Methodology, Investigation; Nayeon Kim, Methodology, Data curation, Resources; Jong-Sup Bae, Conceptualization, Methodology, Supervision, Resources, Resources, Writing- Reviewing and Editing

Funding; This study was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C0001) and by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2020R1A2C1004131).

Ethics approval and consent to participate; This study was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2020-107).

Consent for publication; Not applicable.

Competing interests; Not applicable.

Availability of data and materials; Not applicable.

References

1. Dickson K, Lehmann C. Inflammatory Response to Different Toxins in Experimental Sepsis Models. Int J Mol Sci. 2019;20(18):4341.

2. Ziesmann MT, Marshall JC. Multiple Organ Dysfunction: The Defining Syndrome of Sepsis. Surg Infect (Larchmt). 2018;19(2):184–90.
3. Bae JS. Role of high mobility group box 1 in inflammatory disease: Focus on sepsis. Arch Pharm Res. 2012;35(9):1511–23.
4. Nathan C. Points of control in inflammation. Nature. 2002;420(6917):846–52.
5. Zuo L, Zhong Q, Wang Z, Sun Z, Zhou L, Li Z, et al. Simultaneous determination and pharmacokinetic study of twelve bioactive compounds in rat plasma after intravenous administration of Xuebijing injection by UHPLC-Q-Orbitrap HRMS. J Pharm Biomed Anal. 2017;146:347–53.
6. Zuo LH, Sun Z, Hu YR, Sun Y, Xue WH, Zhou L, et al. Rapid determination of 30 bioactive constituents in XueBiJing injection using ultra high performance liquid chromatography-high resolution hybrid quadrupole-orbitrap mass spectrometry coupled with principal component analysis. J Pharmaceut Biomed. 2017;137:220–8.
7. Yin Q, Li C. Treatment effects of xuebijing injection in severe septic patients with disseminated intravascular coagulation. Evid Based Complement Alternat Med. 2014;2014:949254.
8. Wang P, Song Y, Liu Z, Wang H, Zheng W, Liu S, et al. Xuebijing injection in the treatment of severe pneumonia: study protocol for a randomized controlled trial. Trials. 2016;17(1):142.
9. Jiang M, Zhou M, Han Y, Xing L, Zhao H, Dong L, et al. Identification of NF-kappaB Inhibitors in Xuebijing injection for sepsis treatment based on bioactivity-integrated UPLC-Q/TOF. J Ethnopharmacol. 2013;147(2):426–33.
10. Zheng Y, Xin G, Gong G, Dong TT, Li P, Tsim KWK. Evaluation of Anti-Inflammatory Components of Guizhi Fuling Capsule, an Ancient Chinese Herbal Formula, in Human Umbilical Vein Endothelial Cells. Evid Based Complement Alternat Med. 2020;2020:2029134.
11. Bae JS, Rezaie AR. Protease activated receptor 1 (PAR-1) activation by thrombin is protective in human pulmonary artery endothelial cells if endothelial protein C receptor is occupied by its natural ligand. Thromb Haemost. 2008;100(1):101–9.
12. Yang EJ, Lee W, Ku SK, Song KS, Bae JS. Anti-inflammatory activities of oleanolic acid on HMGB1 activated HUVECs. Food Chem Toxicol. 2012;50(5):1288–94.
13. Jung B, Kang H, Lee W, Noh HJ, Kim YS, Han MS, et al. Anti-septic effects of dabrafenib on HMGB1-mediated inflammatory responses. BMB Rep. 2016;49(4):214–9.
14. Jeong SY, Kim M, Park EK, Kim J-S, Hahn D, Bae J-S. Inhibitory Functions of Novel Compounds from Dioscorea batatas Decne Peel on HMGB1-mediated Septic Responses. Biotechnol Bioproc Eng. 2020;25(1):1–8.
15. Lee I-C, Ryu C-W, Bae J-S. Novel Herbal Medicine C-KOK Suppresses the Inflammatory Gene iNOS via the Inhibition of p-STAT-1 and NF-κB. Biotechnol Bioproc Eng. 2020;25(4):536–42.
16. Lee WH, Choo S, Sim H, Bae JS. Inhibitory Activities of Ononin on Particulate Matter-induced Oxidative Stress. Biotechnol Bioproc Eng. 2021;26(2):208–15.

17. Sim H, Noh Y, Choo S, Kim N, Lee T, Bae JS. Suppressive Activities of Fisetin on Particulate Matter-induced Oxidative Stress. Biotechnol Bioproc Eng. 2021;26(4):568–74.
18. Mai J, Virtue A, Shen J, Wang H, Yang XF. An evolving new paradigm: endothelial cells–conditional innate immune cells. J Hematol Oncol. 2013;6:61.
19. Schulte W, Bernhagen J, Bucala R. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets–an updated view. Mediators Inflamm. 2013;2013:165974.
20. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc. 2009;4(1):31–6.
21. Calixto JB, Campos MM, Otuki MF, Santos AR. Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med. 2004;70(2):93–103.
22. Lu AP, Jia HW, Xiao C, Lu QP. Theory of traditional Chinese medicine and therapeutic method of diseases. World J Gastroenterol. 2004;10(13):1854–6.
23. Langer HF, Chavakis T. Leukocyte-endothelial interactions in inflammation. J Cell Mol Med. 2009;13(7):1211–20.
24. Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol. 2008;83(3):536–45.
25. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. Int J Mol Sci. 2019;20(23).
26. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018;9(6):7204–18.
27. Chaudhry H, Zhou J, Zhong Y, Ali MM, McGuire F, Nagarkatti PS, et al. Role of Cytokines as a Double-edged Sword in Sepsis. In Vivo. 2013;27(6):669–84.
28. Ayala A, Elphick GF, Kim YS, Huang X, Carreira-Rosario A, Santos SC, et al. Sepsis-Induced Potentiation of Peritoneal Macrophage Migration Is Mitigated by Programmed Cell Death Receptor-1 Gene Deficiency. J Innate Immun. 2014;6(3):325–38.
29. Thachil J, Toh CH, Levi M, Watson HG. The withdrawal of Activated Protein C from the use in patients with severe sepsis and DIC [Amendment to the BCSH guideline on disseminated intravascular coagulation]. Br J Haematol. 2012;157(4):493–4.
30. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001;344(10):699–709.

Figures

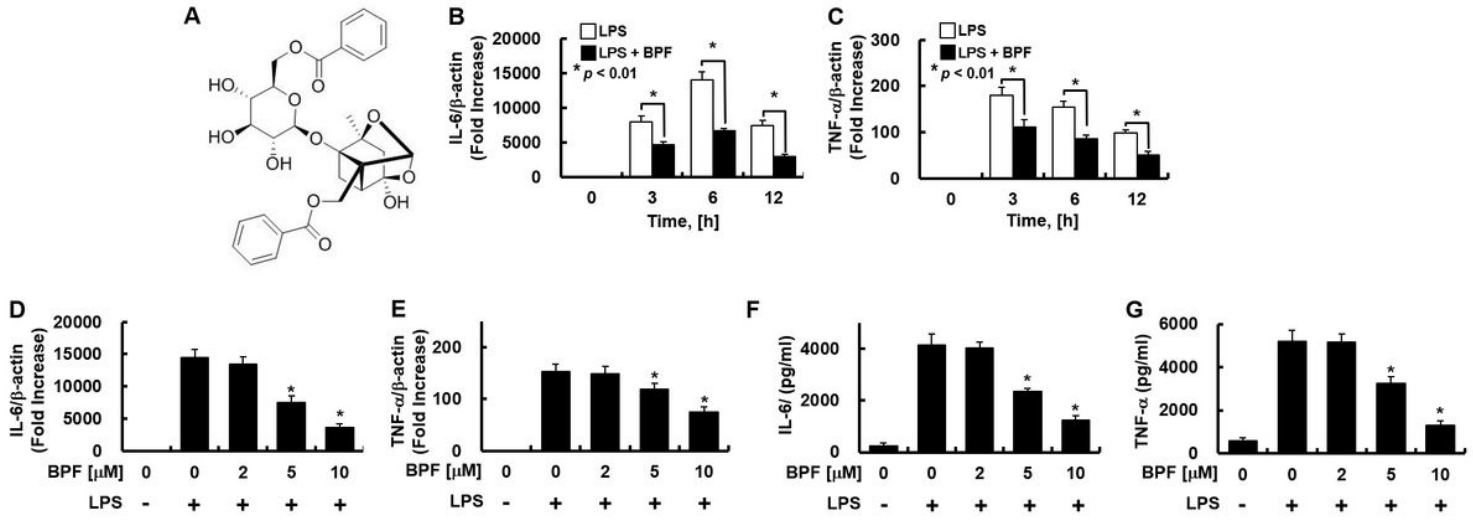

Fig 1

Figure 1

Benzoylpaeoniflorin (BPF) inhibits the expression of inflammatory cytokines in LPS-stimulated HUVECs
(A) Structure of benzoylpaeoniflorin (BPF); (B, C) HUVECs were pretreated with BPF (10 μM for 6 h), then LPS (100 ng/mL) was added. After the indicated times, total mRNA was extracted and the expression level of each cytokine and β-actin were determined using real-time PCR; (D, E) HUVECs were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) was added. After 12 h, total mRNA was extracted and the expression level of each cytokine and β-actin was determined using real-time PCR; (F, G) HUVECs were pretreated with BPF at the indicated concentrations for 6 h, then LPS (100 ng/mL) was added. After 24 h, the cell culture supernatant was harvested, and cytokine levels were determined by ELISA [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.5$ as compared to LPS only (D-G)]. Abbreviations: HUVECs, Human umbilical vein endothelial cells; LPS, lipopolysaccharide

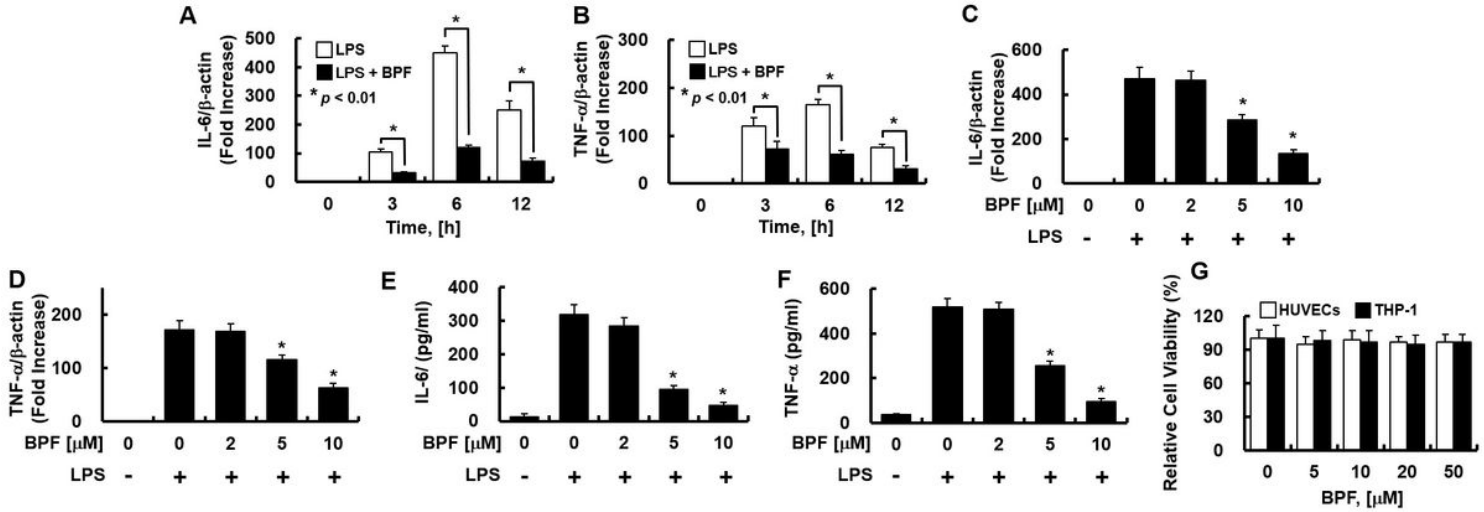

Fig 2

Figure 2

Benzoylpaeoniflorin (BPF) inhibits the expression of inflammatory cytokines in LPS-stimulated THP-1-macrophages (A, B) THP-1-macrophages were pretreated with BPF (10 μM, for 6 h), then LPS (100 ng/mL) was added. After the indicated times, total mRNA was extracted and the expression level of each cytokine and β-actin were determined using real-time PCR; (C, D) THP-1-macrophages were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) was added. After 12 h, total mRNA was extracted and the expression level of each cytokine and β-actin was determined using real-time PCR; (E, F) THP-1-macrophages were pretreated with BPF at the indicated concentrations for 6 h, then LPS (100 ng/mL) was added. After 24 h, the cell culture supernatant was harvested, and cytokine levels were determined using ELISA. (G) HUVECs or THP-1-macrophages were treated with the indicated concentrations of BPF for 48 h. Cell viability was determined using MTT assay [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.5$ as compared to LPS only (D-F)]. Abbreviations: HUVECs, Human umbilical vein endothelial cells; LPS, lipopolysaccharide

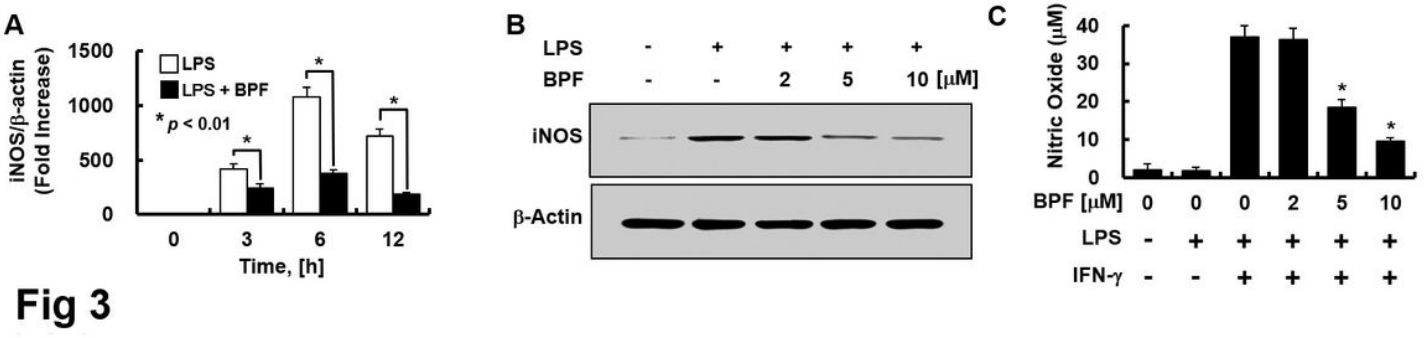

Fig 3

Figure 3

Benzoylpaeoniflorin (BPF) inhibits the expression of iNOS and the production of nitric oxide in HUVECs (A) HUVECs were pretreated with BPF (10 μM for 6 h), then LPS (100 ng/mL) was added. After the indicated times, total mRNA was extracted and the expression level of iNOS and β-actin was determined using real-time PCR; (B) HUVECs were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) was added. After 24 h, the cellular protein was extracted and iNOS and β-actin levels were determined using western blots; (C) HUVECs were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) and IFN-γ (200 ng/mL) were added under the indicated conditions. After 24 h, the cell culture supernatant was harvested, and nitric oxide levels were determined using the Griess reaction assay (A, C) [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.5$ as compared to LPS + IFN-γ (C); (B) The results are from one representative experiment of three independent experiments]. Abbreviations: HUVECs, Human umbilical vein endothelial cells; LPS, lipopolysaccharide

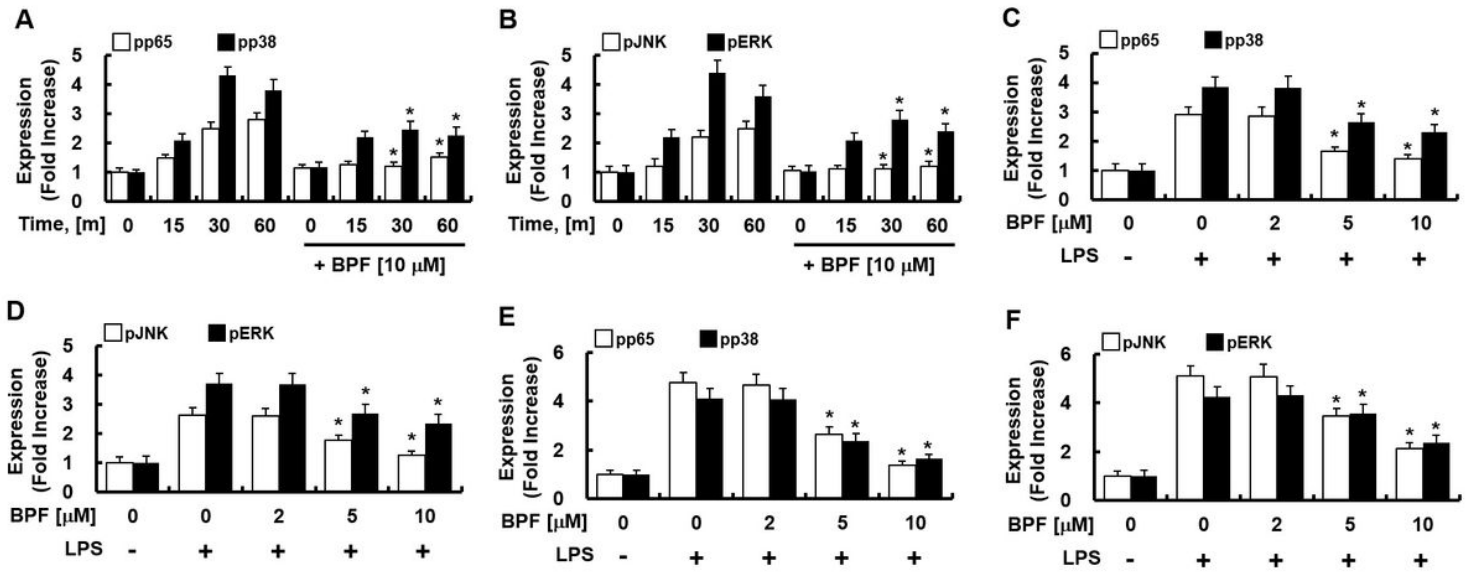

Fig 4

Figure 4

Benzoylpaeoniflorin (BPF) inhibits the phosphorylation of NF-κB and MAP kinase in LPS-stimulated macrophages (A, B) HUVECs were pretreated with BPF (10 μM for 6 h), then LPS (100 ng/mL) was added. Cellular protein was extracted at the indicated times; (C, D) HUVECs were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) was added. After 1 h, the cellular protein was extracted. (E, F) THP-1-macrophages cells were pretreated with the indicated concentrations of BPF for 6 h, then LPS (100 ng/mL) was added. After 1 h, the cellular protein was extracted [(A-C) The levels of the indicated proteins were determined using ELISA. [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.5$ as compared to LPS only]. Abbreviations: HUVECs, Human umbilical vein endothelial cells; LPS, lipopolysaccharide

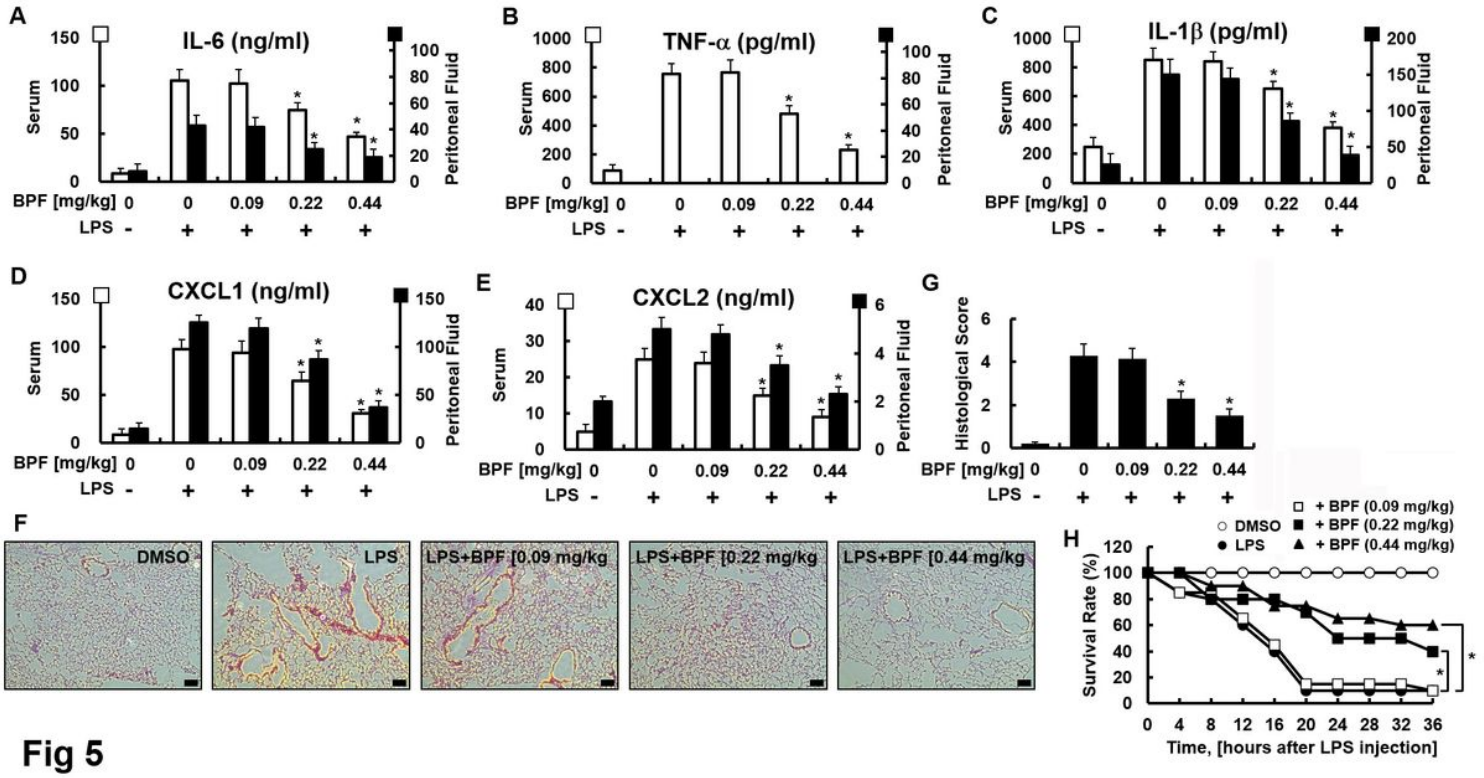

Fig 5

Figure 5

Benzoylpaeoniflorin (BPF) alleviates excessive cytokine production and tissue damage of the lungs and improves survival rate in the LPS-induced mouse septic shock model (A-E) Mice were injected with 2.5 mg/kg of LPS (intraperitoneal injection) and DMSO or BPF (0.09, 0.22 or 0.44 mg/kg, intravenous injection). After 4 h, the mice were euthanized. Blood and peritoneal fluid were collected and the levels of the indicated inflammatory cytokines were determined using ELISA (n = 5); (F, G) Mice were injected with 2.5 mg/kg LPS (intraperitoneal injection) and DMSO or BPF (0.09, 0.22 or 0.44 mg/kg, intravenous injection). After 24 h, the mice were euthanized and the left lung lobes were collected and fixed in formalin, followed by staining with H&E. H&E staining of lung tissues from each group was conducted, and representative images from three independent experiments conducted on three different days are shown. The bar represents 200 μm. Histopathological scores were obtained using an arbitrary scoring index based on the degree of inflammatory cell infiltration and the extent of the lesion area (n = 5); (H) Mice were injected with 25 mg/kg of LPS (intraperitoneal injection) and DMSO or BPF (0.09, 0.22 or 0.44 mg/kg, intravenous injection). The survival of the mice was monitored every 4 h for 36 h and the survival rates were expressed as a percentage (n = 20) [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.5$ as compared to LPS only]. Abbreviations: LPS, lipopolysaccharide

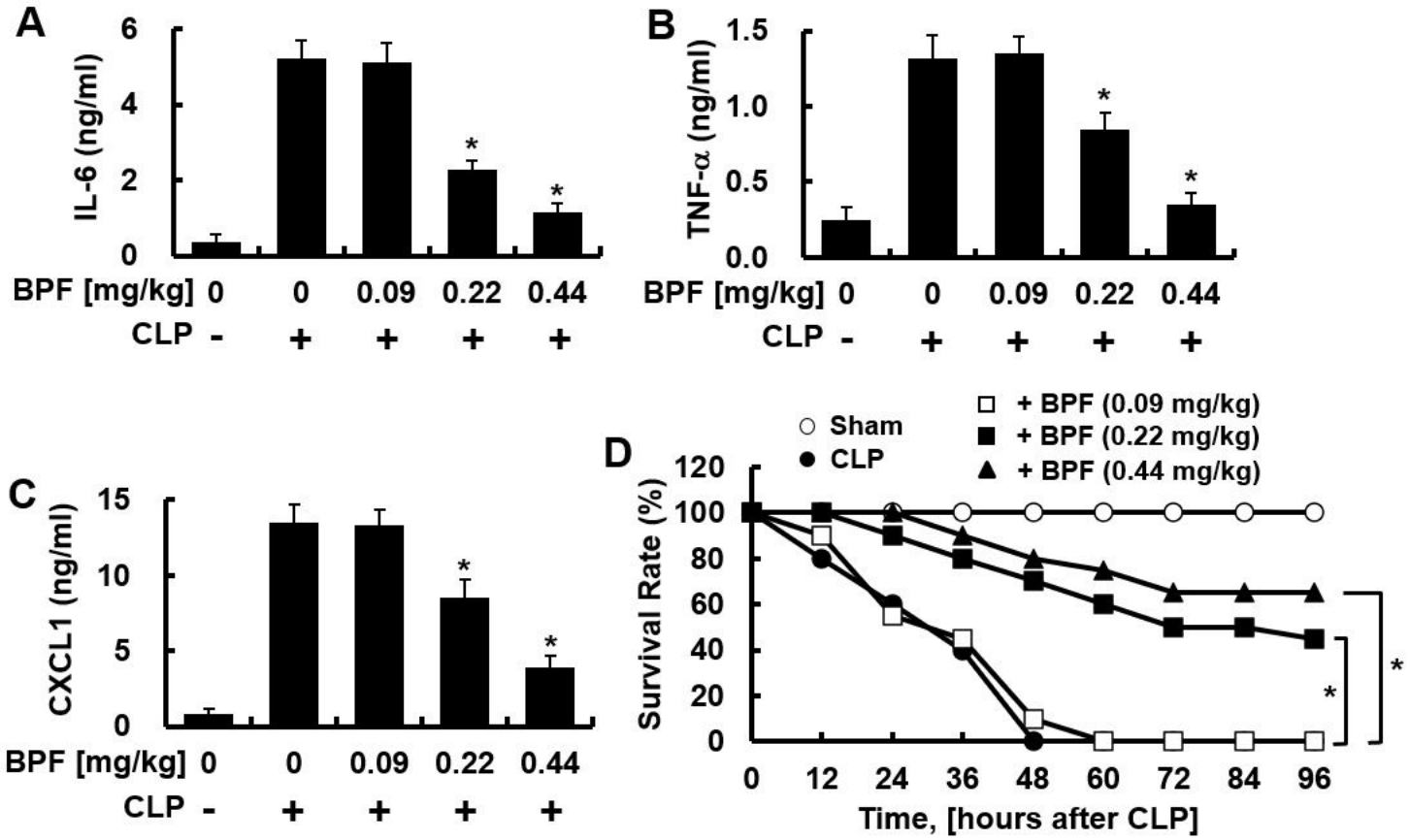

Fig 6

Figure 6

Benzoylpaeoniflorin (BPF) suppresses CLP-induced cytokine production of peritoneal macrophages and improves the mortality of CLP mice (A-C) CLP-operated mice were injected with DMSO or BPF (0.09, 0.22, or 0.44 mg/kg, intravenous injection). After 12 h, the mice were euthanized. Peritoneal macrophages were collected from CLP-operated mice and the levels of the indicated inflammatory cytokines were determined using ELISA (n = 5) [All results are shown as means ± SD of three different experiments with triple samples. *$p < 0.1$ as compared to CLP only]; (D) CLP surgery was done on mice and indicated reagents were administered every 24 h (n = 20). The survival of mice was monitored for 96 h. [*$p < 0.5$]. Abbreviations: LPS, lipopolysaccharide; CLP, cecal ligation and puncture