Beta-naphthoflavone Regulates Inflammation and Coagulation of Sepsis by Raising Nrf2/Ho-1 and Involving AKT/NF- κ B Signaling Pathways

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Abstract

BACKGROUND

During sepsis, macrophages are activated by various irritants, such as Gram-negative bacteria LPS, which leads to the transcription of inflammatory and coagulation factors, including iNOS, COX-2, IL-1 β , TNF- α and TF, PAI-1. Although inflammatory responses play a key role in the host's defense against infection, overactivation of innate immune cells can lead to severe sepsis. In the course of sepsis development, a variety of factors induce inflammatory response while activating coagulation function. Activation of the coagulation system is considered to be a host immune response against infection, however, overactivation of coagulation can cause harm to the host body, resulting in sepsis induced coagulopathy (SIC).

METHODS

In this study, we aimed to evaluate the anti-inflammatory and anticoagulatory effects of BNF on sepsis by constructing in vivo (cecal ligation and puncture (CLP)-induced sepsis) and in vitro (LPS-induced mouse macrophage RAW264.7) sepsis models; the mechanisms behind these effects were also studied. Various aspects of sepsis, including inflammation, coagulation, and lung function, were monitored in the sepsis models.

RESULTS

Results indicated that the administration of BNF in sepsis mice caused reduced expression of pro-inflammatory factors, inhibited activation of coagulation, and lesser lung damage. The levels of LPS-induced macrophages, which lead to inflammation and coagulation, were also reduced. These effects are caused by participation in the Akt/NF- κ B/Nrf2/HO-1 signaling pathway.

CONCLUSION

The results demonstrated that BNF treatment reduced the inflammatory response and coagulation dysfunction caused by sepsis in in vivo and in vitro models. Thus, BNF can be adopted as a novel therapeutic option to reduce sepsis mortality; However, further clinical studies are needed to confirm these effects on human sepsis.

1. Introduction

In 2016, the third international consensus on sepsis and septic shock (sepsis 3.0) defined it as life-threatening organ dysfunction caused by the imbalance of host response to infection[1]. The mortality rate of sepsis increases from 10% in children to 40% in the elderly, and the average mortality rate is about 30%. The mortality of patients with more severe syndrome and septic shock is 50% or higher[2]. If the treatment is not started early and the pathogen is not eradicated, sepsis can lead to organ dysfunction, hypotension, and circulatory failure, often leading to death.

One of the most significant features of sepsis is coagulation activation, during sepsis, many factors activate coagulation function while inducing an inflammatory response [3]. At the same time, the anticoagulation system and fibrinolysis system are down regulated. After the initial trigger event, the inflammatory response is enhanced by several positive feedback circuits, which can activate the coagulation cascade. Therefore, in the process of occurrence and development of many diseases, inflammation and coagulation coexist. Inflammatory factors can promote the production and activation of prothrombin, enhance the procoagulant activity, cause microvascular thrombosis, and promote the occurrence and development of diffuse intravascular coagulation (DIC); whereas coagulation can be widely involved in mediating the inflammatory response [4]. The over activation of coagulation may cause damage to the host body, leading to sepsis-induced coagulopathy (SIC) [5], which has an important impact on the prognosis of sepsis [6].

The host's innate immune system, which consists of neutrophils, macrophages, dendritic cells, natural killer cells, T-cells, and other immune cells, is the first line of defense against infections [7]. Among various immune cells, macrophages play a key role in immune homeostasis and inflammatory response by secreting a series of key cytokines. Macrophages can directly sense the damage-related pattern molecule (DAMP) released during inflammation-induced tissue injury through pattern recognition receptor (PRR), leading to the activation of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammatory bodies [8]. Therefore, it is of great significance to pay attention to and regulate the immune function of macrophages for the treatment of sepsis.

The RAW264.7 cell line is the most commonly used in vitro research model for screening anti-inflammatory active substances and studying inflammation [9]. Under the action of inducers (such as lipopolysaccharide [LPS]), RAW264.7 cells simulate inflammatory response and release or upregulate a variety of inflammatory mediators, such as nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor- α (TNF- α), and Interleukin 6 (IL-6). In Gram-negative bacterial infections, LPS can activate various cells, such as monocytes and vascular endothelial cells, to release a variety of inflammatory mediators, such as TNF, IL-1 β , and IL-6, and produce a series of inflammatory reactions by contacting coagulation factor VII and complement system to resist the invasion of pathogenic microorganisms [10]. While causing inflammation, pathogenic microorganisms and some of their components can also cause coagulation. This in turn up-regulates the expression of tissue factor (TF) in monocytes/macrophages and vascular endothelial cells, leading to coagulation and thrombosis. Studies have indicated that TFs are potential mediators of intracellular signal transduction of established inflammatory pathways [11]. Normal blood cells and vascular endothelial cells do not express TF. The content of TF in normal plasma is very low, whereas the levels of tissue factor pathway inhibitor (TFPI) are about 1000 times higher than that of TF [12]. However, macrophages can be induced to express TF under the action of certain stimuli [13], and are considered important triggers for clotting in diseased states [14]. Many inflammatory substances such as endotoxins and tumor necrosis factors, IL-1, IL-6, IL-8, and C-reactive proteins can cause mononucleocytes/macrophages and vascular endothelial cell expression histokines [15]. TF pathway is not only responsible for the initiation of the coagulation process in physiological hemostasis

but also plays a key role in the process of thrombosis under pathological conditions. Therefore, in the treatment of sepsis, it is of great significance to pay attention to and stimulate macrophage function[16].

It has been observed that toll-like receptors TLR (such as TLR2 and TLR4), microbial products, and other inflammatory mediators initiate signaling pathways leading to NF- κ B nuclear accumulation and enhanced gene transcription which are responsible for the expression of cytokines, chemokines, adhesion molecules, and other mediators of infection-related inflammatory response[17]. Transcription regulator NF- κ B is a core participant in the expression of many immunomodulate media involved in acute inflammatory responses [18]. Nuclear factor-kappa B (NF- κ B), which is widely expressed in the central nervous system, is a protein transcription factor and a key regulator of inflammation and coagulation response, as well as the expression of many genes, such as iNOS and COX2. It also promotes the secretion of a variety of pro-inflammatory cytokines, such as TNF- α and IL-1 β [19].

The combination of NF- κ B with the inhibitory protein I κ B α in the cytoplasm blocks the nuclear positioning sequence of NF- κ B and inhibits its entry into the nucleus[20]. Under physiological conditions, NF- κ B and its inhibitor I κ B α binds and remain dormant in the cytoplasm. Once activated, NF- κ B is transferred to the nucleus to start the transcription of downstream genes[21]. We found that LPS increased the activity of NF- κ B, followed by an increase in the expression of iNOS and COX2. BNF downregulates the increased expression of these two pro-inflammatory mediators by inhibiting the activity of NF- κ B, and the results show a decrease in pro-inflammatory mediator secretion.

Other examples of immune response regulation by specific inhibitory methods involve kinases (such as p38 or Akt) or transcription factors (such as NF- κ B) has shown its pathophysiological importance in experimental models [22]. Protein kinase B (PKB) or Akt is a widely expressed serine/threonine kinase with a PH domain that selectively binds inositol 3-phosphate [23]. Studies have shown that AKT1, which is one of three closely related serine/threonine protein kinases, may play a dual role in carcinogenesis not only by inhibiting apoptosis but also by inhibiting invasion and metastasis. AKT signal imbalance is the underlying cause of several life-threatening diseases, including diabetes, neurodegenerative syndrome, and various cancers. In the immune response of sepsis, the inositol phosphate 3-kinase (PI3K) signaling pathway and Akt play an important role because they regulate the release of inflammatory factors and control downstream signaling molecules[24]. PI3K/AKT pathways are known to be activated by a variety of extracellular and intracellular signals. They are important cell signaling molecules and key survival factors involved in proliferation, differentiation, apoptosis, and several other cellular processes.

Heme oxygenase-1 (HO-1), which is a famous phase II detoxification enzyme, plays a powerful role in maintaining cellular homeostasis and protects cells from damage caused by oxidative stress and inflammatory response[25, 26]. It has been observed that During oxidative stress, HO-1 is upregulated during oxidative stress and inflammatory conditions to enhance cellular defense mechanisms against oxidative damage[27]. In normal cells, HO-1 is expressed at a low levels; however, its expression can be stimulated in cells without stimulation and can be rapidly induced by various oxidation inducers, such as including LPS[28], heme, and hypoxia, etc. Under inflammatory conditions, the enhancement of HO-1

expression plays an important role in cell protection[29]. HO-1 catalyzes the first, rate-limiting step in the degradation of heme to carbon monoxide (CO), biliverdin, and ferrous iron[26]. Biliverdin is converted to bilirubin which is an effective endogenous antioxidant with recognized anti-inflammatory properties[30]. CO has a variety of biological functions, including anti-inflammatory properties[31]. In pure inflammatory models of disease, such as endotoxin exposure, HO-1 deficient mice are vulnerable to oxidant-induced tissue damage and death[32]. HO-1 reportedly inhibits the excessive production of TNF- α and IL-1 β [33] in LPS-induced RAW264.7 cells. In animal models, HO-1 deficient mice exhibit severe inflammation[32], and HO-1 overexpression produces anti-inflammatory effects[27].

The expression of HO-1, like several other phase II detoxifying enzymes, is induced by the binding of upstream activators to the starter region of the antioxidant-response element (ARE) [34]. The nuclear factor erythroid 2-related factor 2 (Nrf2) acts as an upstream activator for is an upstream medium for Phase II detoxifying enzymes (including HO-1)[35]. Apart from being a pleiotropic protein, it is a basic leucine zipper redox sensitive transcription factor that regulates the basic and induced expression of a series of antioxidant and other cryoprotective genes by binding with cis-acting genes s[36]. Under normal conditions, Nrf2 is degraded in the cytoplasm after binding to Keap1, resulting in the decreased accumulation of Nrf2 in the nucleus and reduced transcription of a range of cell-protective genes[37], such as HO-1 [37]. Under inflammatory conditions, Nrf2 is dissociated from Keap1 and is transported to the nucleus to activate the expression of HO-1, thereby resisting inflammatory responses. Based on these and other studies, it is now widely believed that Nrf2/HO-1 plays a key role in regulating the inflammatory response, enhancing the cell's resistance to inflammation.

Beta-naphthoflavone (BNF) is a synthetic derivative of naturally occurring flavonoids that have antioxidant and anti-inflammatory effects. BNF itself is not toxic or carcinogenic, and as a ligand of aromatic hydrocarbon receptors, BNF is widely used in the pharmaceutical industry. In recent years, BNF has been reported to prevent peritonitis by reducing TNF- α induced endothelial cell activation[38], with antioxidant and anti-inflammatory potential. In addition, numerous studies have shown that BNF therapy reduces neonatal hyperoxic lung injury[39] and acute kidney injury by inhibiting inflammation in surrounding tissues[40]. BNF has also been shown to alleviate colitis induced by sodium dextran sulfate[41].

Previous research has indicated that BNF inhibits the activity of NF- κ B and increases the expression of pro-inflammatory mediators, such as iNOS and COX2. However, the role of BNF in sepsis-related inflammatory and coagulation response is still unclear. Therefore, in this study, we utilized LPS-stimulated RAW264.7 cells and cecal ligation and puncture (CLP) animal model to study the anti-inflammatory and anticoagulant activity of BNF and the molecular mechanisms involved.

2. Materials And Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM) (high glucose), fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Gibco, Inc., USA. Beta-naphthoflavone, lipopolysaccharide (LPS), 1,3-bromo chloropropane were purchased from Sigma-Aldrich (St. Louis, Missouri.). Nrf2 inhibitor ML385, AKT inhibitor, HO-1 inhibitor Tin-protoporphyrin IX (SnPPIX) were purchased from MedChemExpress. Total RNA extraction kits were purchased from Tiangen Biochemical Technology Corporation (China). PCR primers were purchased from Shanghai JetBlue (China). HRP Mark II, Western blot Bond™ Primary Antibody Diluent, fast closed liquid, Western blot Stripping buffer, RIPA cell lysate, PMSF protease inhibitor, dithiosulfene alcohol (DTT), Tween20 was purchased from Biyuntian Biotech (China). Phosphatase inhibitors, SYBR Green Master (ROX) were purchased from Roche (Switzerland). BCA Protein Concentration Determination Kit, RevertAid First Strand cDNA Kit was purchased from Thermo Scientific (USA). Glycine, sodium sulphate (SDS) and trihydroxymethylmethane (Tris) were purchased from Solarbio Technologies (China). Rabbit polyclonal antibodies for NF-κB, p-NF-κB, IκB-α, p-IκB-α, p-STAT3, AKT, p-AKT, and rat polyclonal antibodies for STAT3 were purchased from CST Corporation (USA). Rabbit polyclonal antibodies for GAPDH, Beta Tubulin, NRF2, HO-1, COX-2 were purchased from Proteintech (China). Western Blotting ECL Glowing Substrate was purchased from Thermo Scientific Corporation (USA). The anti-mouse PAI-1 DuoSet ELISA test box and the anti-mouse TF DuoSet ELISA test box were purchased from R-D systems (USA). The anti-mouse D-Dimer ELISA test box, the anti-mouse Fibrinogen ELISA test box, and the anti-mouse TAT ELISA test box were purchased from Cusabio Corporation (China).

Real-time quantitative polymerase chain reaction (RT-qPCR).

Total RNA was extracted from cultured cells using TRIzol reagent, and real-time PCR was performed by using the SYBR Green Master (ROX) kit. The following primers were employed: GAPDH, IL-1 beta, IL-6, TNF-α, PAI-1, COX-2. The amplification conditions were: 95°C 30 s, 95°C 10 s, 60°C 30 s, 40 cycles. For internal comparison, loop threshold (Ct) values were compared with GAPDH, and the experimental data were processed in $2^{-\Delta\Delta Ct}$. The experiments were performed in triplicates. The experiments were performed in triplicates.

Table 1
Primer sequences for RT-PCR

Gene	Forward primer	Reverse primer
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
IL-1 β	GCTGCTTCCAAACCTTTGAC	CTTCTCCACAGCCACAATGA
COX2	TGCACTATGGTTACAAAAGCTGG	TCAGGAAGCTCCTTATTTCCCTT
TNF- α	GTGGGTGAGGAGCACGTAGT	CGATCACCCCGAAGTTCAGTAG
IL-6	AGTTGCCTTCTTGGGACTGA	CCTCCGACTTGTGAAGTGGT
iNOS	TGCCACGGACGAGACGGATAG	CTCTTCAAGCACCTCCAGGAACG
PAI-1	GCACAGGCACTGCAAAAGG	GGGCTGAGATGACAAAGGCT

Western blot analysis.

After processing each group of cells, they were washed with 1 mL PBS; the cells were extracted by incubating in lysis buffer for 15 min in ice. The supernatant containing the total protein was removed by centrifugation at 12,000 $\times$ g at 4°C for 15min. Electrophoresis was carried out at a low temperature, and the separated proteins were transferred to polydefluoroethylene membrane. The membrane was then incubated overnight at 4°C with 1:1000 diluted. It was then incubated in a two-resistance incubator at room temperature for 1h with 1:5000 goat anti-rabbit or anti-mouse. The membranes were visualized using image Lab software (Amersham Imager 680), and the analysis was performed using ImageJ software.

Cell viability test based on cell count kit-8 (CCK-8)

The RAW264.7 cells were inoculated in a 96-well culture plate with six compound holes in each group; a 100 μ L culture medium was added to each of the holes. In accordance with the CCK-8 kit instructions, 100 μ L medium and 10 μ L CCK-8 reagents were added to each well. After incubation for 1–2 h at 37°C, the optical density (OD) value was measured at 450 nm using an enzyme-labeled instrument (Thermo Scientific, American). Each group consisted of three parallel holes, whose average value was taken. The experiment was repeated three times. The cell proliferation curve was plotted with time on X-axis and OD value on Y-axis.

ELISA

According to the manufacturer's agreement, IL-6, TNF- α , IL-1 beta ELISA kits are used to quantify the plasma of in vitro cultured RAW264.7 cells or experimental mice.

CLP damage model

The procedure for modeling sepsis in mice is the same as described in previous studies. In short, first anaesthetize the mice with barbiturates. Adequate anesthesia should cause the limbs to be unresponsive, after the lower quadrant of the mouse abdomen hair removal disinfection, and then perform a abdominal incision, thereby locating the blind intestine at a distance of 1 cm from the blind intestine ligation of the blind intestine. Use a needle to pierce the blind intestine with a single penetration between the ligation line and the tip of the blind intestine. Squeeze a small amount (drop) of feces from the intestinal membrane and the intestinal membrane puncture hole to reposition the blind intestine into the abdominal cavity without spreading the feces from the blind intestine to the edge of the abdominal wall wound. Close the peritoneum, fascia, abdominal muscle tissue and skin by applying simple continuous stitching. For animals in the pseudo-surgical group, only switch abdominal procedures are performed. The mice were resuscitated by subcutaneous injections of preheated physiological saline (37°C, 5 ml per 100g body weight). Plasma samples and lung tissue were taken 24 hours after CLP.

Mouse lung tissue H&E staining

Each group of mouse lung tissue after fixation was paraffin-embedded, using a sectioning mechanism into a 3mm sample, and then H&E staining was performed, and the pathology of lung injury was observed under an upright microscope. At least 6 mice per group, each slice randomly selected 10 fields of view for shooting. Lung injury scoring criteria: 0 points for no damage; 1 point for 25% visual field injury; 2 points for 50% field damage; 3 points for 75% field damage; 4 points for full field of view injury.

Immunohistochemistry

Lung tissue slices (3mm) dewaxify in xylene and hydrate in a range of graded ethanol (100%, 95%, 80%, and 75%) for another 5 minutes, then recycle the antigen, wash with PBS, incubate with goat serum for 30 minutes at 37 degree Celsius, then incubate overnight with primary antibodies at 4 degree Celsius, then incubate the slides with primary antibodies overnight at 4 degree Celsius and then perform fluorescent secondary antibodies and cell nucleus staining. The image represents three separate experiments.

Statistical analysis

The data was represented as an average $\pm$ SD and an average $\pm$ SEM. All experiments were performed in triplicates. The normally distributed data were analyzed by one-way analysis of variance (ANOVA) and student's t-test using GraphPad Prism 8.0 software. The P-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of BNF on RAW264.7 Cell Viability

In order to assess the toxicity of BNF, the survival rate of RAW264.7 cells after 24 h of treatment was determined by CCK-8. After culturing with different doses of BNF and CCK-8 solutions, the OD of the cell

suspension was measured at 450 nm. Compared to the control group, no significant changes were found in the OD of groups treated with different concentrations of BNF (0.625 μ M, 1.25 μ M, 2.5 μ M, 5 μ M, 10 μ M and 20 μ M). Thus, the varying concentrations of BNF did not affect RAW264.7 cell viability (figure b). We then measured the expression levels of IL-6 mRNA in RAW264.7 cells after LPS treatment at different concentrations of BNF (2.5, 5, 10, 20 μ M), after which, changes in IL-6 mRNA expression were observed. Results indicated that LPS-stimulated RAW264.7 cells exhibited inhibition in IL-6 mRNA expression after the BNF treatment in a concentration-dependent manner. After analyzing the results, we chose a BNF concentration of 20 μ M for subsequent experiments.

3.2. BNF inhibits the expression of pro-inflammatory and coagulation factor mRNAs in LPS-induced RAW264.7 cells

To further determine whether BNF induces or inhibits the mRNA expression levels of pro-inflammatory, anti-inflammatory, and coagulation genes, RAW264.7 cells were incubated with 1 μ g/mL LPS for 6h with and without BNF (20 μ M), RT-qPCR analysis was performed and total cell protein was extracted for western blot analysis.

Compared to the control group, LPS treatment significantly increased the mRNA expression of PAI-1, COX2, IL-1 beta, IL-6, TNF- α and the protein. expression of COX-2. However, after BNF treatment, the expression of the above-mentioned decreased.

These results confirm that BNF regulates the expression of pro-inflammatory and coagulation genes at the transcription levels in LPS-stimulated RAW264.7 cells, making macrophages more polarized to anti-inflammatory and anticoagulatory effects.

3.3. Enzyme-linked immunosorbent assay (ELISA) of RAW264.7 cells

Protein levels of IL-6, IL-1 beta, and TNF- α in each group of RAW264.7 cells were measured by ELISA. Results indicated that RAW264.7 cells exhibited a significant increase in the expression of IL-6, TNF- α and IL-1 β after LPS stimulation for 6 h. In the BNF-treated cells, the expression of IL-6, IL-1 β and TNF- α decreased significantly compared to the control LPS group.

3.4. BNF inhibits STAT3/NF- κ B/I κ B- α pathway activation in LPS-stimulated RAW264.7 cells

Signal transducer and activator of transcription 3 (STAT3), which is a transcription factor encoded by the STAT3 gene, has been shown to be activated by TLR agonists [42]. The study found that mice with STAT3 conditional knockout were more likely to be induced by LPS to develop sepsis shock, apart from exhibiting increased cytokine production and higher mortality [43]. There is a mutually reinforcing effect between STAT3 and NF- κ B. The activation of NF- κ B induces the production of pro-inflammatory

cytokines, inhibits anti-inflammatory cytokines, and ultimately leads to an inflammatory response. In order to determine whether BNF blocks LPS-induced STAT3/NF- κ B activation, the changes in STAT3/NF- κ B/I κ B- α protein levels were analyzed by western blot. We pretreat RAW264.7 cells with BNF (20 μ M) for 1h and then stimulate with LPS for 6h. As shown in the Fig. 4, after LPS stimulation, the levels of p-STAT3, p-NF- κ B, and p-I κ B- α proteins increased significantly. However, BNF effectively inhibited the rise of LPS-induced p-STAT3, p-NF- κ B, and p-I κ B- α . Therefore, it can be concluded that BNF can block the activation of the STAT3/NF- κ B/I κ B- α signaling pathway in LPS-induced RAW264.7 cells.

3.5. The inhibition of AKT pathway and the activation of Nrf2/HO-1 pathway in LPS-stimulated RAW264.7 cells after BNF treatment

PI3K/Akt regulates BNF-induced Nrf2 expression in RAW264.7 cells. The activation of the Nrf2 pathway is affected by a variety of signal kinases. Recent studies have shown that PI3K/Akt pathways are important upstream regulators for Nrf2 expression. Therefore, we analyzed whether the PI3K/AKT pathways play a central role in Nrf2 expression after BNF induction. BNF pretreated RAW264.7 cells for 1h, and after LPS action for 6h, we used western blot to detect Nrf2/HO-1/AKT protein expression. Western blot analysis revealed that Akt phosphorylation increases in LPS-stimulated RAW264.7 cells after BNF treatment, leading to an increased expression of HO-1. This increased expression of HO-1 under BNF induction may be related to the transmission of PI3K/Akt signals.

To further understand the mechanism, we used a specific PI3K/Akt inhibitor, MK2206. We pretreat RAW264.7 cells with MK2206 (10 μ M) for 2h, BNF pretreatment for 1h, and LPS action for 6h. The results indicated that Nrf2 expression significantly increased after BNF processing; however, in RAW264.7 cells, MK2206 inhibited Akt phosphorylation and significantly reduced Nrf2 activation that was induced by BNF. Therefore, we concluded that PI3K/Akt is essential for the anti-inflammatory and anti-coagulation activity of BNF by regulating Nrf2 expression.

3.6. BNF can raise the expression of HO-1 by activating Nrf2

The above results demonstrated that BNF treatment can promote the expression of Nrf2 and HO-1 proteins. To study whether the increase in the Nrf2 protein was associated with an increase in HO-1, we pretreated RAW264.7 cells with Nrf2 inhibitor ML385 for 2h, followed by BNF treatment for 1h, and then LPS (1 μ g/mL) for 6h. Analysis of Nrf2 and HO-1 protein levels with western blot revealed that ML385 pretreatment inhibited the levels of Nrf2 and HO-1 proteins induced by BNF in RAW264.7 cells, indicating that BNF can improve HO-1 expression by activating Nrf2.

3.7. BNF inhibits the production of LPS-induced pro-inflammatory factors via the HO-1/NF- κ B signaling pathway

The above results show that BNF processing can raise HO-1 expression and inhibit the activation of NF- κ B. To study whether the expression of HO-1 was associated with NF- κ B in RAW264.7 cells treated with BNF, we pretreated RAW264.7 cells with HO-1 inhibitor SnPP-IX for 3h, followed by BNF treatment for 1h, and then LPS (1 μ g/mL) treatment for 6h. Analysis of the phosphorylation level of NF- κ B protein with western blot revealed that after SnPP-IX treatment, the role of BNF in activating the NF- κ B pathway in the LPS-induced RAW264.7 cells was reduced.

3.8 BNF has a protective effect on CLP mice.

To determine the protective effect of BNF, CLP mouse models were used to simulate sepsis. As shown in the Fig. 8, Compared with LPS mice, BNF effectively reduced lung damage characterized by extensive inflammatory cell immersion, destruction of normal alveoli structure and edema, and thickening of the alveoli wall. All of these data suggest that BNF can improve sepsis inflammation and coagulation in CLP mice.

3.9. BNF reduced the amount of fibrinogen in the lungs of mice with CLP-induced sepsis

Fibrinogen (Fib) is a key protein directly involved in the blood coagulation process, and its elevated level can cause imbalances in the body's coagulation and fibrous system[44]. When the body is damaged by various factors, the concentration of Fib increases[45] and plasma fibrin is a sensitive biochemical index, which provides an objective scientific theoretical basis for the clinical selection of treatment options and prognosis[46]. Mice are pretreated with BNF for 1h, and after CLP for 24h, we extract the lung tissue of each group of mice for Fib immunohistochemical staining analysis. The results showed that Fib in the CLP group was significantly higher relative to the SHAM group, while after the BNF intervention, Fib decreased significantly relative to the CLP group. It is suggested that BNF has an improved effect on the coagulation function of mice.

3.10. BNF has a protective effect on sepsis in CLP-induced mice

After BNF pretreatment for 1h, CLP 24h, we extracted plasma from each group of mice. The ELISA method was used to detect the inflammatory indicators of IL-6(fig.a), TNF- α (fig.b), IL-1 β (fig.c) and the coagulation indicators of TF(fig.d), D2D(fig.e), TAT(fig.f), Fib(fig.g) and PAI-1(fig.h) in mouse plasma. We found that the above inflammation and coagulation indicators were significantly higher in the CLP group mice and significantly lower in the CLP + BNF group mice.

3.11. BNF treatment inhibits AKT phosphorylation and promotes the expression of Nrf2/HO-1 in CLP-induced mice

After BNF pretreatment mice for 1h and CLP 24h, the proteins expression of AKT/Nrf2/HO-1 and STAT3 in the lung tissues of each group were evaluated by western blot.

Compared with the SHAM group, the proteins expression of p-AKT and p-STAT3 in the lung tissues of each group were significantly increased; whereas the proteins expression of p-AKT and p-STAT3 were inhibited after BNF treatment. The proteins expression of Nrf2/HO-1 were significantly higher than that of the CLP group after BNF treatment. Therefore, BNF can inhibit the activation of AKT and STAT3 in sepsis lung tissue and increase the Nrf2/HO-1 protein content.

3.12. Schematics of BNF accommodating inflammation and coagulation via AKT/NF- κ B/Nrf2/HO-1 signaling.

4. Discussion

Sepsis is a systemic inflammatory response to external or internal factors, leading to high mortality in intensive care units worldwide[47]. More than one million people die from sepsis in China every year, the death rate being higher in the elderly, men, and people with other health complications[48]. Because of this, there is a need to discover novel and effective therapeutic strategies for sepsis management[49].

Assessing the degree of inflammatory response is very important to address sepsis because insufficient response caused by immune deficiency will further spread infection, and excessive inflammatory response is self-destructive, leading to microcirculation dysfunction, tissue injury, myocardial injury, acute respiratory failure, multiple organ failure, and eventually death. Host factors that regulate innate immunity can prevent inflammatory disorders and help to control inflammation effectively.

Macrophages are the main responders to inflammation; they induce immune responses to endogenous and exogenous stimuli and play a central role in host defense and immune regulation[50]. Macrophages recognize LPS[51], a component of the bacterial cell membrane, through TLR4, thereby producing pro-inflammatory cytokines at inflammatory sites, such as IL-6, IL-1 β , and TNF- α [52]. Previous studies have confirmed that pro-inflammatory cytokines, such as IL-6, TNF- α , and IL-1 β , participate in and promote sepsis. In this study, sepsis was established in mice by CLP[53], which is regarded as the gold standard model of sepsis induction. Results indicated that 24 h after CLP, mouse plasma pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and procoagulant factors (TF, FIB, D-dimer and TAT) increased significantly, and the surviving mice developed sepsis syndrome; this indicates the validity of our sepsis model.

Transcription regulator NF- κ B plays an important role in a variety of processes, including inflammatory response, immune response, apoptosis, brain learning, and bone development[54]. In unstimulated cells, NF- κ B is usually present in the cytoplasm along with the inhibitory family of I κ B α proteins. However, under LPS stimulation, I κ B α proteins are phosphorylated and degraded, leading to the transfer of NF- κ B to the nucleus and the transcription of inflammatory genes. The findings of this study indicated that LPS increased NF- κ B activity, followed by increased iNOS and COX2 expression. We found that BNF can block LPS-triggered I κ B α /NF- κ B signaling pathway in RAW264.7 cells.

Nrf2 is an important anti-inflammatory transcription factor. Under oxidative stress, Nrf2 is phosphorylated and transferred to the nucleus, and then starts the transcription of HO-1 gene[55]. Nrf2 can reduce endotoxin induced sepsis inflammation and coagulation by promoting HO-1 expression. Here, we demonstrated that BNF treatment significantly attenuated the inflammatory and coagulation responses of LPS stimulated RAW264.7 cells. This effect is partly through the inhibition of NF- κ B pathway and activation of Nrf-2/HO-1 pathway .

Nrf2 is a new regulator of innate immune response[56]. It regulates the experimental sepsis phase by preventing dysregulated inflammation and coagulation and improves survival by initiating an appropriate innate immune response. Nrf2 interferes with the transcriptional increase of lipid polysaccharide-induced pro-inflammatory cytokines, including IL-6 and IL-1 beta [57]. In this study, we demonstrate that Nrf2 can reduce LPS-induced sepsis inflammation and coagulation by promoting HO-1 expression, and that BNF therapy significantly attenuates the LPS and CLP-induced inflammation and coagulation responses. This effect is partly effected by inhibiting the AKT/NF- κ B pathway and activating the Nrf2/HO-1 pathway.

Extravascular fibrin deposition is an early and persistent marker of inflammatory response[58]. Fibrinogen is produced by plasma-derived Fib and is known to escape the vascular system in response to the contraction of endothelial cells at the site of inflammation[59]. The acute fibrinolytic response to inflammation is the release of plasminogen activators from the storage sites of vascular endothelial cells, especially tissue plasminogen activator (tPA) and urokinase plasminogen activator (u-PA)[4]. Research has indicated that when challenged by endotoxin, mice lacking plasminogen activator had a more extensive fibrinogen deposition in organs, while PAI-1 knockout mice had no microvascular thrombosis at the endotoxin administration site compared with the wild-type control mice[60]. Suggests the important role of fibrinogen in coagulation.

In this study, we investigated the anti-inflammatory and anti-coagulation effects and potential mechanisms of Nrf2 in mice with sepsis induced by cecal ligation and puncture (CLP) and RAW264.7 cells induced by LPS. We found that Nrf2/HO-1 increased in CLP-induced sepsis mice and LPS-induced RAW264.7 cells, and its overexpression reduced lung injury in sepsis mice and inhibited inflammation and coagulation both in vivo and in vitro.

At present, the prognosis of clinical sepsis is still very poor and the mortality rate is relatively high. Therefore, there is an urgent need to study the mechanism of progression in order to better treat patients. Sepsis is a disease caused by the harmful or destructive response of the host to infection. Many components of innate immune response usually related to host defense against infection can lead to cell and tissue damage in some cases, resulting in multiple organ failure, which is a clinical marker of sepsis.

Due to the high mortality of sepsis under standard treatment, many efforts have been made to improve the understanding of host response disorder in sepsis. Therefore, people have a lot of understanding of the basic principles of controlling bacterial host interaction, and reveal new opportunities for therapeutic intervention.

In our study, it was observed that serum and cellular inflammation and coagulation levels were up-regulated in LPS and CLP-induced sepsis models, while BNF could significantly down-regulate these levels. Our data suggest that BNF affects the early transduction stage of Nrf2/HO-1 and NF- κ B signaling pathways, which is a key process leading to the etiology of many diseases. BNF simultaneously activates Nrf2/HO-1 and inhibits NF- κ B signal transduction, as summarized in the Fig. 12, although some elements of the model need to be further investigated by future research.

Our data do not allow us to exclude the possibility that BNF indirectly affects Nrf2 and NF- κ B signaling through some uncharacterized regulators rather than through direct interaction with Nrf2 and NF- κ B complexes. In addition, we cannot exclude crosstalk between Nrf2 and NF- κ B pathways. Nevertheless, our data provide a basis for further clinical development of BNF as a new drug for sepsis and SIC.

5. Conclusions

In conclusion, the results demonstrated that BNF treatment reduced inflammation and coagulation caused by sepsis in in vivo and in vitro models. These effects involve the activation of Akt/NF- κ B/Nrf2/HO-1 signaling pathways. Therefore, this study provides an important basis for the utilization of BNF as a new treatment option to reduce sepsis mortality. However, further clinical studies are needed to confirm these effects on human sepsis.

Abbreviations

APTT:Activated Partial Thromboplastin Time

BNF: Beta-naphthoflavone

CLP:cecal ligation and puncture

DIC: disseminated intravascular coagulation

D2D:D-Dimer

Fib:Fibrinogen

HO-1:heme oxygenase 1

LPS: lipopolysaccharide

NF- κ B:Nuclear Factor kappa-B

Nrf2:nuclear factor erythroid 2-related factor 2

PAI-1:Plasminogen Activator Inhibitor-1

SIC: sepsis-induced coagulopathy

SnPPIX: Tin-protoporphyrin IX

STAT3: Signal transducer and activator of transcription 3

TF: tissue factor

TAT: Thrombin-antithrombin complex

TFPI: Tissue Factor Pathway Inhibitor

t-PA: Tissue plasminogen activator

u-PA: Urokinase plasminogen activator

Declarations

Ethical Approval and Consent to participate: All mice were maintained in a pathogen-free facility and received food and water ad lib. Animal care and experimentation were conducted with approval from Wenzhou Medical University Institutional Animal Care and Use Committees.

Consent for publication: Yes.

Availability of data and materials: All the data supporting the results are included in the article.

Competing interests: Not applicable.

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Author Contributions: WC, performed the experiments and wrote the paper; XZ, FY, YL and YS collected and analyzed the data; ZF and LP designed the figure legends; HJ, SQ, DL, YH, ZC and XY contributed to the drafting of the manuscript; JP conceived the study. All authors read and approved the final manuscript.

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Figures

Figure 1

(a) The chemical structure of the Beta-naphthoflavone.

(b). Raw264.7 cells vitality was detected by CCK-8. Compared to the CON group, ns>0.05. (n $\geq$ 6).

(c) The IL-6 mRNA levels were analyzed with RT-qPCR. Compared to the LPS group, ****<0.0001, ***<0.0005, **<0.01, *<0.05. (n $\geq$ 4).

Figure 2

The IL-6(a), IL-1β(b), TNF-α(c), PAI-1(d), COX-2(e) mRNA level are quantified by quantifying real-time PCR. Compared to the CON group, #### $p < 0.0001$; compared to the LPS group, **** $p < 0.0001$, ** $p < 0.01$. (f) Compared to the CON group, #### $p < 0.0001$, compared to the LPS group, **** $p < 0.0001$. (g) Protein expression of COX-2.

Figure 3

ELIS A measured IL-6(a), TNF-α(b), IL-1β(c) protein concentrations in RAW264.7 cell supernatant. Compared to the CON group, ****P<0.0001; compared to the LPS group, ****P<0.0001 and **P<0.01.

Figure 4

(a)The pathway expression of STAT 3/NF-κB/IKB-αin RAW 264.7 cells stimulated by LPS. (b,c,d)Protein expression ofSTAT3/NF-κB/IKB-α.Compared to the CON group,####p<0.0001;compared to the LPS group,****p<0.0001, **p<0.01,***p<0.0005. (n≥3).

Figure 5

(a) The protein expression of Nrf2/HO-1/AKT.

(b,c,d) Protein expression of Nrf2/HO-1/AKT. Compared to the CON group, **** $p < 0.0001$, #### $p < 0.0001$; compared to the LPS group, **** $p < 0.0001$, # $p < 0.05$. ($n \geq 3$).

(e) The protein expression of Nrf2/ AKT.

(f)Protein expressionof Nrf2. Compared to the CON group, **p<0.01;compared to the LPS group, p<0.0001;compared to the LPS+MK group and LPS+BNF group, p<0.0001,####p<0.0001. (n≥3).

(g) Protein expressionof p-AKT. Compared to the CON group, ****p<0.0001, @@@p<0.0005;compared to the LPS group, p<0.0001;compared to the LPS+MK2206 group, #p<0.05. (n≥3).

Figure 6

(a) The protein expression of Nrf2/ HO-1 .

(b)Protein expression of HO-1 signal.Compared to the LPS group, \$p<0.05;compared to the LPS+ML385+BNF group, #p<0.05;compared to the LPS+BNF group, *p<0.05. (n≥3).

(c)Protein expression of Nrf2 signal.Compared to the CON group, %p<0.05;compared to the LPS group, p<0.0001;compared to the LPS+ML385+BNF, ###p<0.0005;Compared to the LPS+BNF group, ****p<0.0001. (n≥3).

Figure 7

(a) The protein expression of NF-κB /HO-1.

(b)Protein expression of NF-κB signal. Compared to the CON group, %%%p<0.0005;compared to the LPS group,****p<0.0001;compared to the LPS+BNF group,##p<0.01,\$\$\$p<0.0005. (n≥3).

(a) SHAM group

(b) CLP group

(c) CLP+BNF group

(d) lung injury score

Figure 8

BNF alleviate CLP-induced sepsis of lung damage. Mice were pre-treated with BNF for 1 hour and collected lung tissue 24 hours after CLP. The lung tissue hematoxylin and eosin staining (scale bar: 10μm). (a) sham group mice; (b) CLP treated group mice; (c) BNF treated group mice. (d) Lung injury score. Compared to SHAM group *** $p < 0.005$; Compared to the CLP group, ## $P < 0.01$. ($n \geq 4$).

(a) SHAM group

(b) CLP group

(c) CLP+BNF group

Figure 9

Immunohisto chemical staining of lung tissue of Fib. The results demonstrated that the Fib of the CLP group was significantly higher than that of the SHAM group(a); however, after BNF treatment(c), Fib decreased significantly relative to the CLP group(b). Therefore, it is suggested that BNF has an improved effect on the coagulation function of mice.

Figure 10

Expression of plasma inflammation and coagulation factors in mice. Compared to the CON group, ****p<0.0001, ***p<0.0005, **p<0.01, *p<0.05; Compared to the CLP group, ****p<0.0001, ***p<0.0005, **p<0.01, *p<0.05. (n≥4).

Figure 11

(a) The protein expression of STAT3/AKT/Nrf2/HO-1.

(b) Protein expression of p-AKT. Compared to the CON group, **p<0.01; compared to the CLP group, ***p<0.0005.

(c) Protein expression of p-STAT3. Compared to the CON group, ****p<0.0001; compared to the CLP group ****p<0.0001

(d) Protein expression of Nrf2. Compared to the CON group, ns>0.05; compared to the CLP group, ****p<0.0001.

(e) Protein expression of HO-1. Compared to the CON group, ns>0.05; compared to the CLP group, ***p<0.0005.

Figure 12

Schematic of the regulation of septic inflammation and coagulopathy in RAW264.7 cells by AKT/NF-κB/Nrf2/HO-1 signaling.