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Regular paper

Effects of Baimuxinol on the inflammation and oxidative stress of LPS-induced RAW264.7 macrophages *via* regulating the NF-κB/IκBα and Nrf2/ARE signaling pathway

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Baimuxinol (BAI) is a sesquiterpenoid compound isolated from agarwood. This study aimed to investigate the specific mechanism of BAI on the inflammation as well as oxidative stress of RAW264.7 cells induced by lipopolysaccharide (LPS). The proliferation and cell viability were detected with EdU and MTT assay. The levels of inflammatory factors and antioxidant-related indexes were determined with corresponding kits. The qRT-PCR and western blot assays were performed to detect the expression of the related genes. We found that compared with the control group, cell viability and proliferation of the RAW264.7 cells was increased in the LPS group, while it was decreased in the BAI groups. In addition, in the LPS group, the contents of TNF-α, IL-1β, IL-6, ROS, MDA, PC and 8-OHdG were increased, the activities of T-SOD and CAT were decreased in comparison with the control group. It was reversed after BAI treatment. Finally, we confirmed that the NF-κB/IκBα signaling pathway is inhibited and the Nrf2/ARE signaling pathway is activated after BAI treatment. BAI relieved inflammation and oxidative stress of RAW264.7 macrophages induced by LPS through regulating the NF-κB/IκBα and Nrf2/ARE signaling pathway, which provided a novel insight for the therapy of sepsis.

Keywords: Baimuxinol, oxidative stress, lipopolysaccharide, proliferation, viability

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Abbreviations: BAI, Baimuxinol; CAT, catalase; HO-1, heme oxygenase-1; IL-1β, interleukin 1β; IL-6, interleukin 6; LPS, lipopolysaccharide; MDA, malondialdehyde; PC, protein crbonyal; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α

INTRODUCTION

Sepsis is a systemic inflammatory response syndrome, which refers to organ dysfunction caused by host response disorder. It is a serious life-threatening disease, not only has a high incidence rate and mortality but also takes up a lot of medical resources (Arefian *et al*., 2017). In 2016, the global medical community redefined sepsis, pointing out that sepsis is a fatal organ dysfunction due to the infection (Singer *et al*., 2016). At present, despite the advances in antibiotic therapy (Alhashem *et al*., 2017), mechanical therapy (Zampieri *et al*., 2017), maintaining blood glucose balance (Dellinger *et al*., 2008) and other treatment methods, severe sepsis is still the main cause of death. In addition, in the process of an inflammatory response, macrophages recognize pathogen/injury-related molecular patterns such as lipopolysaccharide (LPS) through toll-like receptor 4 (TLR4) and other pattern recognition receptors expressed on their cell membrane and then activate downstream signaling pathways, such as NF-κb and MAPKs (Lancaster *et al*., 2018). Many studies have confirmed that NF-κB is closely related to inflammation (McDaniel *et al*., 2016; Zaidi *et al*., 2018). The p65/p50 NF-κB usually binds to IκBα and exists in the cytoplasm (McDaniel *et al*., 2016). In response to an inflammatory reaction, IκBα will be phosphorylated and isolated from the NF-κB subunit. Free NF-κB transported to the nucleus and acted as a transcription factor, eventually leads to the secretion of inflammatory factors such as IL-6, IL-1β and TNF-α (Liang *et al*., 2018).

Additionally, there is the interaction between inflammatory response and oxidative stress, the role of oxidative stress in uncontrolled inflammatory diseases has been gradually concerned (Tian *et al*., 2017). Oxidative stress refers to the imbalance of the redox system in the body under the attack of harmful stimulating factors. Excessive reactive oxygen species (ROS) destroy the antioxidant function and lead to the inflammatory response (Newsholme *et al*., 2018; Forrester *et al*., 2018). Studies have found that Nrf2/ARE signaling pathway is closely related to oxidative stress in sepsis (Li *et al*., 2018). When oxidative stress occurs, Nrf2 will enter into the nucleus and interact with ARE to regulate the levels of downstream antioxidant genes, including heme oxygenase-1 (HO-1), catalase (CAT) and superoxide dismutase (SOD). Therefore, up-regulation of Nrf2 expression and activation of ARE-related antioxidant protein can improve oxygenation, inhibit oxidative stress response and alleviate sepsis injury(Wu *et al*., 2020).

Eaglewood, also known as Aquilaria Sinensis (Lour.) Spreng. is a famous traditional Chinese medicine in China. It has the functions of Xiangqi Zhitong, Wenzhong Zhou, Naqi Pingchuan. Clinically, it is commonly used in the treatment of chest and abdominal distension and pain, stomach cold and vomiting hiccup, kidney deficiency and dyspnea, etc. The main active components of Eaglewood are 2-(2-phenylmethyl) chromones and sesquiterpenes. Many studies have found eaglewood extract has anti-inflammatory and antioxidant effects (Zhou *et al*., 2008; Lin *et al*., 2013). Baimuxinol (BAI) is a sesquiterpenoid compound isolated from agarwood. However, as far as we know, there are very limited studies on the pharmacological effects of BAI.

Therefore, in the present study, we aimed to explore the effects of BAI on the proliferation and levels of inflammatory factors and antioxidant-related indexes of RAW264.7 cells stimulated by LPS. We hypothesized that BAI may alleviate LPS induced inflammation and oxidative stress *via* regulating the NF-κB/IκBα and Nrf2/ARE signaling pathway.

MATERIAL AND METHOD

Cell culture

RAW264.7 cells from leukemic mice were provided by the cell bank of Shanghai Chinese Academy of Sciences and cultured in high glucose DMEM medium containing 20% fetal bovine serum. 1% penicillin-streptomycin double-antibody was added to the medium. Then the cells were digested with 0.25% trypsin EDTA, passaged in 1:2, and cultured in a 5% CO_2 incubator at 37°C.

Groups

The cells were divided into Control group (CON), Lipopolysaccharide group (LPS, cultured with 50 mg/L LPS), Low baimuxinol group (L+BAI, cultured with 50 mg/L LPS+10 nM baimuxinol), Middle baimuxinol group (M+BAI, cultured with 50 mg/L LPS+50 nM baimuxinol), High baimuxinol group (H+BAI, cultured with 50 mg/L LPS+100 nM baimuxinol). Then the cells were cultured in a 5% CO_2 incubator at 37°C for 24 hours.

MTT

The cells were placed into 96-well plates (2×10^4 cells/well). The MTT reagent was added to the cells after incubation for 24 h. Then the cells were cultured with dimethyl sulfoxide. Finally, the absorbance was measured at 490 nm by a microplate reader.

5-ethynyl-2-deoxyuridine (EdU) Assay

The trypsin was added to the logarithmic phase cells, and resuspended in a complete medium to obtain cell suspensions, then the cells were placed into a 96-well plate (2×10^4 cells/well) and cultured at 37°C in an atmosphere of 5% CO_2, with the cell concentration adjusted to 2000 cells/well. The proliferation ability was measured with a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) according to the instructions of the manufacturer.

Determination of inflammatory factors antioxidant related indexes

The contents of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), protein carbonyl (PC) and ROS, and CAT and total superoxide dismutase (T-SOD) activities of the cells were determined by the corresponding kits provided by Nanjing Jiangcheng Bioengineering Institute (Nanjing, China).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The RNA was extracted with TRIzol® (Thermo Fisher Scientific, MA, USA). The transcript RT Kit (TIANGEN Biotech, Beijing, China) was conducted to reverse the RNA to cDNA. The mRNA expressions of the target genes were determined with a miScript SYBR Green PCR Kit (Qiagen, Germany) on a 7900 Real-Time PCR System. Reaction conditions: 95°C, 30 s; 95°C, 5 s, 40 cycles; 60°C, 30 s; 95°C, 15 s; 60°C, 30; 95°C, 15 s. The $2^{-\Delta\Delta Ct}$ method was performed to analyze the relative mRNA expressions. GAPDH was selected as the housekeeping gene. The primers used for qPCR were as follows: IL-6, For: TCCAGTTGCCTTCTTGGGAC, Rev: AGA-CAGGTCTGTTGGGAGTG; IL-1β, For: GAAATGCCACCTTTTGACAGTG and Rev: TGGATGCTCTCATCAGGACAG; TNF-α, For: AGCCGATGGGTTGTACCTTG, Rev: ATAGCAAATCGGCTGACGGT.

Western blot

The protein of the cells was lysed with RIPA buffer (Beyotime Biotechnology), and the concentration was detected using the BCA method (Beyotime Biotechnology). The protein samples (give concentration=20μg) were separated by 12% SDS-PAGE, transferred to the PVDF membrane (Millipore) and blocked in TBS (50 mmol/l Tris-HCL [PH7.6], 150mmol/l NaCl) containing 0.1% Tween-20 (TBS-T) and 5% non-fat milk powder for 2 hr. Then the PVDF membranes were incubated with the primary antibody (Nrf2, 1:1000 dilution; HO-1, 1:1000 dilution; NF-κB, 1:1000 dilution; p-NF-κB, 1:1000 dilution; IκBα, 1:1000 dilution; p-IκBα, 1:1000 dilution;GAPDH, 1:500 dilution; Abcam Trading Co. Ltd., Shanghai) at 4°C overnight. The incubation product was further incubated with a secondary antibody (1:2000 dilution) for 1.5h. Finally, the blot was colored with ECL, and then exposed by LAS4000 chemiluminescence imaging analyzer for gray analysis.

Statistical analysis

All data were expressed as mean ± S.D. SPSS22.0 software was performed for statistical analyses. One way analysis of variance (ANOVA) with a Tukey's post hoc test was used for comparison among groups. $P<0.05$ indicated significant difference.

RESULTS

BAI suppressed the viability of the RAW264.7 cells

First, we determined the effect of BAI on the viability of the RAW264.7 cells. The molecular structure of CEP was shown in Fig. 1A. Compared with the CON group, the viability of the cells in the LPS group was significantly increased. After BAI treatment, the viability of the cells was dramatically decreased (Fig. 1B). In addition, the proliferation of the cells was significantly increased after LPS treatment and BAI inhibited the growth of the cells in a dose-dependent manner (Fig. 1C–D).

BAI decreased the levels of inflammatory factors in the RAW264.7 cells

To accurately explore the role of the BAI on the inflammatory response. We analyzed the TNF-α, IL-1β and IL-6 levels of the cells. As shown in Fig. 2, in the LPS group, the levels of TNF-α, IL-1β and IL-6 were markedly up-regulated in comparison with the CON group. In comparison with the LPS group, the TNF-α, IL-1β and IL-6 levels were markedly decreased in L-BAI, M-BAI and H-BAI groups in a dose-dependent manner.

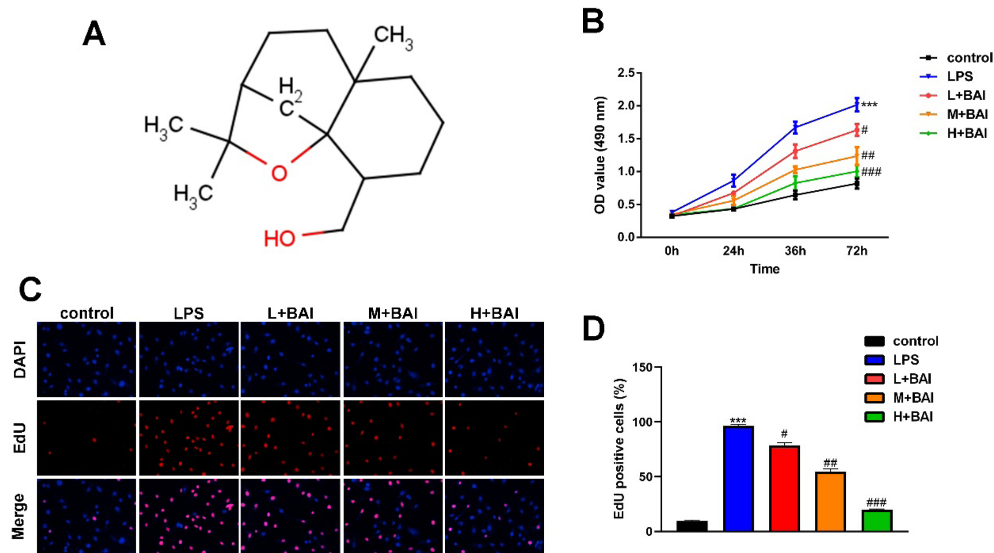

Figure 1. Role of BAI in the viability of RAW264.7 cells induced by LPS.
(**A**) Molecular structure formula of BAI. (**B**) The viability of the cells was detected with MTT. (**B–D**) The proliferation of the cells was detected by EdU. Notes: control, normal cells; LPS, normal cells + 50 mg/L LPS; L+BAI, normal cells + 50 mg/L LPS+10 nM baimuxinol; M+BAI, normal cells + 50 mg/L LPS+50 nM baimuxinol; H+BAI, normal cells + 50 mg/L LPS+100 nM baimuxinol. ****P*<0.001, *vs*. control group; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *vs*. LPS group.

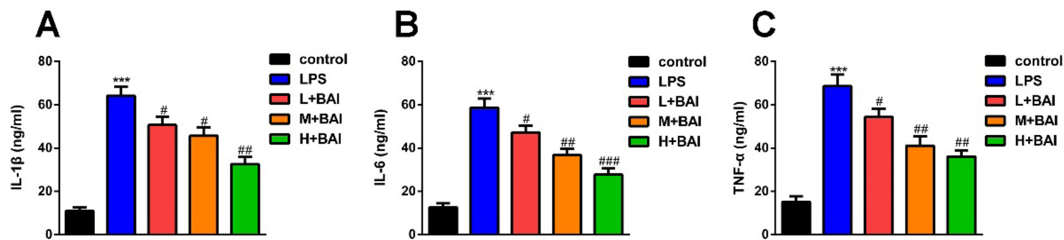

Figure 2. Role of BAI in the levels of TNF-α, IL-1β and IL-6 in RAW264.7 cells induced by LPS.
The levels of IL-1β (**A**), IL-6 (**B**) and TNF-α (**C**) in RAW264.7 cell was detected after LPS and BAI treatment. Notes: Control, normal cells; LPS, normal cells + 50 mg/L LPS; L+BAI, normal cells + 50 mg/L LPS+10 nM baimuxinol; M+BAI, normal cells + 50 mg/L LPS+50 nM baimuxinol; H+BAI, normal cells + 50 mg/L LPS+100 nM baimuxinol. ****P*<0.001, *vs*. control group; #P<0.05, ##*P*<0.01, ###*P*<0.001 *vs*. LPS group.

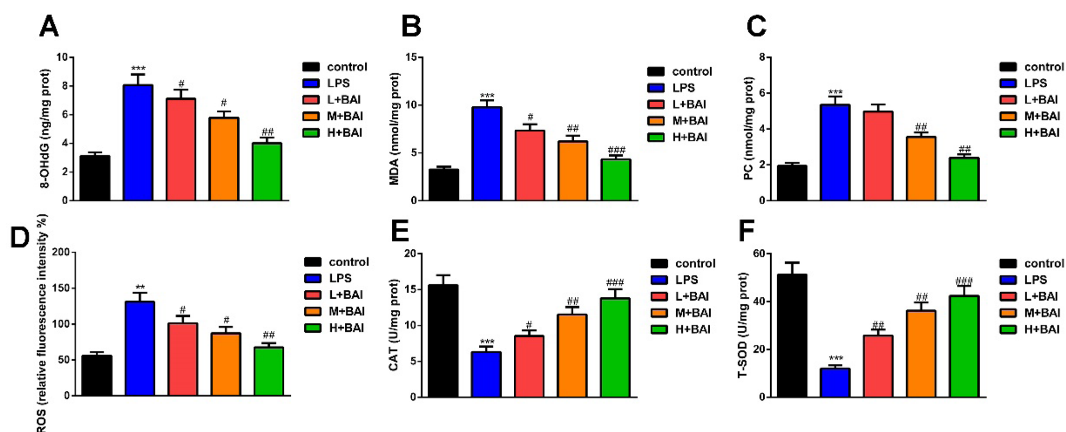

Figure 3. Role of BAI in the antioxidant function of RAW264.7 cells induced by LPS.
(**A**) The levels of 8-OHdG (**A**), MDA (**B**), PC (**C**), ROS (**D**), CAT (**E**) and T-SOD (**F**) in RAW264.7 cell was detected after LPS and BAI treatment. Notes: Control, normal cells; LPS, normal cells + 50 mg/L LPS; L+BAI, normal cells + 50 mg/L LPS+10 nM baimuxinol; M+BAI, normal cells + 50 mg/L LPS+50 nM baimuxinol; H+BAI, normal cells + 50 mg/L LPS+100 nM baimuxinol. ***P*<0.01, ****P*<0.001, *vs*. control group; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *vs*. LPS group.

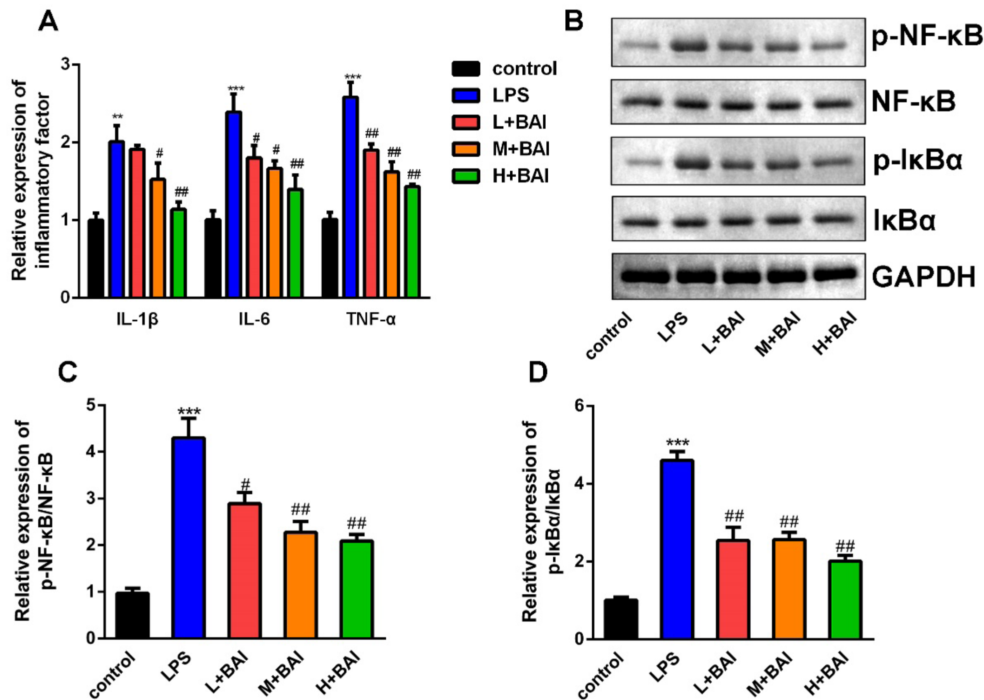

Figure 4. Role of BAI in the mRNA levels of inflammatory factors and NF-κB/IκBα signaling pathway in RAW264.7 cells induced by LPS.
(**A**) The mRNA expressions of TNF-α, IL-1β and IL-6 were measured with qRT-PCR. (**B–D**) The protein expressions of p-NF-κB, NF-κB, p-IκBα and IκBα were detected with western blot. Notes: Control, normal cells; LPS, normal cells + 50 mg/L LPS; L+BAI, normal cells + 50 mg/L LPS+10 nM baimuxinol; M+BAI, normal cells + 50 mg/L LPS+50 nM baimuxinol; H+BAI, normal cells + 50 mg/L LPS+100 nM baimuxinol. **P<0.01, ***P<0.001, *vs*. control group; #P<0.05, ##P<0.01 *vs*. LPS group.

BAI relieved the oxidative stress of the RAW264.7 cells

Subsequently, we explore the effects of BAI on the antioxidant capacity of the RAW264.7 cells. As shown in Fig. 3, in the LPS group, the concentrations of the ROS, MDA, PC and 8-OHdG were dramatically up-regulated, and the T-SOD and CAT activities were significantly down-regulated in comparison with the control group. BAI reversed the effects of LPS on the levels of ROS, MDA, PC, 8-OHdG, T-SOD and CAT in a dose-dependent manner.

BAI suppressed the NF-κB/IκBα signaling pathway

Next, we analyzed the role of BAI in mRNA expressions of inflammatory factors and NF-κB/IκBα signal-

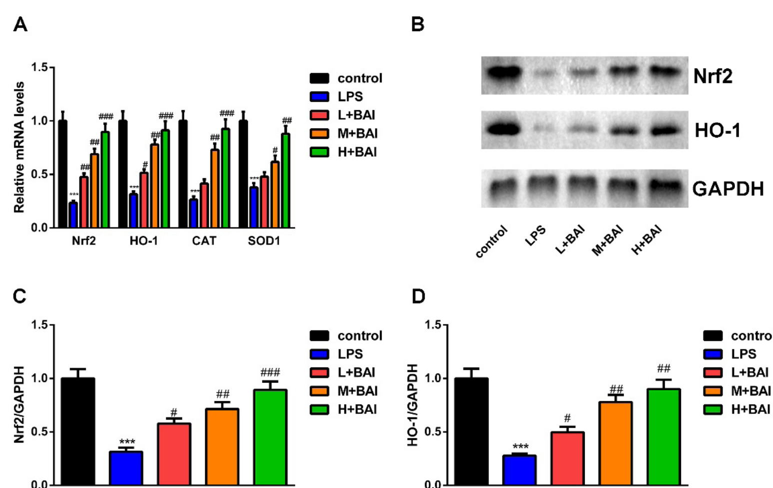

Figure 5. Role of BAI in the Nrf2/ARE signaling pathway in RAW264.7 cells induced by LPS.
(**A**) The mRNA expressions of Nrf2, HO-1, CAT and SOD1 were detected by qRT-PCR. (**B–D**) The protein expressions of Nrf2 and HO-1 were measured by western blot. Notes: Control, normal cells; LPS, normal cells + 50 mg/L LPS; L+BAI, normal cells + 50 mg/L LPS+10 nM baimuxinol; M+BAI, normal cells + 50 mg/L LPS+50 nM baimuxinol; H+BAI, normal cells + 50 mg/L LPS+100 nM baimuxinol. ***P<0.001, *vs*. control group; #P<0.05, ##P<0.01, ###P<0.001 *vs*. LPS group.

ing pathway. In the LPS group. the mRNA expressions of TNF-α, IL-1β and IL-6 were markedly up-regulated in comparison with the control group. Compared with the LPS group, the TNF-α, IL-1β and IL-6 mRNA expressions were markedly down-regulated in L-BAI, M-BAI and H-BAI groups in a dose-dependent manner (Fig. 4A). In addition, in comparison with the control group, the protein expressions of p-NF-κB and p-IκBα in the LPS group were markedly up-regulated. BAI treatment significantly down-regulated the protein expressions of p-NF-κB and p-IκBα in a dose-dependent manner (Fig. 4B–D).

BAI suppressed the Nrf2/ARE signaling pathway

Finally, the role of BAI in the Nrf2/ARE signaling pathway was explored. We found the mRNA expressions of Nrf2, HO-1, CAT and SOD1 were significantly decreased after LPS treatment, while BAI significantly increased the Nrf2, HO-1, CAT and SOD1 mRNA expressions in a dose-dependent manner (Fig. 5A). Additionally, the protein expressions of Nrf2 and HO-1 were increased in the LPS group in comparison with the control group. BAI markedly up-regulated the protein expressions of Nrf2 and HO-1 (Fig. 5B–D).

DISCUSSION

At present, the theory of uncontrolled inflammatory response and oxidative stress are major factors for the pathogenesis of sepsis. In the current study, we found BAI can reverse the excessive proliferation of RAW264.7 cells caused by LPS. Additionally, BAI could relieve inflammation and oxidative stress by regulating the NF-κB/IκBα and Nrf2/ARE signaling pathway.

Macrophages is one of the main cells that participated in inflammation. Stimulated by LPS, macrophages could induce the secretion of inflammatory factors, such as IL-1β, IL-6, TNF-α and so on, and then produce a clinical inflammatory response (Cotran *et al.*, 1999). Excessive inflammation is the major factor in many chronic diseases, including autoimmune diseases and neurodegenerative diseases. Therefore, inhibition of excessive inflammatory response is an effective method for the therapy of chronic diseases (Tabas *et al.*, 2013). TNF-α and IL-1β are the initiating factors in inflammatory mediators cascade reaction (Johnson *et al.*, 2015). Among them, TNF-α is a pro-inflammatory cytokine in regulating immune cells. It can induce fever and apoptosis by producing IL-1β and IL-6 and then induce inflammation. The abnormal content of TNF-α leads to the occurrence of many diseases(Wang *et al.*, 2020; Yosefifard *et al.*, 2019; Johnson *et al.*, 2015). IL-6 is a multifunctional cytokine that regulates immune, inflammatory and other physiological processes (Unver *et al.*, 2018). The expression of IL-6 is positively regulated by LPS and IL-1β. In the present study, we confirmed that BAI decreases the production and mRNA expression of LPS induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in a dose-dependent manner, suggesting BAI has potential anti-inflammatory activity. Previous studies have confirmed that eaglewood or its extract has an anti-inflammatory function. Lin and coworkers found that the ethanol extract of eaglewood leaf has an obvious anti-inflammatory effect on capillary dilation, hyperpermeability, exudation and edema in the early stage of inflammation (Lin *et al.*, 2013). Wu *et al.* showed that the extract of eaglewood can inhibit inflammation by down-regulating the expression of COX-2 and iNOS in the immune signal pathway (Wu *et al.*, 2012). These results were similar to ours and indicated that the extract of eaglewood has an effective anti-inflammatory effect. Therefore, we speculate that BAI, a sesquiterpenoid isolated from eaglewood, maybe a key anti-inflammatory compound.

To further explore the mechanism of BAI, we analyzed the protein expression of the NF-κB/IκBα signaling pathway. NF-κB is critically the control center to regulate inflammatory response (Tak *et al.*, 2001). NF-κB usually binds to IκB in cells in the form of inactive complexes. Once IκB is degraded, NF-κB dimer will transfer to the nucleus and start the transcription of the target gene. A previous study has confirmed that NF-κB can mediate the proliferation, differentiation and production of inflammatory factors of various immune cells(Hayden *et al.*, 2011). In this study, the proliferation ability of the cells was dramatically promoted by LPS, and after treatment of BAI, the over-proliferation of macrophages RAW264.7 was decreased. In addition, we found that LPS activates the NF-κB/IκBα signaling pathway and BAI inhibited it in a dose-dependent manner. Our findings indicated that the inhibitory effect of BAI on over-proliferation may be realized by NF-κB/IκBα inhibiting signal pathway. However, the specific mechanism needs to be further explored.

In addition, ROS is an important product of oxidative stress. When the body or cells are stimulated by endogenous or exogenous harmful substances, the antioxidant capacity will be destroyed, which results in a large amount of accumulated ROS and other oxides. Excessive ROS induces cell damage and promotes the occurrence of inflammatory reactions (Bae *et al.*, 2017). On the other hand, inflammatory cells release many active substances in the inflammatory site, which will also lead to the aggravation of oxidative stress (Kumar *et al.*, 2018). In sepsis, a large number of pro-inflammatory factors and the host's anti-inflammatory factors are released to induce the production of ROS, which leads to the imbalance of oxidation-reduction state in the body, causes oxidative stress reaction, and finally damages tissue cells and organ systems (Prauchner *et al.*, 2017). Previous studies have shown that various plant extracts can relieve oxidative damage in sepsis, such as curcumin (Zhong *et al.*, 2016) resveratrol (Aydin *et al.*, 2016), etc. In the current study, we also confirmed that BAI reduces the excess ROS induced by LPS. Furthermore, excessive ROS can lead to protein, lipid and DNA damage (Aitken, 2017), which was also be confirmed in this study that the MDA, PC and 8-OHdG levels are increased after LPS treatment. It implied that the cells lipid peroxidation, protein and DNA damage occur in the cells. It is worth noting that all these are relieved after BAI treatment. Nrf2/ARE signaling pathway is one of the most important protection systems against oxidative stress. When cells are exposed to oxidative stress, Nrf2 is recognized and combined with the DNA motif (GCTGAGTCA) on ARE, which starts the transcription of antioxidant genes, and then leads to the expression of antioxidants and related enzymes, such as HO-1 (Shaw *et al.*, 2020). Interestingly, we demonstrated that expressions of Nrf2 and HO-1 are up-regulated after BAI treatment, which indicated that BAI may improve the antioxidant function *via* the Nrf2/ARE signaling pathway.

To sum up, our results confirmed that BAI can effectively alleviate the inflammatory response and oxidative stress induced by LPS through regulating the NF-κB/IκBα and Nrf2/ARE signaling pathway. However, there are some limitations in this paper, such as we do not have *in vivo* experiments for validation because the con-

ditions do not permit. Also we hope to validate it on multiple cells in future studies. In conclusion, this study is the first to confirm that BAI has antioxidant and anti-inflammatory functions. Our findings provide a theoretical basis for the clinical application of eaglewood and a new method for the treatment of sepsis.

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