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# 쿠퍼 세포에서 Nrf2 활성화 매개 죽력의 염증 및 인플라마좀 억제 효능

양지혜*

동신대학교 한의과대학 한의예과

# Anti-inflammation and Anti-inflammasome Effects of *Bambusae Caulis* in Liquamen mediated by Nrf2 Activation in Kupffer cells

Ji Hye Yang*

College of Korean Medicine, Dongshin University

## ABSTRACT

**Objectives :** Bambusae Caulis in Liquamen (BCL), a traditional herbal medicine, is a distilled product of condensation from the burning of fresh bamboo stems. We previously identified the anti-oxidant capacity of BCL in hepatocytes and suggested that BCL is a promising therapeutic candidate for treating oxidative stress-induced hepatocellular damage. Despite the importance of the role played by Kupffer cells in liver disease, the efficacy of BCL on Kupffer cells is unclear. Therefore, this study aimed to determine whether BCL could suppress LPS-induced inflammation and LPS+ATP-induced inflammasomes in Kupffer cells.

**Methods :** We used ImKCs, a murine immortalized Kupffer cell line to examined whether BCL inhibited LPS-induced inflammation response and oxidave stress. And, we prepared a total of 18 L of BCL, purchased from Bamboo Forest Foods Co., Ltd. (648 Samdari, Damyang-eup, Damyang-gun, Jeollanam-do, Republic of Korea), was concentrated using a decompression concentrator.

**Result :** The LPS-induced release of inflammatory cytokines was abolished by BCL treatment. Also, BCL treatment suppressed the LPS+ATP-induced expression of inflammasome proteins (NLRP3, IL-1β, and IL-18), and inhibited the release of IL-1β. BCL decreased LPS-or LPS+ATP-induced reactive oxygen species production. In addition, BCL increased nuclear translocation of Nrf2 and the expression of HO-1 in a time-dependent manner.

**Conclusion :** These results suggest the efficacy of BCL with respect to its anti-inflammatory and anti-inflammasome effects mediated by Nrf2 in Kupffer cells.

**Key-words :** *Bambusae Caulis* in Liquame, Kupffer cells, Inflammation, Inflammasome, Nrf2

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## Ⅰ. Introduction

Kupffer cells are found in liver sinusoids or small blood vessels of the liver[1]. They are resident macrophages of the liver and act as first line of defense against pathogens, toxins, and other foreign substances that enter the liver via bloodstream[2]. In liver diseases, Kupffer cells contribute to the inflammatory cascade. In response to injury or infection, they release signaling molecules called cytokines that recruit other immune cells to the site of damage. While inflammation is a normal part of the immune response, chronic inflammation can contribute to liver diseases. Given their role in liver health and disease, Kupffer cells have become targets for therapeutic intervention[3,4].

NOD-like receptor family, pyrin domain-containing 3 (NLRP3) is a protein that plays a critical role in inflammasome formation, specifically the NLRP3 inflammasome[5]. The NLRP3 inflammasome is a multiprotein complex involved in activation of the inflammatory response, particularly in production of interleukin-1 beta (IL-1β) and interleukin-18 (IL-18)[6]. Dysregulation of NLRP3 inflammasome activation has been implicated in inflammatory disorders such as hepatitis. Chronic activation of the NLRP3 inflammasome contributes to sustained inflammation, tissue damage, and disease progression, such as liver fibrosis[7].

Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen, have antimicrobial properties, and help macrophages eliminate engulfed pathogens[8,9]. However, dysregulation of ROS production in activated macrophages can contribute to inflammation and inflammasome activation, leading to the release of pro-inflammatory cytokines such as IL-1β and IL-18[10,11]. Therefore, the regulation of ROS production in cells can attenuate excessive inflammation and inflammasomes.

Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a transcription factor that plays a central role in the cellular defense against oxidative stress[12,13]. It also regulates the expression of various anti-oxidant and detoxification genes. HO-1, heme Oxygenase-1, is one of the genes that can be upregulated by Nrf2[14,15]. HO-1 catalyzes the breakdown of heme, releasing biliverdin, carbon monoxide, and iron. HO-1 activity in macrophage has been shown to modulate the production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[16,17]. Modulation of the Nrf2 pathway has been considered in the development of treatment strategies for inflammatory diseases such as hepatitis[18].

*Bambusae Caulis* in Liquamen (BCL), a traditional herbal medicine, is a processed bamboo product obtained by refining the sap of fresh bamboo stems through process of heating[19]. In a previous study, we found that BCL has an Nrf2-mediated antioxidant capacity in hepatocytes[20]. Using liquid chromatography-mass spectrometry (LC-MS) assay, we found that high amounts of benzoic acid, a scavenger of free radicals, were present in BCL. BCL upregulates glutamate–cysteine ligase (GCL) and NQO1, which are targets for Nrf2. Moreover, the activation of the Nrf2 pathway by BCL was confirmed to be mediated through protein kinase Cδ. In addition, BCL inhibits effects of BCL on oxidative stress. Treatment with BCL reduced t-BHP-induced cell death and expressions of Bax and Bad in a dose-dependent manner. These findings suggest that BCL is a promising therapeutic candidate for treating oxidative stress-induced hepatocyte damage. However, although the role of Kupffer cells in liver disease is important, their anti-inflammatory and anti-inflammasome effects

*Corresponding author: Ji Hye Yang. College of Korean Medicine, Dongshin University, 67, Dongshindae-gil, Naju-si, Jeollanam-do, 58245, Republic of Korea.
Tel : +82-61-330-3518, E-mail : uranus2k@dsu.ac.kr
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have not yet been elucidated. Based on the findings of previous studies, this study aimed to determine whether BCL can suppress LPS-induced inflammation and LPS+ATP-induced inflammasomes in ImKCs, a murine immortalized Kupffer cell line. In addition, we verified that the anti-inflammation and anti-inflammasome effects of BCL are related to its anti-oxidant effects.

## 2. Materials and Methods

### 2.1. Materials

The antibodies against iNOS, Nrf2, and NLRP3 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against lamin A/C was obtained from Cell Signaling Technology (Danvers, MA, USA). The IL-1$\beta$ antibody was provided by R&D Systems (Minneapolis, MN, USA) and HO-1 antibody was obtained from Enzo Life Sciences (Plymouth Meeting, PA, USA). Horseradish peroxidase-conjugated goat anti-rabbit, anti-goat, and anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA, USA). ATP, LPS (*Escherichia coli* 055:B5), MTT, DCFH-DA, dimethylsulfoxide, sodium nitrite, and $\beta$-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). siNrf2 was purchased from Dharmacon (Lafayette, CO, USA).

### 2.2. Preparation of Bambusae Caulis in Liquamen (BCL)

*Bambusae Caulis* in liquid (BCL) was prepared as described in a previous study [20]. A total of 18 L of BCL, purchased from Bamboo Forest Foods Co., Ltd. (648 Samdari, Damyang-eup, Damyang-gun, Jeollanam-do, Republic of Korea), was concentrated using a decompression concentrator.

### 2.3. Cell Culture

ImKCs (SCC119) were obtained from Sigma-Aldrich was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (high glucose) supplemented with 50 units/mL penicillin/streptomycin and 10% fetal bovine serum at 37° C in a humidified 5% $CO_2$ atmosphere.

### 2.4. MTT Assay

To measure cell viability, cells were plated in 48-well plates and treated for 24 h. Viable cells were then stained using 0.2 mg/mL MTT for 1 h. The media was then removed and formazan crystals produced in the wells were dissolved using 200 $\mu$L of dimethyl sulfoxide. The absorbance at 540 nm was measured using a microplate reader (Spectramax; Molecular Devices, Sunnyvale, CA, USA).

### 2.5. NO Production Assay

NO production was evaluated using a Griess reagent (Sigma, St. Louis, MO, USA). Following pretreatment with BCL for 1 h, the cells were incubated with LPS for 15 h. The cell medium (100 $\mu$L) was transferred into a microplate, and then 100 $\mu$L Griess reagent (0.04 g/mL dissolved in distilled water) was added and allowed to react at 37° C for 30 min. Absorbance at 540 nm was analyzed using a microplate reader.

### 2.6. Measurement of ROS Generation

Diacetyldichlorofluorescein (DCFH-DA) is a cell-permeable, non-fluorescent probe that is cleaved by intracellular esterases and converted to highly fluorescent dichlorofluorescein upon reaction with $H_2O_2$. After treatment with LPS (100 ng/mL, 3 h) or LPS (100 ng/mL, 6 h) + ATP (5 mM, 30 min), ImKC cells were stained with 10 μM DCFH-DA for 30 min at 37° C. $H_2O_2$ generation was determined by measuring the fluorescence intensity of dichlorofluorescein using a fluorescence microplate reader (Jemini; Molecular Devices) with excitation and emission wavelengths of 485 and 530 nm, respectively.

### 2.7. Immunoblot Analysis

Protein extraction, subcellular fractionation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described[21]. Briefly, the samples were separated using 7.5% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were visualized using an ECL chemiluminescence detection kit (Amersham Biosciences). Equal protein loading and the integrity of subcellular fractionation were verified by β-actin expression in immunoblots.

### 2.8. RT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared using a cDNA synthesis kit (Bioneer, Daejeon, Korea) and a thermal cycler (Bio-Rad, Hercules, CA, USA). To obtain cDNA, total RNA (2 μg) was reverse transcribed using the oligo $(dT)_{18}$ primer. Primers were synthesized by Bioneer, Korea. The following primer sequences were used: mouse iNOS sense, 5′-CCTCCTCCACCCTACCAAGT-3′ and antisense, 5′-CACCCAAAGTGCTTCAGTCA-3′; mouse IL-1β sense, 5′-TGGACGGACCCCAAAAGATG-3′ and antisense, 5′-AGAAGGTGCTCATGTCCTCA-3′; mouse IL-18 sense, 5'-DNA-AGGACACTTTCTTGCTTGCC-3′ and antisense, 5'-DNA-CACAAACCCTCCCCACCTAA-3′; mouse GAPDH sense, 5′-TGCCCCCATGTTTGTGATG-3′ and antisense, 5′-TGTGGTCATGAGCCCTTCC-3.' GAPDH was used as a control for real time-PCR.

### 2.9. Enzyme-Linked Immunosorbent Analysis

IL-6, IL-1β, and TNF-α levels were quantified using an ELISA kit (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions. IL-6, IL-1β, and TNF-α levels in the cell supernatant were analyzed via ELISA using anti-mouse IL-1β, TNF-α, or IL-6 antibodies and biotinylated secondary antibodies, according to the manufacturer's instructions.

### 2.10. Statistical Analysis

One-way analysis of variance (ANOVA) was used to assess the statistical significance of differences among groups. For each statistically significant treatment effect, Newman–Keuls test was used for comparisons between multiple groups. The data were expressed as means ± standard error (S.E.).

## 3. Results

### 3.1. *Bambusae Caulis* in Liquamen inhibits an inflammatory response in ImKCs

Lipopolysaccharide (LPS) is found in the outer membrane of Gram-negative bacteria[22]. On detecting LPS, immune cells trigger an inflammatory response and activate inducible nitric oxide synthase (iNOS), leading to the production of nitric oxide, which in turn serves as a signaling molecule having various effects including antimicrobial activity against invading pathogens. In addition, in the presence of LPS, immune cells release pro-inflammatory cytokines, such as TNF-α and IL-6[23]. These cytokines help orchestrate the immune response, recruit other immune cells to the site of infection, and promote inflammation[24,25]. Thus, we investigated whether *Bambusae Caulis* in Liquamen (BCL) inhibited an LPS-induced inflammatory response in ImKCs, a murine immortalized Kupffer cell line. First, we checked the cytotoxicity of BCL in ImKCs using MTT assay and found no cytotoxicity at BCL concentrations of up to 30 mg/mL in cells. Consequently, following experiments were conducted with a maximum concentration of 30 μg/mL (Fig. 1A). Next, we examined whether BCL inhibited LPS-induced NO production and iNOS expression in ImKCs. LPS-stimulated NO production was decreased by BCL in a dose-dependent manner, and BCL suppressed the LPS-induced protein expression

and mRNA levels of iNOS (Fig. 1B-D). We investigated whether BCL inhibited the production of inflammatory cytokines using ELISA. The release of cytokines (TNF-$\alpha$ and IL-6) by LPS into the medium was abolished by BCL (Fig. 1E and F). Our results confirmed that BCL alleviated the LPS-induced inflammatory responses in Kupffer cells.

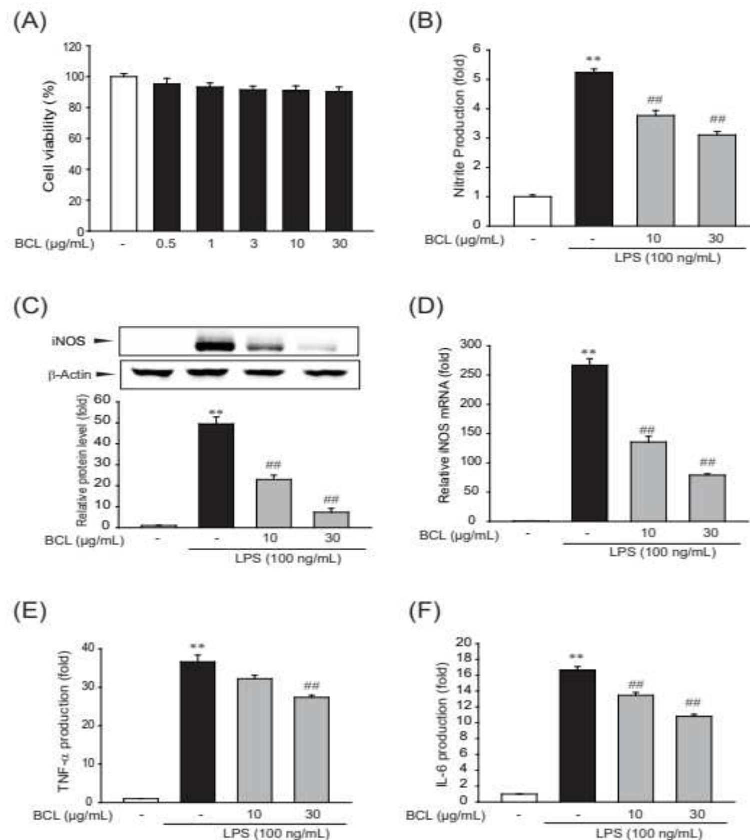


**Figure 1.** LPS-induced nitric oxide (NO) and pro-inflammatory cytokine expression were suppressed by *Bambusae Caulis* in Liquamen in Kupffer cells. (A) The cytotoxicity measurement of BCL in ImKCs using MTT. BCL was treated at concentrations ranging from 0.5 μg/mL to 30 μg/mL, and incubated for 24 h. (B) Nitrite production assay. BCL (10 μg/mL to 30 μg/mL) was pre-treated for 1 h, and then LPS was treated at 100 ng/mL for 15 h. We measured nitrite production in the culture supernatant using Griess reagent. (C) The measurement of iNOS protein expression in ImKCs using western blot. BCL was pre-treated for 1 h, and then LPS was treated at 100 ng/mL for 12 h. Protein levels of iNOS in the cell lysates were measured. (D) The measurement of iNOS mRNA level in ImKCs using real time PCR assays. BCL were pre-treated for 1 h, and then LPS was treated at 100 ng/mL for 6 h. (E,F) The measurement of TNF-$\alpha$ and IL-6 productions in ImKCs. BCL were pre-treated for 1 h, and then LPS was treated at 100 ng/mL for 6 h. We measured cytokine production in the culture supernatant using ELISA. Data are presented as mean ± S.E. of three replicates; $^{**}P < 0.01$ significant versus vehicle-treated control; $^{\#\#}P < 0.01$, significant versus LPS alone.

### 3.2. *Bambusae Caulis* in Liquamen inhibits an inflammasome in ImKCs

Inflammasomes are multiprotein complexes that play key roles in the innate immune system, particularly in inflammatory responses. NLRP3 is a sensor protein that forms part of the inflammasome[5]. It acts as a sentinel within cells and detects various signals associated with cellular stress and cell damage[26]. When NLRP3 is activated by these signals, it triggers the assembly of the inflammasome complex, leading to the activation of caspase-1. Active caspase-1 then cleaves pro-IL-1$\beta$ and pro-IL-18 into its active form, IL-1$\beta$ and IL-18[27]. They also play a role in enhancing the activities of natural killer cells and T cells. Therefore, we examined whether BCL affects the anti-NLRP3 inflammasome induced by LPS+ATP. As a result LPS+ATP increased the mRNA levels of NLRP3, IL-1$\beta$ and IL-18, whereas BCL treatment decreased their mRNA levels (Fig. 2A–C). In addition, BCL treatment suppressed the LPS+ATP-induced protein expression of NLRP3 and IL-1$\beta$ (Fig. 2D and E) and inhibited the release of IL-1$\beta$ into the culture supernatant (Fig. 2F). These results suggest that BCL can inhibit LPS+ATP-induced inflammasomes in ImKCs.

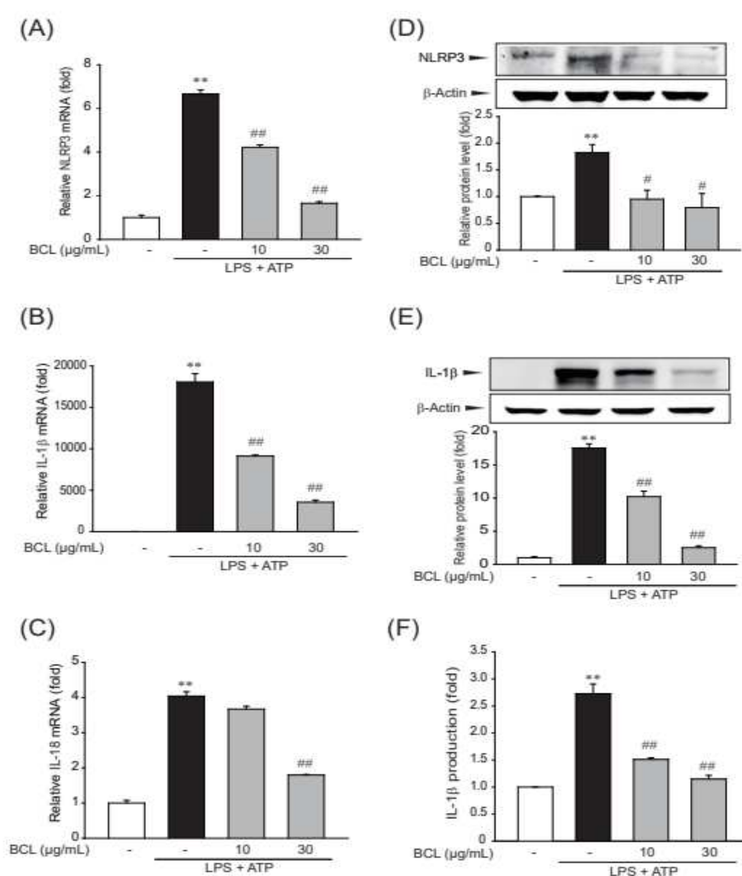


Figure 2. LPS+ATP-induced inflammasome was suppressed by *Bambusae Caulis* in Liquamen in Kupffer cells. BCL were pre-treated at concentrations ranging from 10 μg/mL to 30 μmg/mL for 1 h. Then, LPS was treated at 100 ng/mL for 6 h and ATP at 5 mM for 30 min (A–C). The measurement of

NLRP3, IL-1β, and IL-18 mRNA levels in ImKCs using real time PCR assays. (D,E) The measurement of NLRP3 and IL-1β protein expression in ImKCs using western blot. (F) The measurement of IL-1β production in ImKCs. We measured cytokine production in the culture supernatant using enzyme-linked immunosorbent assay (ELISA). Data are presented as mean ± S.E. of three replicates; $^{**}P < 0.01$ significant versus vehicle-treated control; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significant versus LPS + ATP.

### 3.3. *Bambusae Caulis* in Liquamen suppressed ROS production and activated Nrf2 in ImKCs

Various stimuli that activate the NLRP3 inflammasome, such as microbial products and cellular stress, can also induce ROS[28]. We previously found that BCL has excellent anti-oxidant efficacy and Nrf2 activation capacity[19]. Activation of Nrf2 in macrophages is a cellular defense mechanism against oxidative stress. This can lead to the upregulation of HO-1[14]. HO-1, when expressed in macrophages, can modulate the inflammatory response by producing anti-inflammatory molecules such as carbon monoxide[29]. Therefore, we examined whether BCL exhibits anti-oxidant efficacy and Nrf2 activation capacity in Kupffer cells. First, we confirmed that BCL decreased LPS- and LPS+ATP-induced ROS production in ImKCs (Fig. 3A and B). Subsequently, we examined whether BCL had Nrf2 activation capacity. BCL treatment increased the nuclear translocation of Nrf2 in a time-dependent manner and induced the expression of HO-1, a Nrf2 target (Fig. 3C and D).

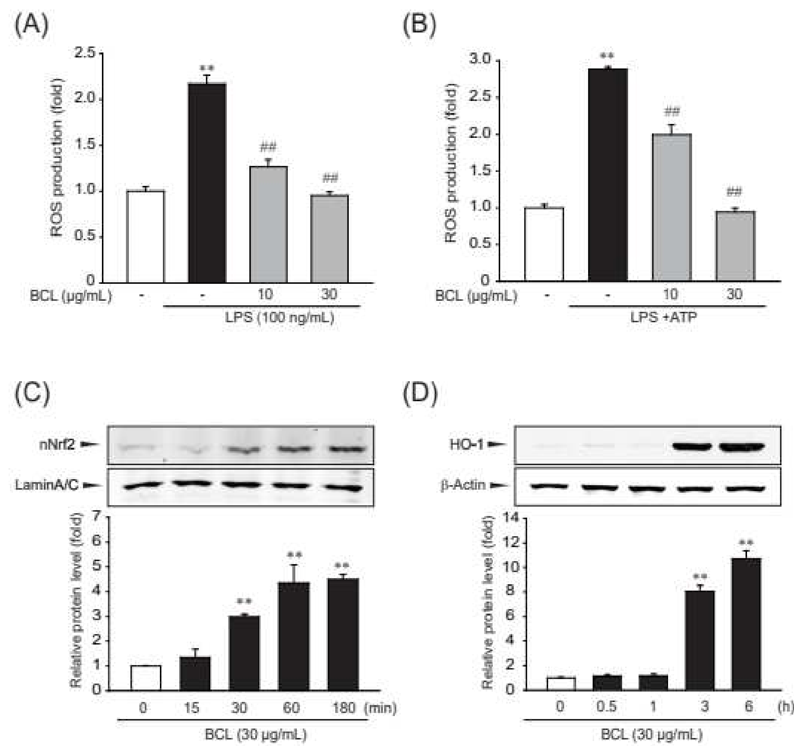


**Figure 3.** LPS or LPS+ATP induced-ROS productions were decrease by *Bambusae Caulis* in Liquamen in Kupffer cells. (A,B) The measurement of ROS production using DCFH-DA. BCL were pre-treated

at concentrations ranging from 10 μg/mL to 30 μg/mL for 1 h, and then LPS was treated at 100 ng/mL for 3 h (A). After BCL were pre-treated at concentrations ranging from 10 μg/mL to 30 μg/mL for 1 h, we treated LPS (100 ng/mL) for 6 h and ATP (5 mM) for 30 min (B). Then, cells were stained with DCFH-DA (10 μM) for 30 min. Intracellular fluorescence intensities were measured using a fluorescence microplate reader. (C) BCL induced-nuclear translocation of Nrf2 was measured in nuclear fraction of ImKCs using immunoblot. Cells were stimulated with BCL (30 μg/mL) for 15-180 min. (D) BCL induced-protein expression of HO-1 was measured in the lysates of ImKCs. Cells were stimulated with BCL (30 μg/mL) for 0.5-6 h. HO-1 protein expression was determined by immunoblotting. Data are presented as mean ± S.E. of three replicates; $^{**}P < 0.01$ significant versus vehicle-treated control; $^{\#\#}P < 0.01$, significant versus LPS alone or LPS+ATP.

### 3.4. Effects of *Bambusae Caulis* in Liquamen in ImKCs were associated with Nrf2.

Through the experiments in this study, we found that BCL can suppress inflammation and inflammasomes and has anti-oxidant efficacy and Nrf2 activation capacity in ImKCs. However, we are yet to determine whether the anti-inflammatory and anti-inflammasome effects of BCL are directly related to Nrf2 activation. Therefore, we used siRNAs for Nrf2 (siNrf2) to confirm the association between BCL and Nrf2 activation. siNrf2 reversed that BCL reduced iNOS protein expression and NO production (Fig. 4A and B). In addition, siNrf2 suppressed the effects of BCL in reducing IL-1β protein expression and release of IL-1β (Fig. 4C and D). These results suggest that the anti-inflammation and anti-inflammasome effects of BCL mediate by Nrf2.

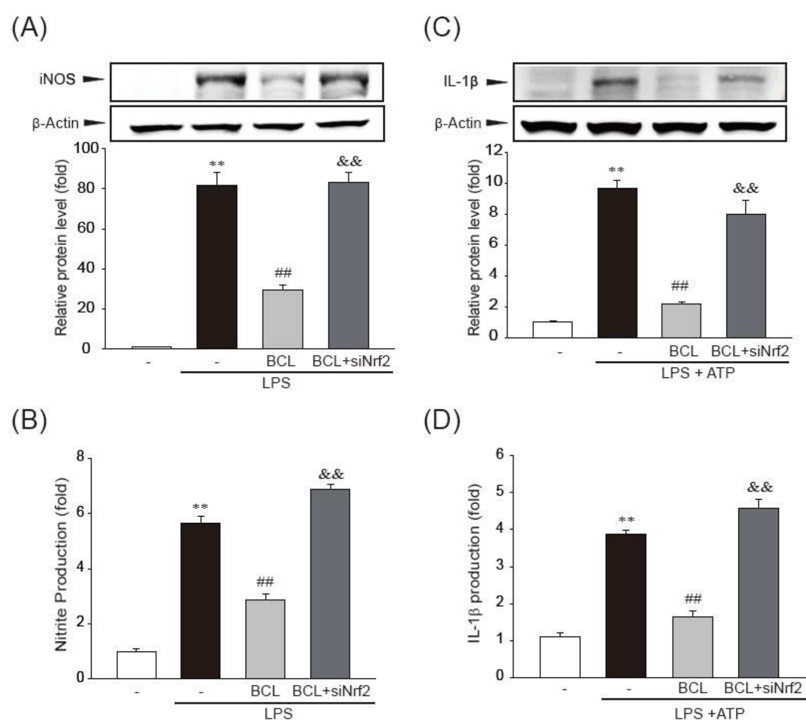


**Figure 4.** Anti-inflammation and anti-inflammasome effects of *Bambusae Caulis* in Liquamen is associated with

Nrf2 in Kupffer cells. (A) The measurement of iNOS protein expression using immunoblot. After siNrf2 transfection in ImKCs for 24 h. Cells were pre-treated with BCL (30 μg/mL) for 1 h and then LPS was treated at 100 ng/mL for 6 h. (B) Nitrite production assay. After siNrf2 transfection for 24 h in ImKCs, BCL was pre-treated for 1 h and then LPS was treated at 100 ng/mL for 15 h. We measured nitrite production using Griess reagent. (C,D) The measurement of IL-1β protein expression and IL-1β production. After siNrf2 transfection for 24 h in ImKCs, BCL were pre-treated for 1 h. Then, LPS was treated at 100 ng/mL for 6 h and ATP was treated at 5 mM for 30 min. IL-1β protein expression was determined by immunoblotting (C). IL-1β production was measured by ELISA kit (D). Data are presented as mean ± S.E. of three replicates; $^{**}P < 0.01$ significant versus vehicle-treated control; $^{\#\#}P < 0.01$, significant versus LPS+ATP; $^{\&\&}P < 0.01$, significant versus LPS+ATP and BCL.

## 4. Discussion

Based on previous findings, we investigated whether BCL could suppress inflammation and inflammasomes in Kupffer cells. Kupffer cells are specialized macrophages found in the liver and are the main effector cells that kill hepatocytes in various forms of hepatitis[30,31] through the production of proinflammatory cytokines, reactive oxygen species, nitric oxide, phospholipases, and lysosomal enzymes. First, we confirmed that BCL can inhibit LPS-induced NO production and iNOS expression in ImKCs (Fig. 1), and that BCL has anti-NLRP3 inflammasome effects induced by LPS+ATP (Fig. 2).

Subsequently, we examined whether BCL has anti-oxidant activity and Nrf2 activation capacity in Kupffer cells. LPS triggers endocytosis of the TLR4, leading to endothelial ROS production through NAD(P)H oxidase (NOX) activation[32,33]. LPS-induced macrophages produce pro-inflammatory cytokines such as TNF-α and IL-6. Treatment of macrophages with ATP results in the production of ROS, which stimulate the PI3K pathway and subsequent Akt and ERK1/2 activation[34,35]. ATP-mediated ROS-dependent PI3K is required for the activation of caspase-1 and secretion of IL-1β and IL-18[36]. Therefore, we found that BCL decreased LPS- or LPS+ATP-induced ROS production, increased nuclear translocation of Nrf2, and induced the expression of HO-1 in ImKCs (Fig. 3).

Nrf2 plays a central role in the regulation of cellular antioxidant and other cytoprotective genes in cells, including macrophages. Activation of Nrf2 in macrophages has been associated with anti-inflammation and anti-inflammasomes[37,38]. Nrf2 helps maintain the redox balance within macrophages, ensuring that ROS levels are controlled and do not lead to excessive oxidative stress or inflammatory responses[39]. Therefore, the modulation of Nrf2 activity in Kupffer cells may have therapeutic implications, especially in conditions characterized by inflammation and oxidative stress, such as hepatitis. In the present study, we used siNrf2 to confirm the association between BCL and Nrf2 activation. siNrf2 inverted effects of BCL in reducing inflammation and inflammasome (Fig. 4).

These results suggested that BCL is a potential therapeutic drug candidate for the prevention and treatment of hepatitis.

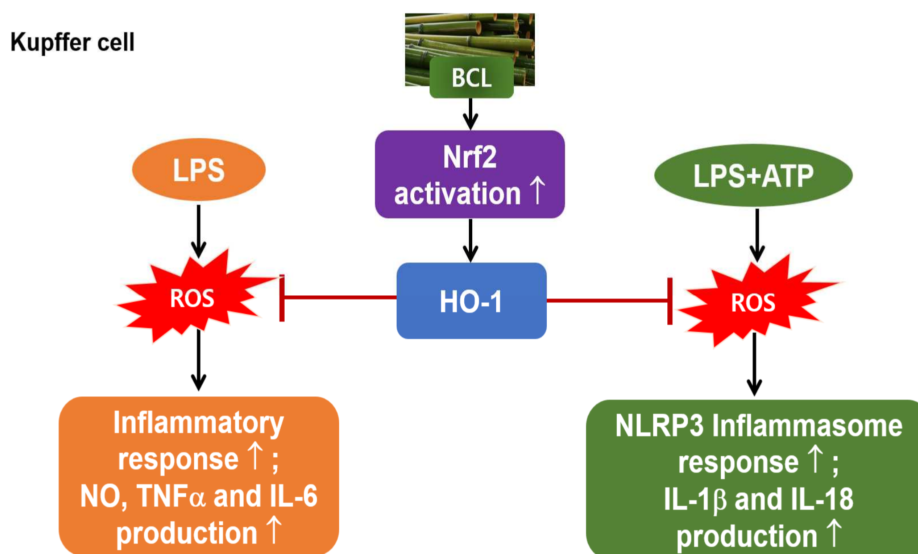


Figure 5. Schematic diagram illustrating the mechanism of BCL effects.

## 5. Conclusions

The study results showed that BCL inhibited LPS-induced inflammation and the LPS+ATP-induced inflammasome in Kupffer cells. BCL decreases LPS-or LPS+ATP-induced ROS production and increases the nuclear translocation of Nrf2. In addition, effects of BCL in reducing inflammation and inflammasome were reversed by siNrf2. These results suggest that BCL is effective in Kupffer cells and that the anti-inflammatory and anti-inflammasome effects of BCL are mediated by Nrf2 (Fig. 5). This study suggests that BCL is a promising therapeutic drug for the treatment of liver injuries.

## 6. A written apology

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