

# BuyangHuanwu Decoction Inhibits LPS-induced Inflammation in BV2 Microglial Cells by NF- $\kappa$ B/NLRP3 Pathway BYHWD Inhibits LPS-induced Inflammation in BV2 Cells

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## Research

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# Abstract

**Background:** Neuroinflammation has been implicated in the pathogenesis of various neurological and cerebrovascular diseases. Recently, microglia has become a promising target to treat neuroinflammation-related diseases. BuyangHuanwu Decoction (BYHWD), a traditional Chinese medicine recipe, is a representative prescription for the treatment of neuroinflammation, showed great therapeutic potential in neuroinflammation-related cerebrovascular disorders including cerebral ischemia. However, its mechanism of action remains unclear.

**Methods:** In the present research, we studied the therapeutic effects of BYHWD on LPS-induced inflammatory response in BV2 microglia cells. The inflammatory cytokines include IL-6, TNF- $\alpha$ , NO were detected by the commercial kit. The inflammatory-related protein was measured by western blot and immunofluorescence.

**Results:** Our findings showed that BYHWD could significantly reverse LPS-induced morphological changes in BV2 microglial and inhibits the local synthesis of proinflammatory cytokines and free radical via directly regulating NF- $\kappa$ B activity and inflammasome assembly. Our data showed that BYHWT inhibits LPS-induced TNF- $\alpha$ , IL-6, and NO production, Also can inhibit iNOS protein expression and the activation of NF- $\kappa$ B according to inhibit NLRP3 activation.

**Conclusions:** In conclusion, our results demonstrated that BYHWD is a potential candidate for treating neuroinflammation-related diseases. Certainly, more comprehensive researches on the precise mechanism of action of BYHWD in cerebrovascular disease is required in future vivo studies.

## 1. Introduction

Neuroinflammation has been implicated in the pathogenesis of various neurological and cerebrovascular diseases, such as Alzheimer's disease, depression, stroke and cerebral ischemia etc.<sup>1,2</sup> The immune response within the central nervous system (CNS) involves local immune cells and signaling pathways. Microglia, which is the resident macrophage in the CNS, are versatile cells involving in triggering inflammatory pathways and oxidative stress<sup>3</sup>. In response to exogenous stimuli, activated microglia transmit inflammatory reactions by releasing pro-inflammatory cytokines and mediators. Thus, recently, microglia has become a promising target to treat neuroinflammation-related diseases.

BuyangHuanwu decoction (BYHWD) was originally recorded in the traditional herbal literature of Yi-Lin-Gai-Guo written by Qing-Ren Wang in 1830 during the Qing Dynasty. According to the Chinese Pharmacopoeia <sup>4</sup>, the decoction is comprised of seven commonly used Chinese herbal drugs: 1. Radix Astragali (*huangqi*), the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var., mongholicus (Bge.) Hsiao; 2. The carda part of Radix Angelicae Sinensis root (*guiwei*), the dried lateral roots of *Angelica sinensis* (Oliv.) Diels; 3. Radix Paeoniae Rubra (*chishao*), the dried roots of *Paeonia lactiflora* Pall.; 4. Rhizoma Chuanxiong (*chuanxiong*), the dried rhizomes of *Ligusticum chuanxiong* Hort; 5. FlosCarthami (*honghua*), the dried flowers of *Carthamus tinctorius* L.; 6. Semen Persicae (*taoren*), the dried seeds of

*Amygdalu spersica* L.; and 蛲. *Pheretima (dilong)*, the dried bodies of *Pheretima aspergillum* (E. Perrier), in the ratio of 120:6:4.5:3:3:3 on a dry weight basis, respectively. According to the traditional Chinese medical literature, this formula has been shown to provide neuroprotective effects for neuroinflammation related conditions such as brain ischemia<sup>5,6</sup>, stroke<sup>7,8</sup>. A previous study has found that BYHWT have the potential improvement for ischemic stroke and extended lifespan, primarily by regulating neuroinflammation, apoptosis, angiogenesis and blood coagulation, as well as by mediating neurogenesis and nervous system development<sup>9</sup>. However, the nothing is known about the effects of BYHWD in regulating neuroinflammation. Thus, in the present study, we focused on the effects of BYHWD on LPS-induced inflammatory response in BV2 microglia cell, and aimed to determine the possible mechanism underlying its anti-inflammatory action.

## 2. Methods

### 2.1 Drug Administration

Buyang Huanwu Decoction is a granule made up of *Radix Astragali seu Hedysari*, *Radix Angelicae Sinensis*, *Radix Paeoniae Rubra*, *Lumbricus*, *Semen Persicae*, *Flos Carthami*, *Rhizoma Ligustici Chuanxiong*, according to the ratio of 120 : 6 : 4.5 : 3 : 3 : 3. The decoction was made by boiling the mixture in distilled water at 100°C for 30 min three times, then the drug solution was cooled and dried to give the drug powder, weigh 400mg powder and dissolve in 40ml DMEM medium to make 10mg/ml mother liquor.

### 2.2 Cell Culture

BV2 mouse microglia cell come from Hunan University of Traditional Chinese Medicine. Cells were maintained in DMEM medium with 10% FBS (Procell), 10000u/mL penicillin and 10000µg/mL streptomycin(Solarbio) at 5%CO<sub>2</sub>, 37°C incubator.

### 2.3 Drugs and Treatment

BYHWT was dissolved in DMEM medium to a concentration of 10mg/mL, LPS(Sigma) was dissolved in PBS(Procell) to a concentration of 1mg/mL, then pass 0.22um sterile filter. In all experiment, cells were pretreated with BYHWT(1-8mg/ml) for 2h before stimulated with LPS(400ng/mL) for 24h.

### 2.4 CCK-8 Assay

BV2 cells were seeded in 96-well plates at a density of 8\*10<sup>3</sup> cells per well(100µL per well). The cells were processed as described above, and 6 parallel holes were set for each group. After 24 hours of stimulation, remove the cell supernatant and add medium containing 10% CCK-8(Biosharpe) and the culture was continued for 3h, The absorbance was measured with a microplate reader (5LX800,Biotek)at 450 nm.

### 2.5 NO Assay

BV2 cells were seeded in 96-well plates at a density of  $8 \times 10^3$  cells per well(100 $\mu$ L per well). The cells were processed as described above, and 6 parallel holes were set for each group. After incubation for 24h, the supernatant of culture was collected and the concentration of NO was measured by commercial kit (Beyotime) according to the manufacturer's instruction.

## 2.6 ELISA Assay

BV2 cells were seeded in 6-well plates at a density of  $1 \times 10^5$  cells per well(2mL per well). The cells were processed as described above, and 3 parallel holes were set for each group. After incubation for 24h, the supernatant of culture was collected, and analyzed by ELISA according to the manufacturer's protocol(MULTI SCIENCES)

## 2.7 Western Blotting

BV2 cells were seeded in 6-well plates at a density of  $1 \times 10^5$  cells per well (2mL per well), incubated and treated as mentioned above. Cultured cells were lysed with RIPA buff(add PMSF at 100:1),After lysing the cells on ice for 30 minutes, The supernatants were collected and then quantitated for protein determination using a BCA Protein Assay kit and the cells protein(30 $\mu$ g) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 2h at 80V, and transferred to polyvinylidene fluoride (PVDF) membranes(Immobilon, USA) for 120 min at 200 mA. After blocking with 5% non-fat milk or BSA for 1h at 37°C, the membrane was incubated overnight with primary antibodies. (GAPDH, Proteintech 1:5000;NF- $\kappa$ B p65,Abcam,1:1000;p- NF- $\kappa$ B p65,Abcam,1:2000;IkB-a, Cell Signaling Technology, 1:1000; p-IkB-a, Cell Signaling Technology, 1:1000; NLRP3, Abcam, 1:1000) in antibody dilution(Solarbio) at 4°C overnight. Then the membrane was washed three times for 15 min each with TBST buffer, and incubated for 60 min at 37°C with HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) and HRP-conjugated Affinipure Goat Anti-Mouse IgG(H + L)(Proteintech),secondary antibodies diluted in TBST buffer (1:8000). Finally, the membrane was washed three times for 15 min with TBST buffer. The protein bands were detected with enhanced chemiluminescence (Millipore), The GAPDH protein level and  $\beta$ -actin protein level was used as protein loading control.

## 2.8 Immunofluorescence staining

BV2 cells were seeded in 24-well plates at a density of  $3 \times 10^4$  cells per well(500 $\mu$ L per well), incubated and treated as mentioned above. After fixed with 4% paraformaldehyde for 20 min at room temperature, cell were permeabilized with 0.1% Triton X-100 for 20 min. Then blocking with 3% BSA for 1h at 37°C, and were incubated overnight at 4°C with primary antibodies. (iNOS, Cell Signaling Technology, 1:400;NLRP3,1:50,Boste). On the flowing day, After washed with PBS for three times, cells were incubated with secondary antibodies (Fluorescein(FITC)-conjugated Affinipure Goat Anti-Rabbit IgG(H + L),1:400;Alexa Flour 594- conjugated Affinipure Goat Anti-Rabbit IgG(H + L),1:400;Donkey Anti-Goat IgG H&L (Alexa Fluor® 555),1:200, Proteintech )in 1%BSA dilution for 1h at 37°C. The cells were treated with DAPI(Solabio) for 10 min. All images were captured with a fluorescence microscope (Olympus)

## 2.9 Statistical analysis

All analyses were performed using SPSS version 23.0 (IBM, USA). Data were represented as the means ± SEM. The data were analyzed with a one-way analysis of variance (one-way ANOVA) or repeated-measures analysis of variance (RM ANOVA) where statistically appropriate. When significant effects were detected, post hoc multiple pairwise comparisons were made with the LSD comparisons test after ANOVA. The analysis results were only reported when a significant difference was found. Statistical significance was defined as  $P < 0.05$ .

### **3. Results**

#### **3.1 Screening of the best dose for BWT and LPS**

To determine the effect of BYHWT on the toxic effect of BV2 microglia, the CCK-8 assay was performed 24 h after treatment with various concentrations of BYHWT ranging from 1 mg/mL to 9mg/mL. Results(Fig1A) show that the dose of the drug shows obvious cytotoxicity at 9mg/ml. Thus, the concentration of BYHWT range of 1-8mg /mL for the subsequent experiments. According to Fig1B and Fig1C, 400ng/ml of LPS is not significantly toxic to cells, and has the highest level of NO production for cells. After the cells were pretreated with different concentrations of BYHWT for 2h, 400ng/mL LPS was added to stimulate the cells for 24h, the addition of LPS and BYWHT had no significant effect on cell viability(Fig1D), indicating that subsequent experiments can be performed at this concentration. As indicated in Fig1E, the morphological changes in BV2 cells were evaluated after treatment with BYHWT with or without LPS, microscopic showed that the resting microglia were spindle shaped with small cell bodies and long cellular pseudopods. After stimulated with LPS, the cells have reduced pseudopods, and cell bodies become larger and rounder, but BYHWT effectively reduced this change, And as the drug dose increases, the number of cells tending to a resting state increases. These results show that BYHWT can significantly decrease the change of cell morphology induced by LPS.

#### **3.2 BYHWT inhibit LPS-induced TNF- $\alpha$ , IL-6, and NO production, And can inhibit iNOS protein expression**

To investigate the anti-inflammatory effects of BYHWT, the expression of inflammatory mediators were detected in this study by ELISA. As shown in Fig2A and 2B, the IL-6, TNF- $\alpha$  levels increased in the cell culture media in the LPS treated alone group, and pre-treatment with different concentration of BYHWT dramatically

decrease of cytokine production, same as NO production level(Fig2C). Similarly, iNOS protein from cells for Western Blot and Immunofluorescence detection also showed the same results (Fig2E).

#### **3.3 BYHWT effectively inhibits LPS-induced activation of NF- $\kappa$ B**

NF-κB activation mediates many inflammatory responses in the central nervous system. To investigate the anti-inflammatory mechanism of BYHWT, Western blot analysis used to detect the protein expression of NF-κB and IκB-α expression level. As shown in Fig3A-B, the LPS stimulation resulted in phosphorylation levels of NF-κB p65 and IκB-α, and the effect were significantly inhibited by the treatment of BYHWT. The decrease of IκBα protein level and IκBα increased significantly after compared with control group. However, The molecular mechanisms of BYHWT on neuroinflammation involved significantly inhibited IκB phosphorylation and degradation following LPS activation.

### **3.4 BYHWT decrease inflammatory response through inhibits NLRP3 activation**

To investigate whether BYHWT affects the NLRP3 inflammasome activation in LPS-activated BV2 cells, The expression protein levels of NLRP3 were examined by western blot analysis and Immunofluorescence detection. As shown in Fig4A-4B, the NLRP3 level were markedly elevated after LPS stimulation, and treatment with significantly decreased LPS-induced NLRP3 level in BV2 cells. It indicates that the anti-inflammatory effect of BYHWT may be achieved by regulating the activation of NLRP3.

## **4. Discussion**

BuyangHuanwu decoction (BYHWD), a traditional Chinese medicine (TCM) prescription, has long been used clinically to aid neuroprotective effects after stroke. According to experimental reports, BHD improves blood circulation, controls pain, regenerates neuronal cells<sup>10-12</sup>. And protects against the neuronal damage caused by ischemic and oxidative stress<sup>13,14</sup>. BYHWT have been traditionally used to treat cognitive deficits and other brain disorders in TCM owing to the virtues of neuroprotection, anti-oxidative stress anti-apoptosis, anti-inflammation and neurotrophic actions<sup>5,14-17</sup>. Our research it is, or at least partially, helpful to better comprehend the effective underlying mechanisms of classical prescription.

In the present research, we found that BYHWD was effectively to inhibit LPS induced inflammatory responses in BV2 microglia cells. We noted that BYHWD under 9 mg/ml showed no toxicity to BV2 microglial cells. It could significantly reduce LPS-induced morphological changes and inhibit the production of pro-inflammatory cytokines and free radical. LPS is the most widely used inflammatory mediator, which can activate microglia and trigger the pro-inflammatory signaling cascade.

In the present research, we found that BYHWT was effectively to inhibit LPS induced inflammatory responses in BV2 microglia cells. We noted that BYHWT showed no toxicity to BV2 microlial cells. It could significantly reduce LPS-induced morphorlogical changes and inhibit the production of pro-inflammtory cytokines and free radical. LPS is the most widely used inflammatory mediator, which can activate microglia and trigger the pro-inflammatory signaling cascade<sup>18</sup>. In normal states, microglia surveils the microenvironment whilst maintains the homeostasis in the brain. While in disease states, in response to

injury, harmful toxins, infection or inflammation, microglial cells secret pro-inflammatory mediators, which are regulated by the transcription factor nuclear factor kappa B (NF- $\kappa$ B), to amplify neuroinflammation and result in pathological changes. NF- $\kappa$ B is the central regulator of inflammation that controls the gene transcription of chemokines, cytokines, proinflammatory enzymes, adhesion molecules and proinflammatory transcription factors<sup>19,20</sup>. Consistently, we observed that NF- $\kappa$ B was activated in BV2 microglia cells and resulted in the generation of proinflammatory cytokines (IL-6 and IL-1 $\beta$ ) and inducible enzymes (iNOS), as well as nitric oxide (NO). In resting cells, the inhibitors of  $\kappa$ B ( $I\kappa$ B) family sequesters NF- $\kappa$ B in the cytoplasm. LPS activates a complex of  $I\kappa$ B kinases (IKK) and results in the phosphorylation of  $I\kappa$ B protein, which is rapidly ubiquitinated and degraded<sup>21</sup>. This alteration leads to the phosphorylation of NF- $\kappa$ B p65 at Ser536 and the release of NF- $\kappa$ B from  $I\kappa$ B. Liberated NF- $\kappa$ B then translocates to the nucleus and binds to specific gene promoter elements to initiate transcription<sup>22</sup>. Therefore, the regulation of NF- $\kappa$ B is crucial in neuroinflammation-related diseases control<sup>23</sup>. Our data displayed that SYG was effective in regulating the activity of NF- $\kappa$ B.

Moreover, activated NF- $\kappa$ B enhances the transcriptional level of cytosolic innate immune signaling receptor NOD-, LRR- and pyrin domain-containing 3 (NLRP3), a pivotal mediator of IL-6-associated neuroinflammation<sup>24,25</sup>. Targeting NLRP3 pathway is also a promising strategy to develop pharmacotherapy for inflammation-associated diseases. Our finding indicated that BYHWT could inhibit local synthesis of IL-6 by suppressing the formation of NLRP3 inflammasome and overexpression.

In conclusion, our results demonstrated that BYHWD is a potential candidate for treating neuroinflammation-related diseases via directly regulating NF- $\kappa$ B activity and inflammasome assembly. Certainly, more comprehensive researches on the precise mechanism of action of BYHWD in cerebrovascular disease is required in future vivo studies.

## Conclusions

BYHWT inhibits LPS-induced TNF- $\alpha$ , IL-6, and NO production, Also can inhibit iNOS protein expression and the activation of NF- $\kappa$ B according to inhibit NLRP3 activation. Our findings provided a new strategy that BYHWD is a potential candidate for treating neuroinflammation-related diseases via directly regulating NF- $\kappa$ B activity and inflammasome assembly.

## Abbreviations

BYHWD:BuyangHuanwu Decoction;LPS:Lipopopolysaccharide;CNS:Central Nervous System;TCM:Traditional Chinese Medicine; FBS:Fetal Bovine Serum;PBS:Phosphate Buffer Saline;ELISA:Enzyme-linked immunosorbent assay;PMSF:Phenylmethanesulfonyl Fluoride;CCK-8: Cell Counting Kit-8 assay;NF- $\kappa$ B:Nuclear Factor Kappa B; iNOS:Inducible Nitric Oxide synthase;NO:Nitric Oxide; $I\kappa$ B:Inhibitors of  $\kappa$ B; NLRP3:NOD-, LRR- and pyrin domain-containing 3;IL-6, Interleukin-6;TNF- $\alpha$ :Tumor Necrosis Factor aerfa

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable

### **Consent to publish**

Not applicable

### **Availability of Data and Materials**

All the data used to support the findings of this study are available from the corresponding author upon reasonable request.

### **Competing interests**

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

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### **Authors' contributions**

ZS designed the study and wrote the draft together; ZP and SP performed the study; LL analyzed the results and reviewed the manuscript, all authors approved the final version.

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Not applicable

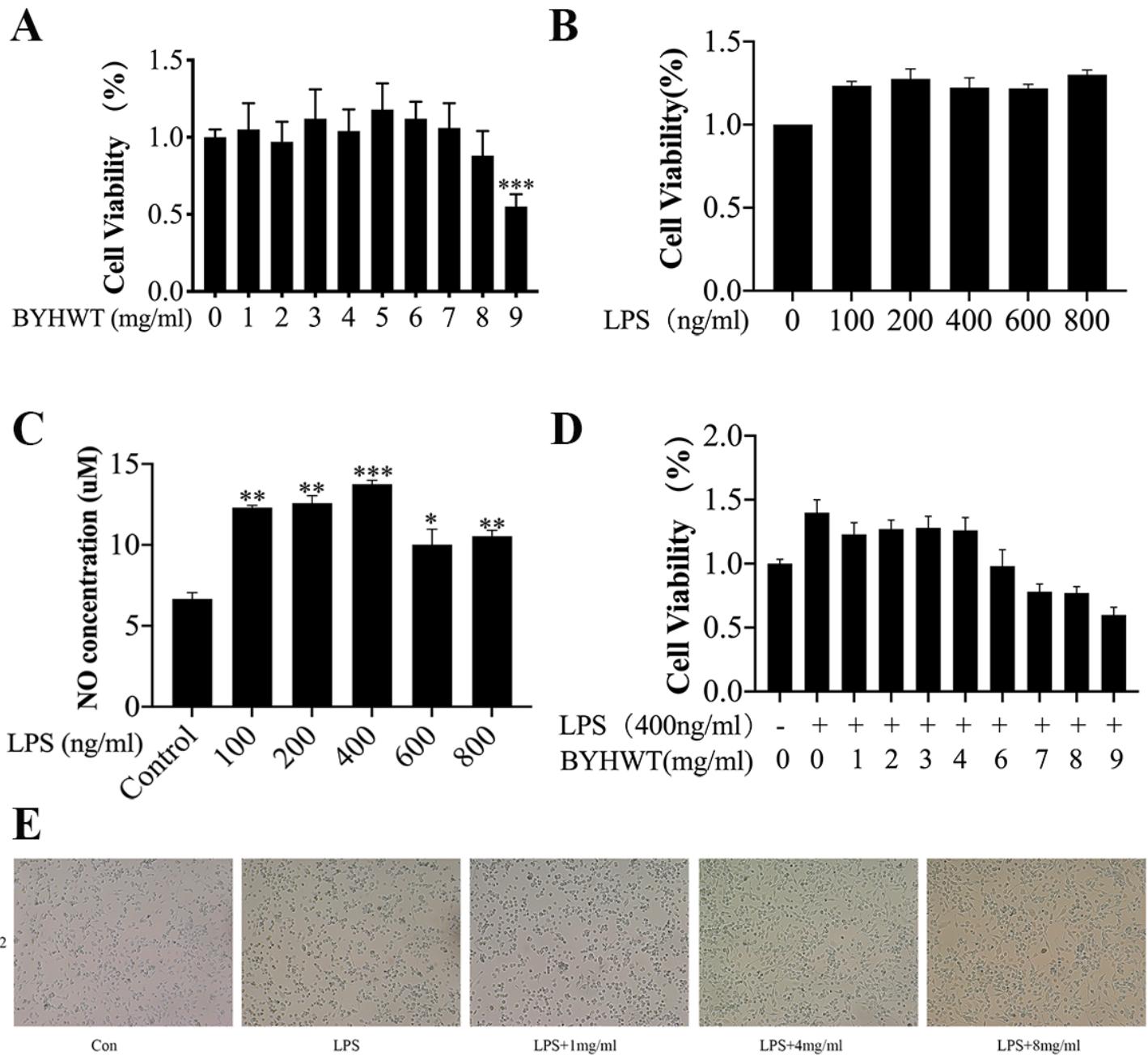
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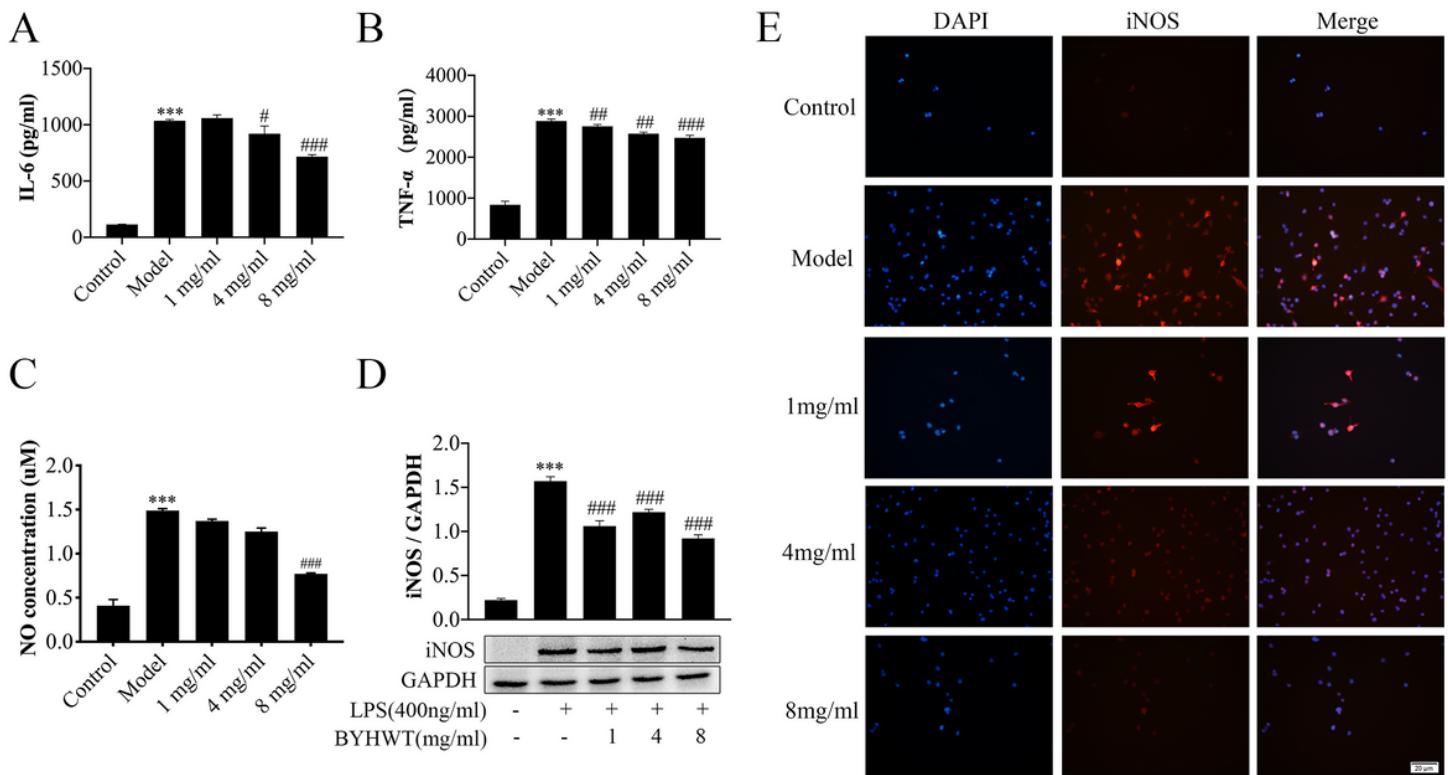
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## Figures



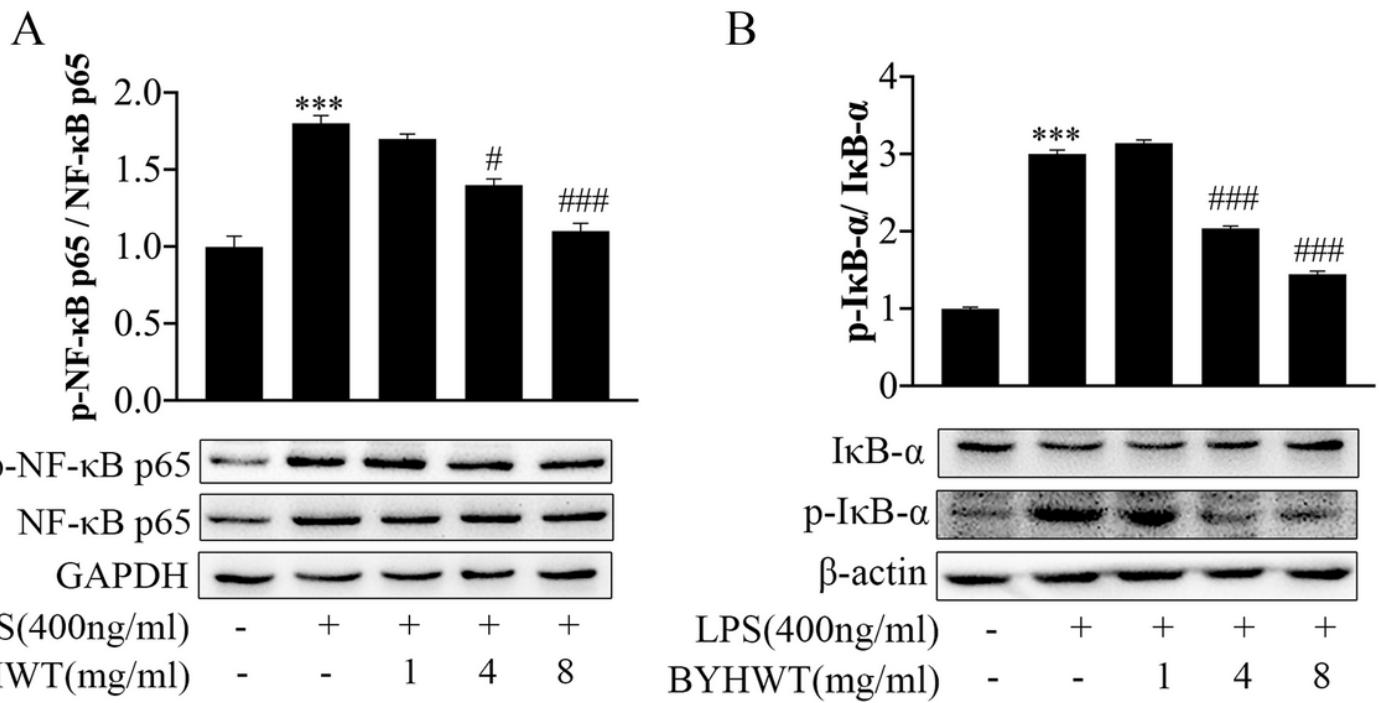
**Figure 1**

Effect of BYHWT on the viability of BV2 microglial cells and microglial activation. A. Effects of different concentrations of BYHWT alone on cell viability, untreated cells were used as control; B. Effects of different concentrations of LPS alone on cell viability, untreated cells were used as control; C. Changes in NO production levels after LPS stimulation; D. BV2 cells were pretreated with different concentrations of BYHWD for 2 h and incubated with or without LPS (400ng/mL) for 24 h. Cell viability was determined using CCK-8 assay; E. cell morphology was observed with an optical microscope; Experimental treatments were analyzed in triplicate and quantitative data are expressed as the mean  $\pm$  SD of three independent experiments. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  VS. control.



**Figure 2**

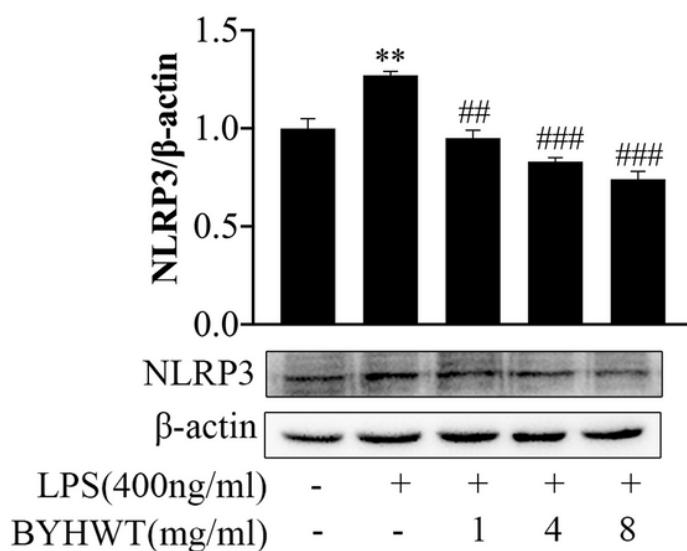
BYHWT inhibit LPS-induced TNF- $\alpha$ , IL-6, and NO production. And can inhibit iNOS protein expression. BV2 cells were pretreated with various concentrations of BYHWT for 2 h and incubated with or without LPS (400ng/mL) for 24 h. A. Levels of IL-6 in the supernatants were determined by ELISA. B. Levels of TNF- $\alpha$  in the supernatants were determined by ELISA. C. NO concentration in the supernatant was measured using the Griess reaction. D. iNOS expression in BV2 cells. Total cellular protein samples were collected and examined by western blotting. E. Representative fluorescent microscopy images showing the expression of iNOS (red) in LPS-activated BV2 cells. The nuclei were stained with DAPI (blue). Experimental treatments were analyzed in triplicate and quantitative data are expressed as the mean  $\pm$  SD of three independent experiments. \*p<0.05, \*\*\*p<0.001 VS. control; #p<0.05, ##p<0.01, ###p<0.01 VS. LPS treated group.



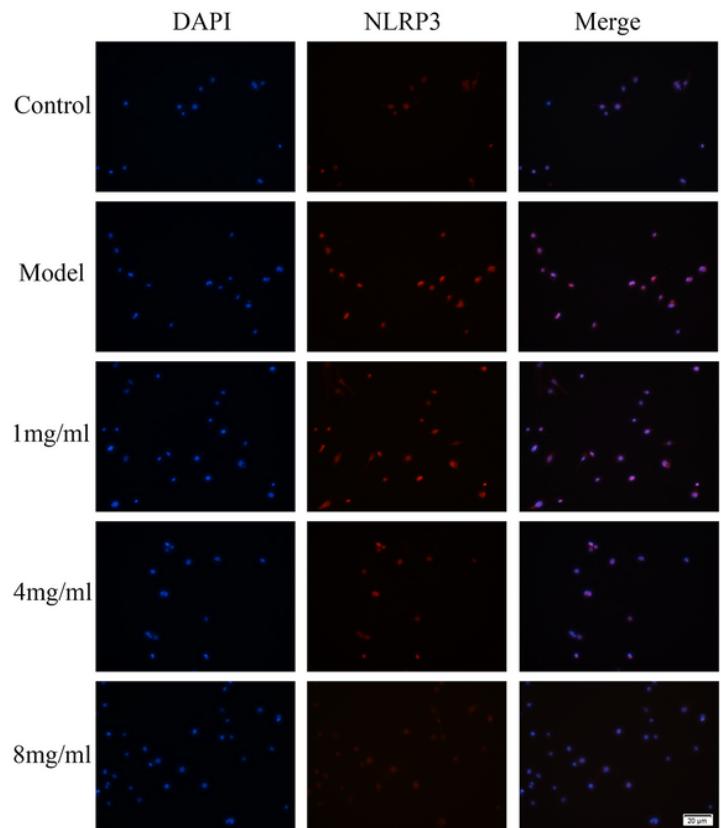
**Figure 3**

Effects of BYHWT on NF-κB activation by LPS-induced BV2 cells. BV2 cells were pretreated with various concentrations of BYHWT for 2 h and incubated with or without LPS (400ng/mL) for 24 h. A. Western blot analysis of NF-κB p65, phospho-p65 $\alpha$ B. Western blot analysis of I $\kappa$ B $\alpha$ , phospho-I $\kappa$ B $\alpha$ . Quantitation was performed on three independent experiments. Experimental treatments were analyzed in triplicate and quantitative data are expressed as the mean  $\pm$  SD of three independent experiments. \*\*\*p<0.001 VS. control; #p<0.05, ###p<0.001 VS. LPS treated group.

A



B

**Figure 4**

Effects of BYHWT on NLRP3 activation by LPS-induced BV2 cells. BV2 cells were pretreated with various concentrations of BYHWT for 2 h and incubated with or without LPS (400ng/mL) for 24 h. A. Western blot analysis of NLRP3. B. Representative fluorescent microscopy images showing the expression of NLRP3 (red) in LPS-activated BV2 cells. The nuclei were stained with DAPI (blue). Experimental treatments were analyzed in triplicate and quantitative data are expressed as the mean  $\pm$  SD of three independent experiments. \*\*p<0.01 VS.control; ##p<0.01,###p<0.001 VS. LPS treated group.