Buyanghuanwu Decoction inhibited TLR4 induced activation of nuclear factor-kappaB in acute carbon monoxide poisoning rats

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Abstract: Acute carbon monoxide poisoning (ACMP) is one of the most common types of poisoning worldwide, and may result in delayed encephalopathy, however, its pathogenesis remain obscure and there is no optimal treatment strategy for the patients with ACMP. Here, we developed the ACMP rat model to observe the protective effects of Buyanghuanwu Decoction (BYHWD) on hippocampal neuron. BYHWD (per 5 g/kg) were intragastric administration to rats twice each day for 28 days after ACMP. In ACMP +BYHWD group rats, the neuronal injury in the hippocampal region was significantly less than that of ACMP group's. BYHWD of intragastric administration also markedly decreased the expression of the TLR4, MyD88, NF-κB P65 after acute carbon monoxide poisoning (P<0.05). TNF-α、IL-1β protein level in ACMP + BYHWD group was lower than that of ACMP group (P<0.05).Our results suggested that decreased the activation of TLR4- NF-κB signal pathway due to BYHWD may partially account for its effect of neuroprotection standing against ACMP, and inhibited the inflammatory reaction to promote the ability recovery of learning and memory in ACMP rats.

Key word: Buyanghuanwu Decoction; Acute carbon monoxide poisoning; hippocampal neuron; Rat

1. Introduction

The acute carbon monoxide poisoning (ACMP) is a clinical emergency, which is harmful, especially to the brain [1, 2]. If treatment is not given timely, it may endanger life with serious complications. Some patients showed the nerve dysfunction with dementia, mental and extrapyramidal symptoms after 2 ~ 28 days "pseudo-recovery period", which is called delayed encephalopathy after acute carbon monoxide poisoning (DEACMP)[3]. There were no effective treatment measures for the disease with high morbidity and poor prognosis [3, 4].

The pathogenesis of DEACMP remains obscure. Researches showed that the occurrence of the disease is the outcome of combined action of a variety of mechanisms including ischemic, anoxia, reperfusion and free radical damage mechanism, excitatory amino acids toxicity, etc[5-8]. In recent years, immunologic mechanisms for DEACMP are receiving more and more people's attention. Thom et

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al[9, 10] proposed immune factors involved in the pathogenesis of delayed encephalopathy process, and detected $CD4^+$T lymphocytes and macrophages in the rat brain. MHC Ⅱ、CD4 and IFN-γwere observed in the brain tissue of the rat model with ACMP, and indicated that immune reaction plays an important roles on CO-mediated neuropathology [11]. Furthermore, ACMP could activate neurons by N-methyl-D-aspartic acid, then secondary neuronal nitric oxide synthase released into the blood vessels around, making the neutrophil activation to produce reactive oxygen species (ROS), thus resulting in lipid peroxidation of brain tissue[12].

Recently, a wide range of research were done on the TLR4- NF-κB signaling pathway in identifying Pathogen Associated Molecular Patterns (PAMPs)triggering the body's immune response[13, 14]. TLR4 being activated causes the combination of autospecific dimerization and MyD88. MyD88 combines with interleukin-1 receptor-associated kinase(IRAK), which result in the self-phosphorylation of IRAK, thus activate tumor necrosis factor receptor-associated factor 6 (TRAF6), making the mitogen-activated protein kinase (MAPK) family activated, in which nuclear factor κB (NF-κB) induces kinase activate kinaseα,β of I-κB family, making I-κB degrade because of phosphorylation and NF-κB shift to cell nucleus, then switch on the transcription of some inflammatory cell factors, such as TNF-α, IL-1βet al[15-19]. It has not been reported whether the inflammation process play a role in the pathogenesis of DEACMP.

To date, the strategies of ACMP treatment are still limited. Hyperbaric oxygen is the main method for treating ACMP's patients [20, 21]. However, due to some methodological limitation, the role of hyperbaric oxygen in the therapy of ACMP remains controversial [5, 22, 23].

Buyanghuanwu Decoction (BYHWD) is a classical prescription for benefiting qi and activating blood circulation in Chinese traditional medicine, comprised of Radix Astragalis Mongolici, Radix Angelicae Sinensis, Radix Paeoniae Rubra, Szechwan Lovage Rhizome, Pheretima Aspergillum, Flos Carthami, Semen Persicae[24]. It has been hundreds of years for its treatment of stroke in china[25]. Experimental studies showed that BYHWD can promote the proliferation of rat cortical neurons under both

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normal and hypoxia conditions[26],and protect neurons from hypoxia-induced apoptosis[27]. BYHWD also significantly restrains the up-regulated activity of nNOS after focal cerebral ischemia to protect the cerebral ischemic lesion from the early stage following the onset of ischemia[28], and act as neuroprotective effects [24, 29, 30] to promote the ability of recovery of learning and momory. Furthermore, Chen et al showed that the BYHWD can significantly suprress the expression of inflammatory cytokines and ROS level[31]. Yi et al demonstrated that BYHWD could reduce the protein and mRNA expressions of IL-1β and TNF-α in levels after cerebral infarction[32]. However, There have not been reported that the BYHWD inhibit the TLR4-NF-κB signal pathway to reach the role of anti-inflammatory.

In our study, we established the ACMP rat model to observe the effects of BYHWD on the TLR4-MyD88-NF-κB signal pathway, to explore treatment mechanism of BYHWD on delayed encephalopathy after ACMP.

2. Material and method

2.1 BYHWD preparation

Radix Astragalis Mongolici, Radix Angelicae Sinensis, Radix Paeoniae Rubra, Szechwan Lovage Rhizome, Pheretima Aspergillum, Flos Carthami, Semen Persicae were purchased from Beijing tongrentang, they were mixed in proportion of 120:6:4.5:3:3:3:3 and soaked for 30min in 5 times volume of distilled water, then decocted 30min. The final concentration was 5mg/ml through boiling, cooling and filtration, and conserved at 4 ℃ before use.

2.2 Animal model preparation

All experimental procedures were reviewed and approved by the Animal Ethics Committee of the Capital Institute of Pediatrics. Adult healthy male Sprague-Dawley rats (SPF grade) weighing 250-300g were purchased from Vital River Laboratories (Beijing, China). Rats were selected as a normal control group (n=25), a ACMP group (n=40), and BYHWD treatment group (ACMP+BYHWD group n=40) at random. Rat in ACMP group and ACMP+BYHWD group were treated with CO(120ml/kg body weight) by intraperitoneal injection for the first time, then added CO 60ml/kg body weight every 4hours for 4 times. The control group was treated with an intraperitoneal

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injection of air. About 0.3 ml whole blood was drawn from the left femoral artery for carboxyhemoglobin (HbCO) assay after intraperitoneal anesthesia by 3% pentobarbital injection with a Blood Gas Analyzer (RapidLab, BayerHealthCare, Leverkusen, Germany). Rats with coma and high HbCO concentration (>50%) were regarded as the successful models of ACMP. During the whole experiment, rats were kept in a homeothermic blanket control unit (Shandong Apparatus, China) and their core body temperature maintained at 36 – 37℃. Normal control group and ACMP group were given saline by gavage twice a day, ACMP+BYHWD group were given BYHWD 5 g/kg/d by gavage twice a day for 28days.

2.3 Morris water maze test

Morris Water Maze tracking and analyzing system (MT-200) were used to test the spatial memory of rats. The method entails placing the rat in a quadrant other than where a hidden platform is located and then the time taken (latency) for the rat to find the hidden platform is recorded. Each rat was released from three different quadrants and was allocated 120 seconds to find the platform. If they were unable to do so, the rat was guided to the platform and allowed to remain on the platform for 15 seconds. The time latency to reach the platform and also the length of swimming path were compared among groups. Probe trial was conducted to examine the rats had learned the exact location of the platform. The time spent in the target quadrant (Q1) was compared among groups.

2.4 Hematoxylin and eosin staining

The rats were sacrificed after behavioral study, the brain were removed and dissected on an ice-cold surface, and fixed in 10% neutral buffered formalin overnight, dehydrated in graded solutions of alcohol, cleared in xylene and paraffin embedded. Sections were sequentially cut at 7mm increments and stained with hematoxylin and eosin (H & E).

2.5 Western Blot Assay

The appropriate amount of CelLytic MT reagents (Sigma-Aldrich, Saint. Louis, Missouri, USA) (1 g of tissue/20 mL of reagent) were added to the tissues. The samples were then transferred (with lysis/extraction reagent) to a pre-chilled

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microhomogenizer and centrifuged at 12,000 × g for 10 minutes at 4 ℃. Protein levels were measured using the bicinchoninic acid method. Proteins were separated by polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes that were then placed in 5% skim milk powder solution at room temperature for 1 hour. The TLR4 antibody (1:1000), MyD88 antibody (1:500), NF-κBP65 antibody (1:500) was added to the membranes overnight at 4°C and incubated with anti-rabbit secondary antibody (1:2000) at room temperature for 2 hours. Detection was performed using the ECL reagent. Results were analyzed with a gel image processing system.

2.6 RT-PCR

Total RNA was extracted using Trizol reagent(Invitrogen, Carlsbad, CA) and determine by Nanodrop 2000 Spectrophotometer at 260/280nm(Thermo Fisher Scientific). The primers were designed using Primer 3.0 (http://frodo.wi.mit.edu/primer3) (Table S1). Quantitative real-time PCR experiments were performed on an ABI prism7900HT sequence detection system (Applied Biosystems, Foster city, CA) according to the following conditions: 1 cycle at 95℃ for 10 min, 45 cycles at 95℃ for 15 s and 60℃ for 1 min. Melting curve and sequence analysis were performed to verify PCR product specificity. Data are reported as values normalized by the housekeeping gene Gapdh. Gentic relative quantitative was calculated using the 2－▲▲Ct method.

2.7Enzyme Linked Immunosorbent Assay (ELISA)

Hippocampus tissues were transferred (with RPIA lysis buffer) to a pre-chilled microhomogenizer and centrifuged at 5000 × g for 10 minutes at 4 ℃. Supernatants was collected for further analysis.The quantity determination of TNF-α, IL-1βin hippocampus tissues was performed by enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO BIOTECH, China), following the manufacturer's protocol. Developed color reaction was measured as OD units at 450 nm. The concentration of TNF-α, IL-1βwas determined by using the standard curve constructed with the kit's standards and was expressed in pg/mL.

2.8 Statistical Analysis

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All data were presented as mean ±SD. Differences among groups were analyzed using ANOVA. P<0.05 was considered statistically significant. SSPS20.0 was used for all statistical analyses.

3 Results

3.1 Spatial learning and memory

All groups of rats were examined by Morris Water Maze test to observe the escape latency and target quadrant at 3, 7, 28 after giving BYHWD to evaluate hippocampus-dependent spatial learning and memory. Traveled distance to find the platform is one of the parameters evaluated in Morris Water Maze, which describes the learning process. The results showed that the traveled distance in ACMP+BYHWD group were significantly shorter than the ACMP group on day 28(P<0.05). No significant difference was observed among the normal group, ACMP group, ACMP+BYHWD group on day 3 and 7(Figure1A). Escape latency is the time required by a rat to find the target platform. No considerable difference was found among the normal group, ACMP group, ACMP+BYHWD group on day 3 and 7 with respect to escape latency, but on day 28, the escape latency in control group and ACMP+BYHWD is significantly shorter than that of ACMP group(Figure1B)(P<0.05;P<0.05).The time spent in the target quadrant was test. Rat receiving BYHWD treatment displayed a less swimming time in the target quadrant than the ACMP group on day 28 (Figure 1C) (P<0.05). These findings suggest that the oral administration of BYHWD could improve learning and long-term memory.

3.2 Morphology change of hippocampal neuron

The influence of BYHWD on the hippocampus was assessed by the H&E method. As shown in Figure2, Hippocampus neuron cells grew in order, cell morphology was in good condition in normal control group. However, hippocampus neuron cells were shrunken and the nuclei were condensed on day 28 in ACMP group. Pyknotic cells were less in ACMP+BYHWD group on day 28. No significant difference was observed among the normal group, ACMP group, ACMP+BYHWD group on day 3 and 7 with respect to hippocampus neuron in H&E staining (data not shown).

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3.3 The expression of NF-κBp65

NF-κB is an important nucleus transcription factors in Rel protein family, The NF-κB/Rel family is composed of five subunits-NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (P65), RelB and C-Rel, which can form various homo- or hetero-dimers, however, the most studied form is a heterodimer of the p50 and p65 subunits predominant in many kinds of cells [33, 34]. P65 subunit phosphorylation could cause the change of NF-κB structure to adjust the NF-κB transcriptional activity [35, 36]. We try to detect the NF-κB-p65 protein expression levels to explore the NF-κB activity in this study. The results found that the expression of NF-κB-p65 is low in normal control group, ACMP+BYHWD group and higher in ACMP group. ACMP+BYHWD group at 28 day NF-κBp65 expression levels were significantly lower than ACMP group（P<0.05）and higher than normal control group(P<0.05)(Figure 3).

3.4 The expression of TLR4，MyD88

We used the western blot method to test the protein level of TLR4 and MyD88 in rat at 28 days after delivery. The result showed that TLR4, MyD88 protein expression level were significantly lower than the ACMP group on 28 days (P<0.05; P<0.05, Figure 4).

3.5mRNA detection

In order to further determine the TLR4, MyD88, NF-κBp65 whether at the mRNA level has decreased in BYHWD group, we used the RT-qPCR method to verify the expression of mRNA, the results found that the expression levels of TLR4, MyD88, NF-κBp65 in ACMP+BYHWD group are lower than the ACMP group (P < 0.05) (Figure 5).

3.6TNF-α，IL-1βexpression

In order to determine whether the activity of the NF-κBp65 reduced affected the activity of its downstream factors, we determined the activity of its downstream factor TNF-α, IL-1β. Results showed that of TNF-α, IL-1β activity in BYHWD group are lower than the ACMP group (P < 0.05) on day 28 after drug delivery (Figure 6).

4 Discussions

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In this study, we established the model of ACMP and determined the spatial learning and memory by morris water maze test. we found that traveled distance, escape latency and time spent in the target quadrant decreased significantly in ACMP+BYHWD group compared to ACMP group on day 28 ($P < 0.05$). Many pyknotic cells were observed in the ACMP group compared with the normal control group with H&E staining. BYHWD is a classical prescription for benefiting qi and activating blood circulation in Chinese traditional medicine[25]. With BYHWD treatment after ACMP, hippocampus neurons in ACMP+BYHWD group survival number is more than the ACMP group, and pyknotic cells significantly reduce. TLR4, MyD88, NF-κBp65 expression level was lower in ACMP+BYHWD group compared to ACMP group ($P<0.05$). TNF-α, IL-1β activity were also significantly decreased in ACMP+BYHWD group ($P<0.05$).

More and more reports showed that even though there is no infection, the signal given by damage and stringent state cells can cause immune reaction[37, 38]. Toll like receptors (TLRs) are crucial for pathogen recognition and downstream signaling to induce effective immunity[39]. TLR4 (Toll like receptor4, TLR4) is a pattern recognition receptors (PRRs). It can recognize pathogen and switch on inherent immunity when pathogen begins to invade the organism [40]. In our study, TLR4 protein expression level in ACMP group was obviously higher than that of ACMP+BYHWD group and control group. The results showed that TLR4 expression level was excessive in ACMP group, and the BYHWD may inhibit the expression of TLR4 in ACMP+BYHWD groups. TLR4 mRNA expression levels significantly lower than the ACMP group, in accordance with TLR4 protein expression level.

MyD88 (myeloid differentiation protein 88) is a key molecule in TLR4 signal pathways[39]. TLR4 mediated MyD88 signal transduction pathways plays an important role in resisting pathogens invasion of the immune response process[41]. It can regulate the expression of a variety of inflammation related genes[41]. TLR4 being activated causes the combination of autospecific dimerization and MyD88, and completed the signal transduction [42]. Based on the observation of TLR4 expression decreased in ACMP+BYHWD group, we detected the intracellular protein MyD88

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expression in hippocampal neurons. The results showed that MyD88 protein and mRNA expression levels in ACMP+BYHWD group was obviously lower than that of ACMP group. These demonstrated that TLR4 inhibited caused the expression of MyD88 decreased. The study results suggest BYHWD inhibited the expression of TLR4 and down regulate the expression of MyD88.

NF-κB as an important transcriptional regulation factor, exists in vascular endothelial cells, nerve cells and glial cells, has the function of transfer the signal inside and outside the cells, involved in many of the regulation of gene expression and inflammatory reaction[43]. MyD88 combines with interleukin-1 receptor-associated kinase(IRAK), which leads to the self-phosphorylation of IRAK, thus activate tumor necrosis factor receptor-associated factor 6 (TRAF6), making the mitogen-activated protein kinase (MAPK)family activated, in which NF-κB induces kinase activate kinaseα,β of I-κB family, making I-κB degrade because of phosphorylation and NF-κB shift to cell nucleus, then switch on the transcription of some inflammatory cell factors, such as TNF-α, IL-1βet al [44]. There, we used western blot and RT-PCR to detect the expression of NF-κBp65、TNF-α、 IL-1β in hippocampus. Results showed that expression of NF-κBp65、TNF-α、 IL-1β decreased in ACMP+BYHWD group compared to the ACMP group (P<0.05). The results suggested that NF-κB is activate, caused the inflammation factors IL-1 and TNF-αexpression, and BYHWD can reduce the activity of NF-κB, thereby inhibiting the expression inflammatory cytokines IL-1βand TNF-α, to achieve the goal of treatment.

In ACMP model, TLR4-MyD88 signal pathway is activated, then NF-κB is activated and caused the expression of IL-1β, TNF-α. BYHWD inhibited the activity of TLR4, result in the decreased expression of MyD88, caused the reduced activity of NF-κB, thereby lead to the low activity of inflammatory cytokines IL-1βand TNF-α, then to reduce the inflammatory response and achieve the goal of treatment.

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Figure captions

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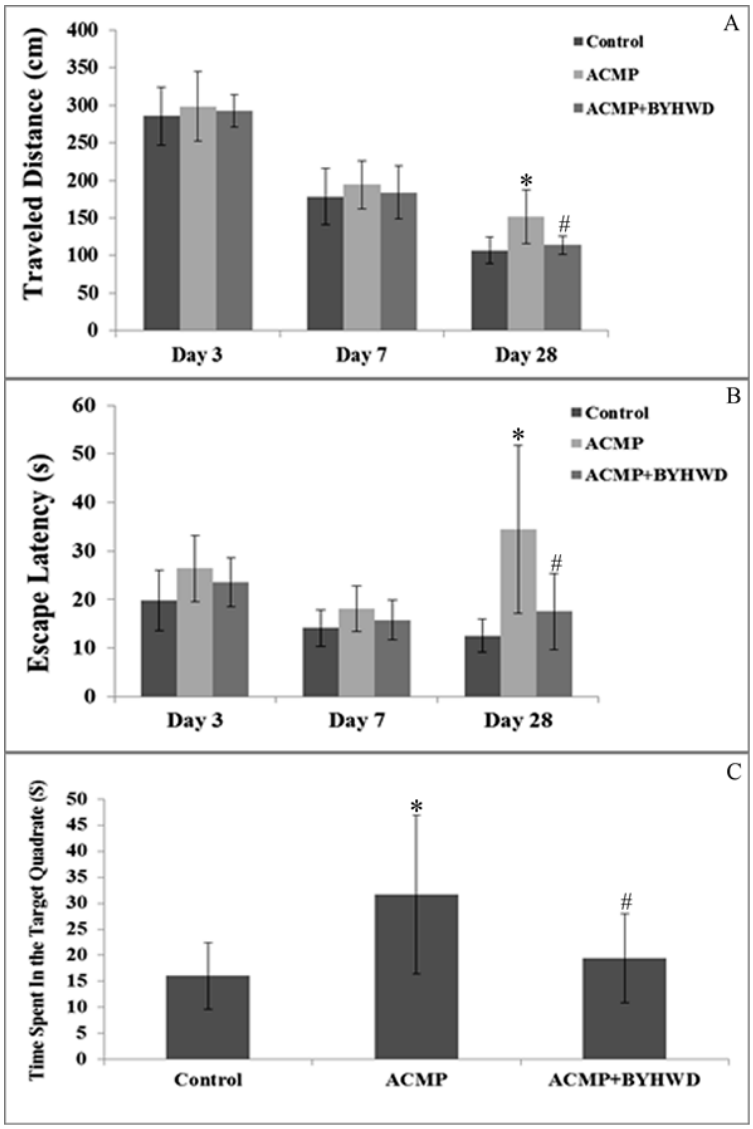

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390 **Figure1 Spatial learning and memory.** A:Traveled distance on days 3,7,28 after
391 ACMP. B: The means of escape latency on days 3,7,28 after ACMP, C: Comparing
392 the mean time spent in the target quadrant on day 28 after ACMP in the probe test .
393 The values are the mean±SD (n=7or8).*P<0.05 versus normal control group, #P<0.05
394 versus ACMP group.

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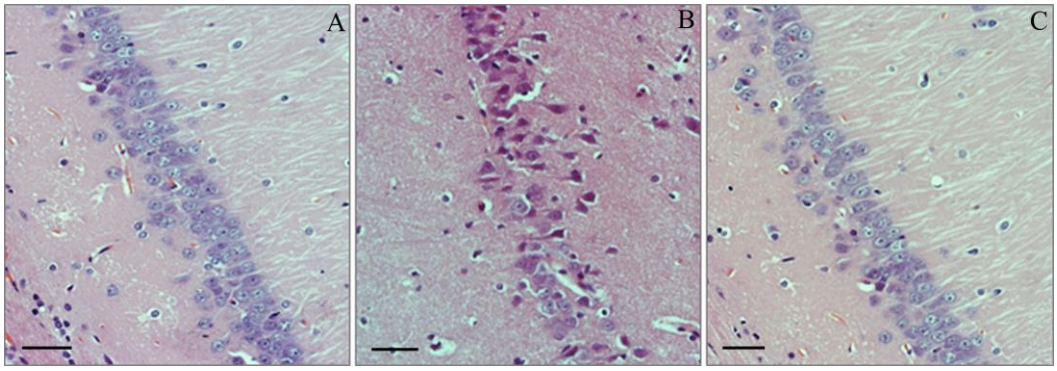
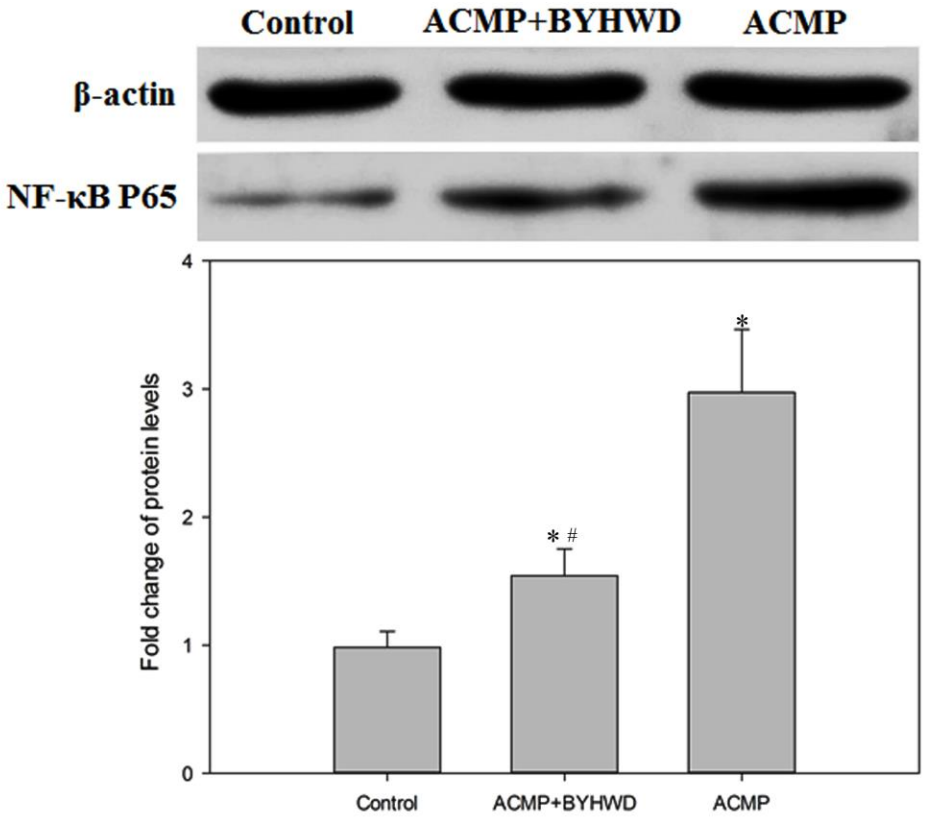


395 **Figure2 H&E staining for hippocampal neuron on day 28.** A, control group; B,

396 ACMP group; C, ACMP+BYHWD group. Bar:500px.



397

398 **Figure 3. BYHWD regulated the NF-κBp65 expression.** Results are presented as

399 the mean±SD (n=6 for protein expression assay) *P<0.05 versus normal control group,

400 #P<0.05 versus ACMP group.

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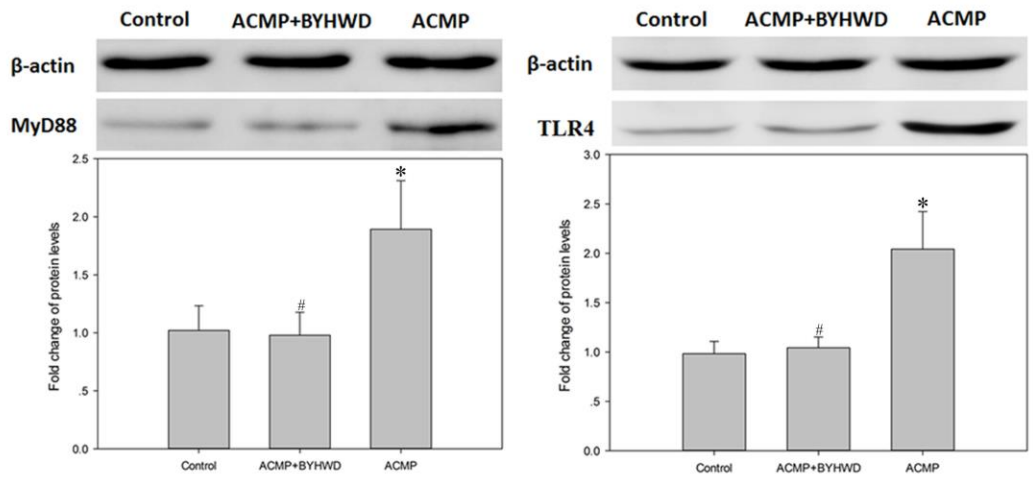

Figure4. BYHWD regulated the TLR4, MyD88 expression. Results are presented as the mean±SD (n=6 for protein expression assay) **P*<0.05 versus normal control group, #*P*<0.05 versus ACMP group.

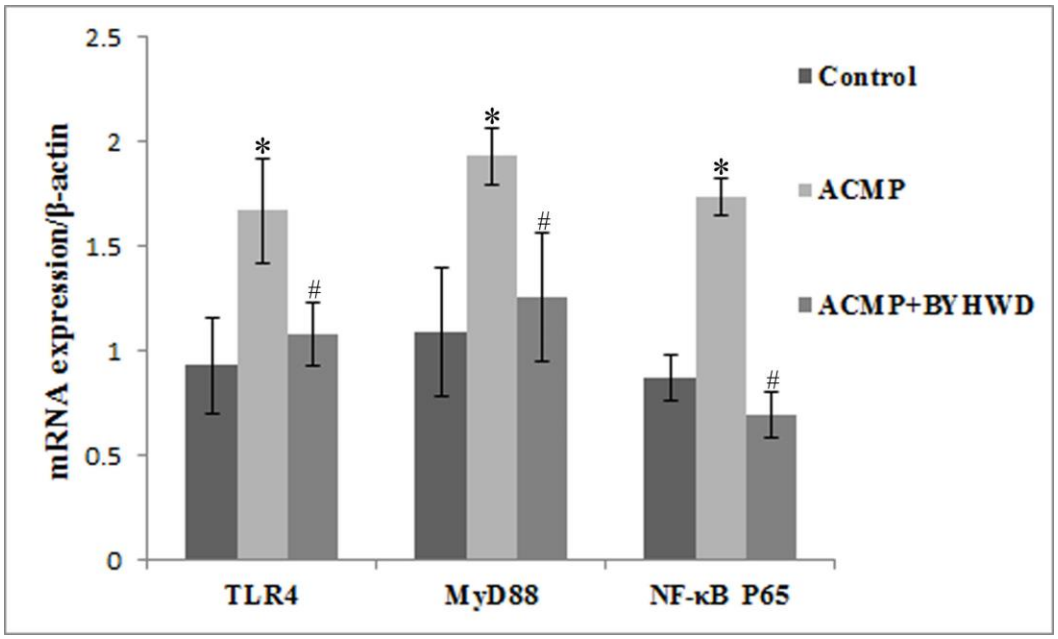

Figure 5. BYHWD regulated the NF-κBp65, TLR4, MyD88 mRNA expression. Results are presented as the mean±SD (n=6 for mRNA expression assay) **P*<0.05 versus normal control group, #*P*<0.05 versus ACMP group.

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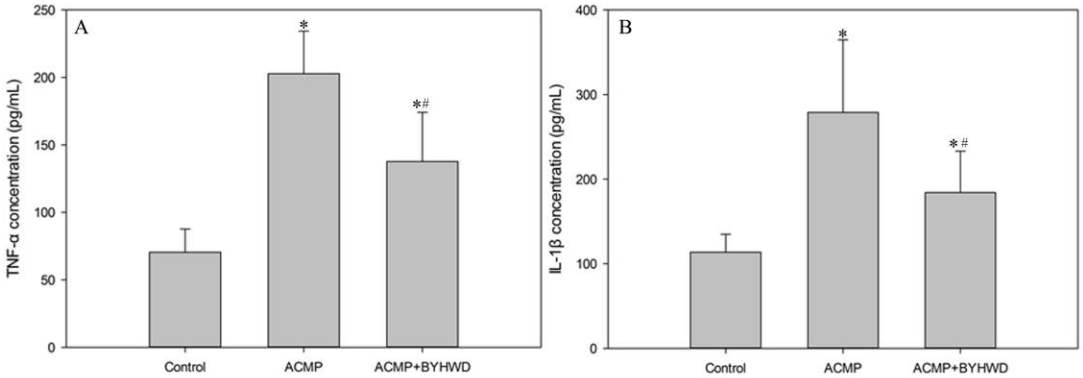

409

410 **Figure 6. BYHWD regulated the TNF-α, IL-1βexpression.** Results are presented as

411 the mean±SD (n=6 for protein expression assay) **P*<0.05 versus normal control

412 group, #*P*<0.05 versus ACMP group.

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