

Original Research

The Chinese herb *Fructus Broussonetiae* aids learning and memory in chronic cerebral hypoperfusion by reducing proinflammatory microglia activation in rats

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The neuroprotective role of *Fructus Broussonetiae* in a model of chronic cerebral hypoperfusion with cognitive decline was focused on neural plasticity and microglia/macrophage polarization. Chronic cerebral hypoperfusion was induced by bilateral common carotid artery ligation. *Fructus Broussonetiae* shortened escape latency and added the number of platform crossings of rats, up-regulated the expression of synaptophysin in the gray matter and increased myelin basic protein expression in the white matter. Further mechanistic experiments were conducted to examine microglia activation and M1/M2 polarization. It was shown that *Fructus Broussonetiae* reduced the activation of microglia revealed by decreased expression of ionized calcium-binding adapter molecule-1, inhibited M1 polarization of microglia and improved microglial M2 polarization shown by down-regulated the expression of inducible nitric oxide synthase and Fc fragment of IgG receptor IIIa and up-regulated the expression of arginase-1. In conclusion, the Chinese herb *Fructus Broussonetiae* can improve cognitive function following chronic cerebral hypoperfusion by down-regulating the activation of microglia, inhibiting microglial M1 polarization, and improving neural plasticity.

Keywords

Fructus Broussonetiae; chronic cerebral hypoperfusion; macrophages/microglia polarization; inducible nitric oxide synthase; arginase-1; neuroprotective effect; rats

1. Introduction

Chronic cerebral hypoperfusion (CCH) is a pathological state that usually occurs in brain diseases, such as Alzheimer's disease and vascular Parkinsonism, leading to cognitive impairment

(Saggu et al., 2016). Recent studies have highlighted two typical pathological features of the chronically hypo-perfused brain are white matter injury and neuronal loss in the cerebral cortex and hippocampus (Damodaran et al., 2019; Roman et al., 2002; Yao et al., 2019). Further research found that in bilateral common carotid artery surgery (BCAS) mice, inhibiting the microglial inflammatory activation or altering microglial polarization decreased white matter injury and improved cognitive function (Miyano-hara et al., 2018; Qin et al., 2017; Yao et al., 2019). Microglia can be activated by diverse signaling pathways and polarized to pro-inflammatory or anti-inflammatory response, thus exacerbating brain damage or promoting brain repair in the CCH (Qin et al., 2018). Therefore, microglia represent a potential therapeutic target for chronic cerebral hypoperfusion-related diseases.

Traditional Chinese Medicine has been applied for thousands of years in the therapy of cognitive impairment, and at least 34 herbs have definite citations for improving memory impairment (May et al., 2013). The possible mechanism of herbal benefits include: (a) promote neural progenitor proliferation; (b) extend neurite outgrowth; (c) increase the synaptic regeneration; (d) enhance the anti-inflammatory and antioxidant capacity and so on (Cakova et al., 2017; Liu et al., 2017; Wang et al., 2017; Yang et al., 2017). In traditional Chinese medicine, *Fructus Broussonetiae* was often used for the therapy of dementia amnesia (Yang et al., 2017). It was recently demonstrated that *Fructus Broussonetiae* has a neuroprotective effect on APP/PS1 mice (Li et al., 2020b). However, the effect and mechanism of *Fructus Broussonetiae* on chronic cerebral hypoperfusion remain unknown until now.

2. Materials and Methods

2.1 Model of chronic cerebral hypoperfusion

The model was built by bilateral common carotid artery (BCCA) ligation. Animal tests were carried out according to the

guidelines of the Institutional Animal Care and Use Committee of Xuanwu Hospital of Capital Medical University. Male Sprague-Dawley rats (weighing 280 to 300 g) were purchased from Beijing Vital River Lab Animal Technology (PRChina). Rats were fed under a standard laboratory condition (12-h light/dark cycle, at $22 \pm 2^\circ\text{C}$) with free access to water and food. BCCA occlusion surgery (Volgyi et al., 2018) was performed on the rats to induce chronic cerebral hypoperfusion. A ventral midline incision was done on the neck of isoflurane-anesthetized rats (3–5% in 70% nitrogen and 30% oxygen). Common carotid arteries were gently separated from the vagus nerve. Firstly, the right common carotid artery was ligatured by 2 silk sutures (4-0), and 30 min later, the left carotid was occluded. The sham-operation group underwent the same process without ligatures. Body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ during the surgery by a temperature-controlled heating device (CMA 150; Carnegie Medicin, Stockholm, Sweden). Then the rats were put back to their home cages. Animals were divided randomly into 3 groups: the sham-operated group, the Model group, and the *Fructus Broussonetiae* group ($n = 12$, 6 for western blot analysis and 6 for immunofluorescent staining). Drug treatment or vehicle was administered from day 1 to day 42 following surgery by intragastric administration. The Chinese herb *Fructus Broussonetiae* was prepared by an experienced pharmacy technician from the Department of Pharmacy (Dongfang Hospital, Beijing University of Chinese Medicine) according to the procedures recorded in the Chinese Pharmacopoeia. The Chinese herb *Fructus Broussonetiae* was boiled in distilled water for 40 min and extracted twice, and then it was filtered and concentrated to 0.58 g/mL. Drug dosage in rats is calculated basing on the body surface area according to the human dose. The body weight of rats on day 0, 3, 7, 14, 28, and 42 post-surgery was recorded.

2.2 Morris water maze test

The Morris Water Maze (MWM) experiment is done from day 36 to day 42 post-surgery (Coppi et al., 2018). The water maze consists of a black circular water tank (120 cm diameter, 40 cm height). The water was kept at a temperature of $22 \pm 1^\circ\text{C}$ and a depth of 20 cm. The tank was divided into four cardinal points: East, South, West, and North. And a platform (diameter, 10 cm; height, 19 cm) in one of four locations was submerged approximately 1 cm below the surface of the water and hidden from the rat's view. Visible cues outside the tank were provided for orientation. The test includes two phases, acquisition training and probe trial. In the acquisition training, the rats were trained for 5 consecutive days, 4 trials per day to search for the platform for 120 sec. Once a rat found the platform, it was permitted to remain on it for 30 sec. If a rat did not find the platform within 120 sec, it was guided to the platform for a 30 sec rest. On day 6, a probe trial was performed. The platform was removed, and rats were put in the tank for 120 sec with the starting location farthest from the platform. The escape latency, pathlength, and swim speed were recorded semi-automatically by a video tracking system (SMART; Pan-Lab, Barcelona, Spain). To evaluate the role of *Fructus Broussonetiae* in cognitive impairment, the MWM test, including escape latency (s), time in the target quadrant, frequency of platform crossing, and swimming speed, was evaluated.

2.3 Western blotting

Rat brains were dissected 42 days after BCCA. Samples are processed for western blot analysis, as described previously (Li et al., 2019). The membrane was then incubated with primary antibodies as follows: microtubule-associated protein 2 (MAP2, 1:1000), synaptophysin (1:1000), Fc-gamma receptor III (CD16, 1:1000), inducible nitric oxide synthase (iNOS, 1:1000), macrophage mannose receptor (CD206, 1:1000; all from Abcam, Cambridge, MA, United States), Arg1 (1:1000; all from Cell Signaling Technology, Danvers, MA, United States). Anti- β -actin rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. After incubation with horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology), the immunoreactivity was detected using a commercially available enhanced chemiluminescence kit (Millipore, MA, United States). The integrated density values were analyzed by ImageJ software (Rawak Software, Inc., Germany).

2.4 Immunofluorescence staining

Immunostaining procedure in the brain was performed as described previously (Nishikawa et al., 2018). The brain sections were incubated overnight at 4°C with the primary antibody, followed by incubation with the fluorescent-labeled secondary antibody. Primary antibodies included MAP2, synaptophysin, CD16, Arg1, myelin basic protein (MBP, Abcam, Cambridge, MA, United States), NeuN (Millipore, Burlington, MA, United States), Iba1 (Wako Pure Chemical Industries, Osaka, Japan). All sections were counterstained with DAPI. Images were acquired by a fluorescence microscope (Carl Zeiss, Germany).

2.5 Statistical analysis

Data are present as mean \pm SEM. All statistical analyses were performed using SPSS v11.0 (SPSS Inc., Chicago, IL, United States). The results were analyzed using the Student's t-test or two-way analysis of variance followed by Newman-Keuel's test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 *Fructus Broussonetiae* lightened cognitive impairment induced by BCCA without influencing the bodyweight

In general, BCCA is followed by a decrease in body weight. On postoperative days 3, there is a remarkable loss in body weight in both the Model and *Fructus Broussonetiae* (FB) groups. After that, the difference in body weight among Sham, Model, and FB group began to decrease (Fig. 1A). Until days 14, there was no noticeable difference between the three groups.

In the Morris water maze test, the escape latency of the rat in the Model group was significantly longer compared with the Sham group at each time point, which can be abrogated by *Fructus Broussonetiae* (Fig. 1B). In spatial probe trials, the percentage of time in the target quadrant displayed no significant difference in all groups (Fig. 1C). The frequency of platform crossing decreased significantly after BCCA, which was suppressed by *Fructus Broussonetiae* (Fig. 1D,F). The results of swimming speed, which could assess sports ability, suggested that the above difference was not due to a difference in the sport ability of different groups (Fig. 1E). These results demonstrated that *Fructus Broussonetiae* attenuates cognitive impairment in rats induced by BCCA.

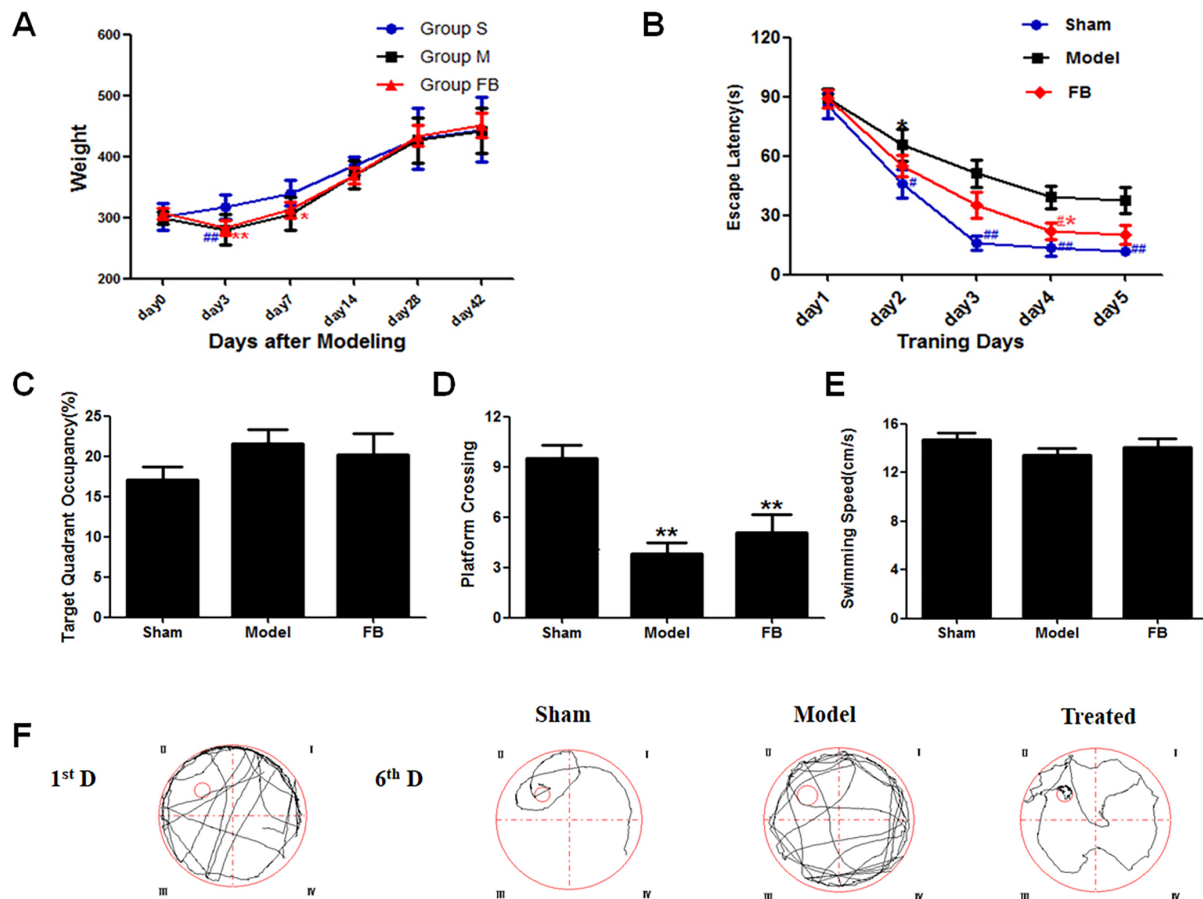


Figure 1. The Chinese herb *Fructus Broussonetiae* ameliorated cognitive impairment induced by bilateral common carotid artery occlusion on the Morris Water Maze with no effect on body weight. (A) Comparison of weight for each group on the 0th, 3rd, 7th, 14th, 28th, and 42nd day. (B) The escape latency (%) of rats in the training trials of hidden platform task. (C) Percentage of time spent in the target quadrant in the probe trial. (D) Frequency of platform crossing in the probe trial. (E) Swimming speed in the probe trial. (F) Representative pathways in the first and last training day of hidden platform task for each group. Values are expressed as mean \pm SE. Sham group: $n = 12$; Model group: $n = 12$; FB group: $n = 12$. * $P < 0.05$ vs. Sham, ** $P < 0.01$ vs. Sham, # $P < 0.05$ vs. Model, ## $P < 0.01$ vs. Model.

3.2 *Fructus Broussonetiae* protected the brain against BCCA injury

To determine the protective effects of *Fructus Broussonetiae* on the dendritic and synaptic proteins, the protein level of MAP2 and synaptophysin from the cortex and hippocampus were quantified by immunofluorescence and western blot. In the normal cortex, hippocampal CA1 and CA3, MAP2 and synaptophysin co-localized with NeuN, while this co-localization becomes discontinuous and disorders in Model group, which was partially abrogated by both *Fructus Broussonetiae* (Fig. 2A,C). Among the sham, model, and FB groups, the protein levels of MAP2 were similar (Fig. 2B). BCCA exposure decreased the level of synaptophysin, which was also markedly reversed by *Fructus Broussonetiae* treatment (Fig. 2D). These data indicate the neuroprotective effect of *Fructus Broussonetiae* against the grey matter damage following CCH.

To evaluate the role of *Fructus Broussonetiae* on white matter injury after BCCA, MBP level from the cortex and basal ganglia were quantified by immunofluorescence staining. The results indicated that the MBP level was reduced in the Model group

compared with the Sham group, which was upregulated by *Fructus Broussonetiae* treatment (Fig. 3A,B). These data indicate that *Fructus Broussonetiae* can protect the brain against white matter damage following CCH.

3.3 *Fructus Broussonetiae* reduced the microglia activation and regulated M1/M2 polarization after BCCA

Firstly, to investigate the role of *Fructus Broussonetiae* on the activation of microglia/macrophages after BCCA, we detected the expression of Iba1 in the cortex, hippocampal CA1, CA3, dentate gyms (DG), corpus callosum (CC) by immunofluorescence. Compared with the sham group, activated microglia acquired the amoeboid morphology in the model group, while this effect was partially inhibited by *Fructus Broussonetiae* (Fig. 4). This suggests that *Fructus Broussonetiae* could down-regulate the activation of microglia.

Activated microglia could display two functional states of polarization, namely pro-inflammatory M1-like phenotype and anti-inflammatory M2-like phenotype. To confirm whether *Fructus Broussonetiae* impacts the polarization of microglia/macrophages,

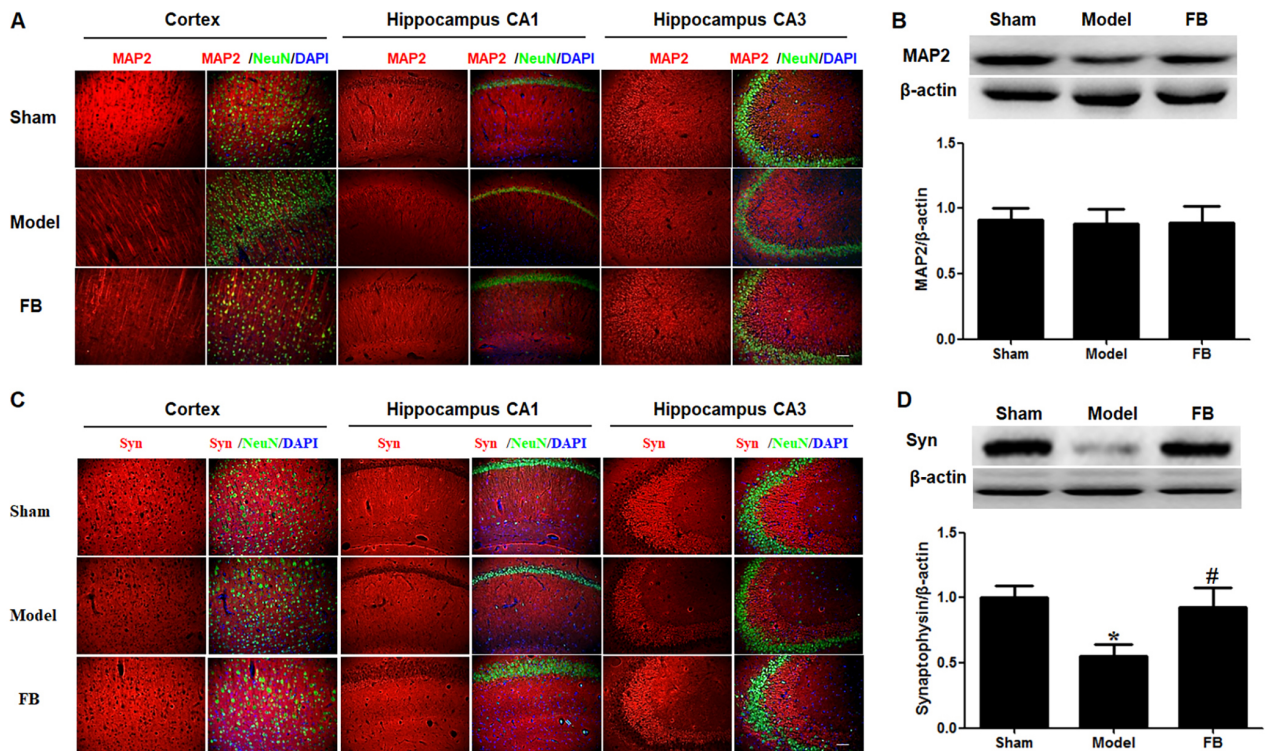


Figure 2. The Chinese herb *Fructus Broussonetiae* improved gray matter injured and upregulated the level of synaptophysin after bilateral common carotid artery occlusion. (A) Representative immunofluorescence images showing colocalization of MAP2 (red) and NeuN (green) in the cortex, Hippocampus CA1, and Hippocampus CA3, DAPI indicates blue nuclear. (B) Western blot detection and quantitative analysis of MAP2. (C) Representative immunofluorescence images showing colocalization of synaptophysin (red) and NeuN (green) in the cortex, Hippocampus CA1, and Hippocampus CA3, DAPI indicates blue nuclear. (D) Western blot detection and quantitative analysis of synaptophysin. Scale bar: 50 μ m. Sham group: n = 12; Model group: n = 12; FB group: n = 12. *P < 0.05 vs. Sham, #P < 0.05 vs. Model.

the M1 markers (iNOS, CD16) and the M2 markers (Arg1, CD206) were measured. The results of western blot showed that both iNOS and CD16 were upregulated after BCCA, which could be abrogated by *Fructus Broussonetiae* treatment (Fig. 5A,B); $P < 0.05$). Similar changes in CD16 expression double-labeled with Iba1 were observed in the cortex by immunofluorescence (Fig. 5C). On the other hand, Arg-1 protein expression was decreased after BCCA, which was partially inhibited by *Fructus Broussonetiae* treatment (Fig. 6A). Consistent with this, the number of microglia/macrophages double-labeled with Arg-1 and Iba1 in the cortex, the anti-inflammatory and protective M2-like phenotype, was notably lower in the Model group compared with Sham operation group, which was also reversed by *Fructus Broussonetiae* treatment (Fig. 6C). CD206 protein levels were similar in the three groups (Fig. 6B). These data indicate that *Fructus Broussonetiae* inhibits the shift towards the M1-like phenotype of microglia/macrophages and promotes the microglia/macrophages towards the M2-like phenotype.

4. Discussion

This was an investigational study designed to demonstrate the therapeutic action and mechanisms of *Fructus Broussonetiae* on the chronic cerebral hypoperfusion induced cognitive disorder. Our results have shown interesting findings: (a) *Fructus Broussonetiae* exhibited a protective effects against cognitive deficits

induced by BCCA without influencing the body weight; (b) *Fructus Broussonetiae* might protect against both the brain gray matter and white matter damage following CCH; (c) the action mechanisms of *Fructus Broussonetiae* includes attenuating the activation of microglial cells and facilitating microglia towards an anti-inflammatory M2 polarization.

Chronic cerebral hypoperfusion is a pathological state involved in the generation of dementia. Consistent with the previous study (Choi et al., 2016), spatial learning and memory impairment testing by the Morris water maze is observed in our model of CCH (Ghasemi et al., 2020). We proved for the first time that *Fructus Broussonetiae* could improve long-term memory revealed by increased frequency of platform crossing and shortened escape latency time. Similarly, it was recently demonstrated that *Fructus Broussonetiae* has a neuroprotective effect on APP/PS1 mice (Li et al., 2020b). In addition to *Fructus Broussonetiae*, it was recently demonstrated that another edible medicinal plant-Mallotus oblongifolius extracts in China could alleviate cerebral ischemic injury by promoting the proliferation of neural stem cell (Li et al., 2020a). Bilateral common carotid artery occlusion has been used to induce the white and gray matter damage associated with CCH widely (Choi et al., 2016; Yao et al., 2019). In our model, white matter and gray matter damage were consistent with previous results of studies on CCH. In the normal cortex and hippocampal CA1 and CA3, MAP2 and synaptophysin co-localized

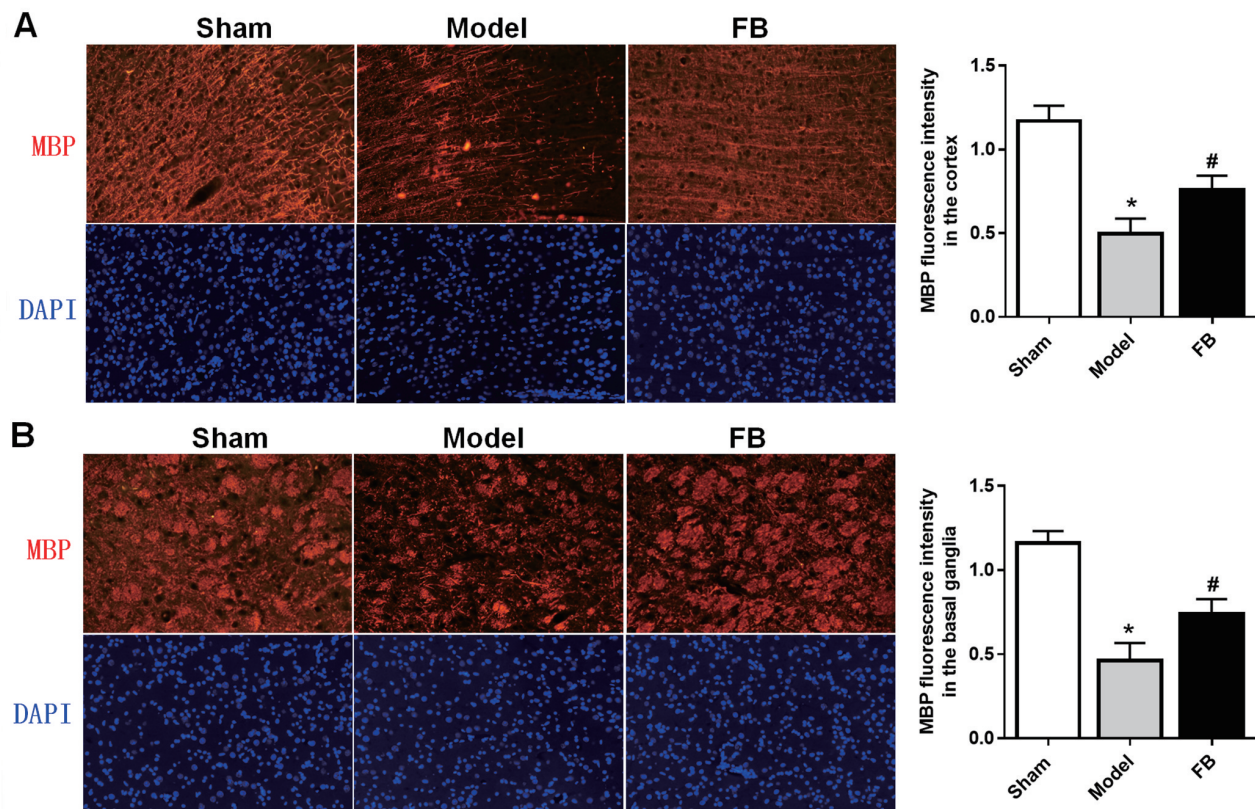


Figure 3. The Chinese herb *Fructus broussonetiae* improved white matter injured induced by bilateral common carotid artery occlusion. Immunofluorescence staining and quantitative analysis of MBP in the cortex (A) and basal ganglia (B). Scale bar: 50 μ m. Sham group: n = 12; Model group: n = 12; FB group: n = 12. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Model.

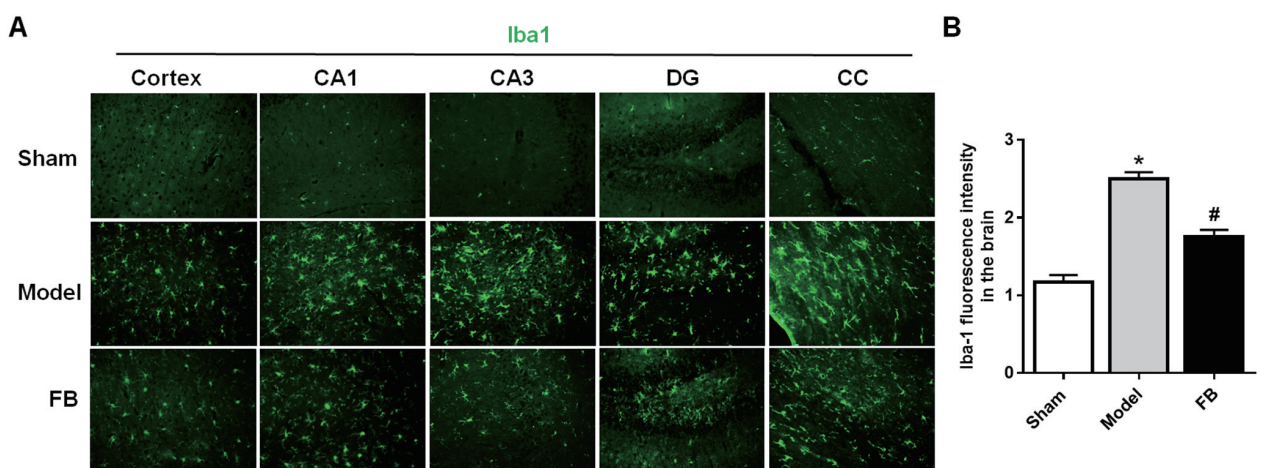


Figure 4. Influence on microglia activation of bilateral common carotid artery occlusion and *Fructus Broussonetiae*. (A) Representative immunofluorescence images showing Iba1 (green) in Cortex, Hippocampe CA1, CA3, and DG, CC. DAPI indicates blue nuclear. Scale bar: 50 μ m. Abbreviations: DG, dentate gyms; CC, corpus callosum. (B) Quantitative analysis of immunofluorescence. Sham group: n = 12; Model group: n = 12; FB group: n = 12. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Model.

with NeuN, while this co-localization becomes discontinuous and reduced in the Model group. Also, MBP expression was decreased in the Model group. And the protective effect of *Fructus Broussonetiae* in both gray matter and white matter is confirmed by up-

regulated the protein expression of MAP-2, Synaptophysin, and MBP.

Microglia can be activated by diverse signaling pathways and polarized to pro-inflammatory or anti-inflammatory state, there-

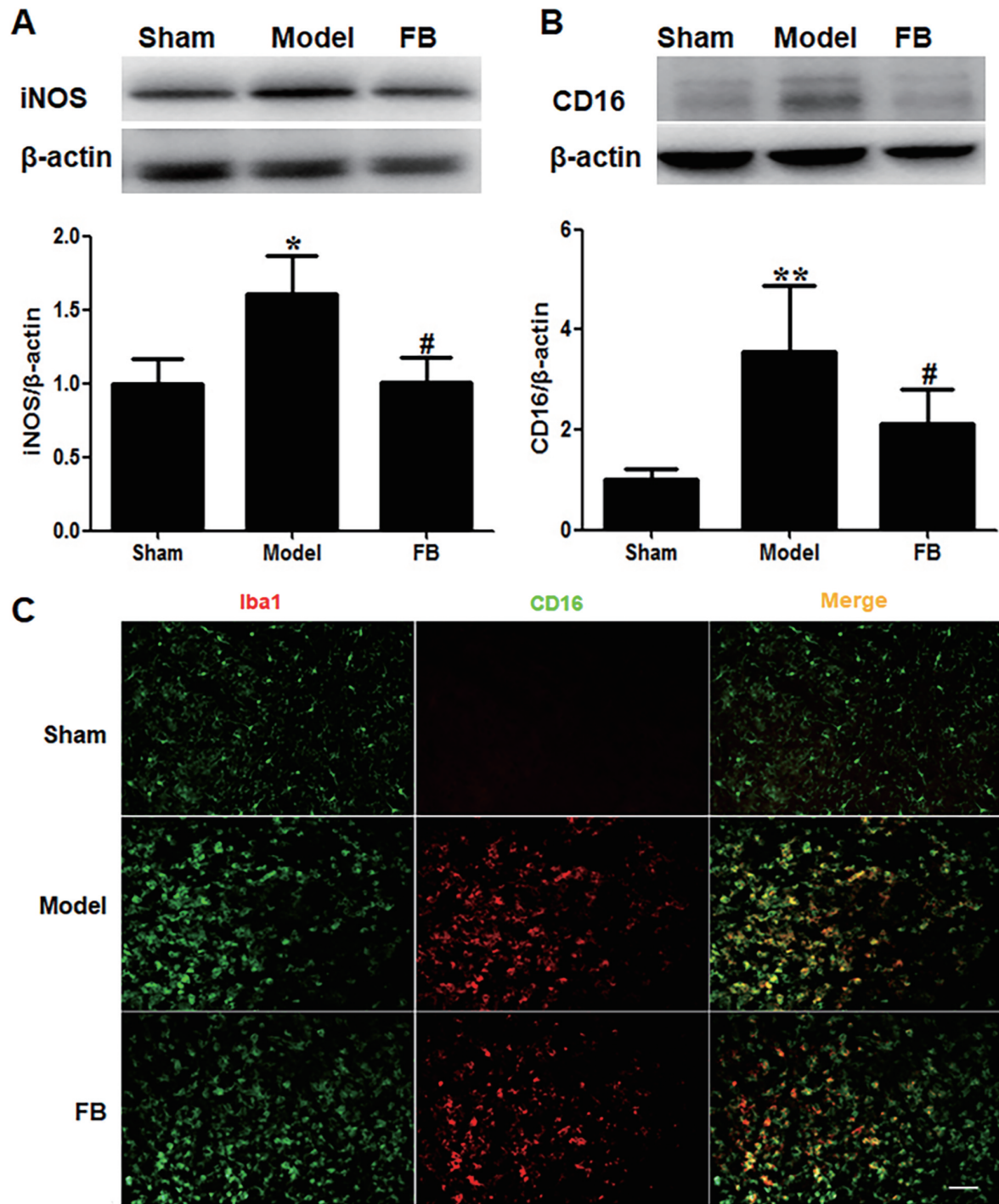


Figure 5. The Chinese herb *Fructus Broussonetiae* inhibited the polarization of M1/M2 macrophages/microglia toward an M1-like phenotype induced by bilateral common carotid artery occlusion. (A) Western blot detection and quantitative analysis of iNOS. (B) Western blot detection and quantitative analysis of CD16. (C) Representative immunofluorescence images showing colocalization of CD16 (red) and Iba1 (green) in Cortex. Scale bar: 50 μ m. Sham group: n = 12; Model group: n = 12; FB group: n = 12. * P < 0.05 vs. Sham, ** P < 0.01 vs. Sham, # P < 0.05 vs. Model.

fore aggravating brain injury or advancing brain repair in the CCH (Qin et al., 2018). It was suggested that microglia play a lethal role in the progress of white matter injury and cognitive impairment induced by CCH, and depletion of microglia alleviates white matter damage and cognitive disorder in a mouse CCH model (Kakae

et al., 2019). Therefore, microglia represent a latent therapeutic target for neurodegenerative diseases caused by chronic cerebral hypoperfusion. Recent research has observed that the BCCA operation preferentially induce the M1 polarization of microglia (Qin et al., 2017b), which was proved by our results. We showed that

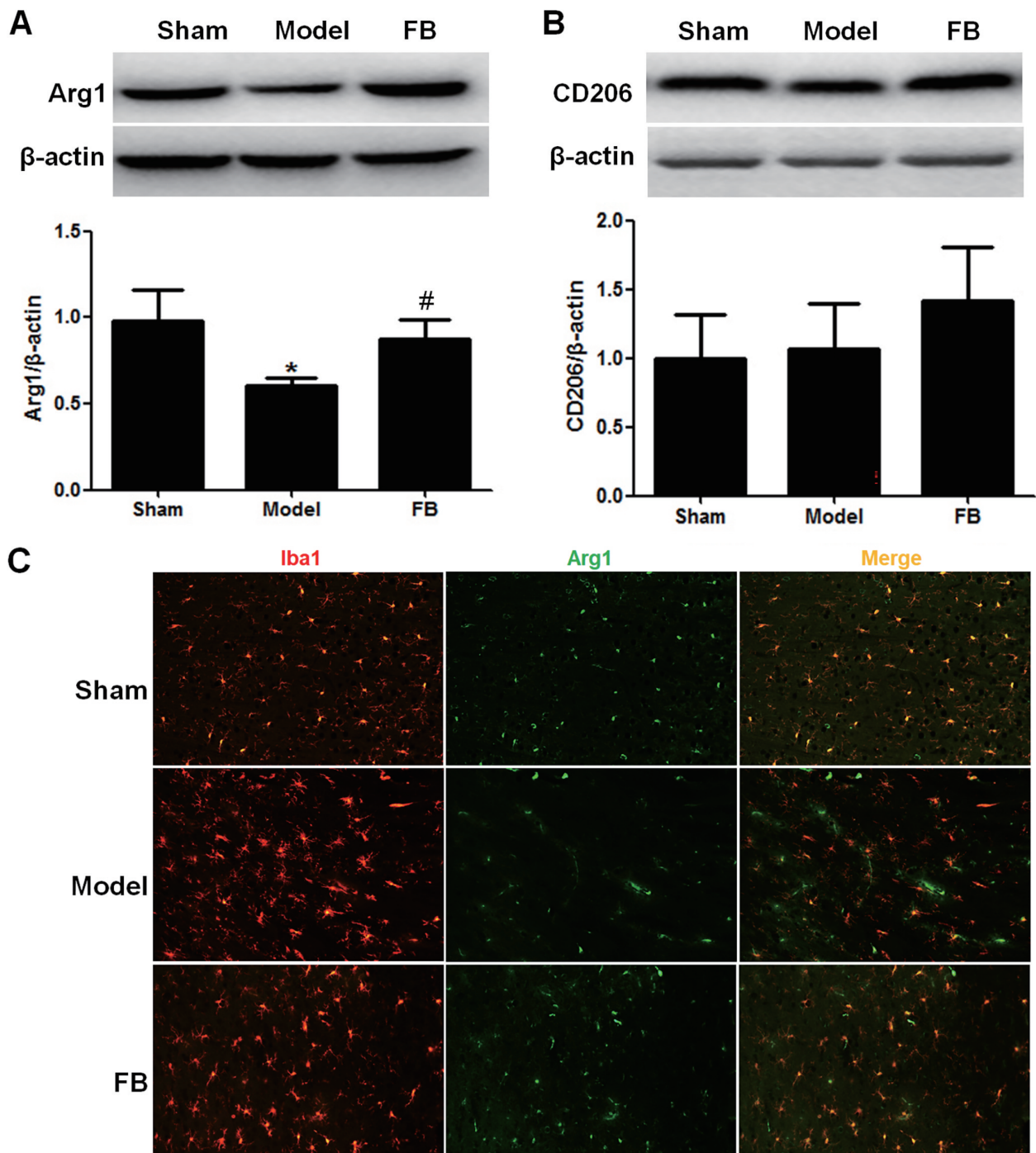


Figure 6. The Chinese herb *Fructus broussonetiae* promoted the polarization of M1/M2 macrophages/microglia toward an M2-like phenotype induced by bilateral common carotid artery occlusion. (A) Western blot detection and quantitative analysis of Arg1. (B) Western blot detection and quantitative analysis of CD206. (C) Representative immunofluorescence images showing colocalization of Arg1 (red) and Iba1 (green) in Cortex. Scale bar: 50 μ m. Sham group: n = 12; Model group: n = 12; TCC group: n = 12. * P < 0.05 vs. Sham, # P < 0.05 vs. Model.

the M1 markers (iNOS, CD16) were upregulated after BCCA occlusion, and the M2 markers (Arg1) were decreased after BCCA occlusion.

Our data provided the first demonstration of *Fructus Broussonetiae* acting as a modulator of microglial activation and polar-

ization after CCH by inhibiting the microglial activation, inhibiting polarization toward M1 state (pro-inflammatory activation) and promoting M2 shift (anti-inflammatory activation). Similarly, it was demonstrated that minocycline mitigates depressive-like behavior and demyelination by reducing microglial activa-

tion in the model of transient global cerebral ischemia (Du et al., 2019). These results demonstrate that *Fructus Broussonetiae* positively regulates the microglial activation and polarization, thus protecting neuroinflammation during chronic cerebral hypoperfusion. Further work is needed to clarify the underlying mechanisms on the regulation of microglial activation and polarization by *Fructus Broussonetiae*.

The present work proved that *Fructus Broussonetiae* could protect against chronic cerebral hypoperfusion in vivo via reducing the microglial activation and promoting microglial M2 polarization. The Chinese herb *Fructus Broussonetiae* is, therefore, a promising therapeutic tool for CCH to protect the cognitive function. Although it is far away from the clinical application, nevertheless, we showed that *Fructus Broussonetiae* could alleviate the brain injury of CCH by adjusting the microglial activation and polarization, which provides a scientific basis for the future clinical use of *Fructus Broussonetiae*.

Abbreviations

Chronic cerebral hypoperfusion, CCH; BCCA, bilateral common carotid artery; MWM, Morris Water Maze; Fructus Broussonetiae, FB; MAP2, microtubule-associated protein 2; Iba-1, ionized calcium-binding adapter molecule-1; iNOS, inducible nitric oxide synthase; CD16, Fc fragment of IgG receptor III; CD206, macrophage mannose receptor; Arg-1, arginase-1; MBP, myelin basic protein.

Author contributions

PL, LYW, YQW, RLW, FFL, SJZ, ZT, HPZ, and ZPH performed the experiments and analyzed the data, YML, and ZGC wrote the paper.

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee of Capital Medical University.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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