



## Original Article



# The Effects of Baicalin and Geniposide in Combination on Microglial Activation and 5-Lipoxygenase Expression in Rats during Recovery from Cerebral Ischemia

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## Abstract

**Background and objectives:** This study aimed to analyze the effects of baicalin (BC) and geniposide (GP) in combination (7:3) on the activation of microglia (MG) and 5-lipoxygenase (5-LOX) expression in rats during the recovery period after cerebral ischemia and to determine whether inhibition of the 5-LOX pathway is beneficial for M1-to-M2 polarization of MG.

**Methods:** Sprague Dawley rats were divided into five groups: control, model, and BC/GP (7:3) at 30, 45, and 60 mg/kg. A permanent middle artery occlusion model was established using the thread embolism method, and recovery after cerebral ischemia was monitored for 5 weeks. The effects on microglial activation and 5-LOX expression were evaluated by the neurofunctional score and immunofluorescence double labeling. The gene expression of 5-LOX in MG was determined by quantitative polymerase chain reaction before and after drug administration. The gene expression of tumor necrosis factor alpha, inducible nitric oxide synthase, interleukin 10, and cluster of differentiation 206 in MG was determined after the administration of zileuton (depressor). Western blotting was performed to determine the protein expression of 5-LOX, cysteinyl leukotriene receptor 1, cysteinyl leukotriene receptor 2, leukotriene B4 receptor 1, and leukotriene B4 receptor 2 before and after the administration of zileuton.

**Results:** The activation of MG and the expression of 5-LOX were both increased after cerebral ischemia. BC/GP in combination inhibited the activation of microglia-reduced expression of 5-LOX and induced M2 polarization of MG.

**Conclusion:** The combination of BC and GP downregulates the 5-LOX inflammatory pathway by inhibiting activation of MG and promotes M1-to-M2 polarization of MG. Neurons are protected by the M2-type polarization, resulting in alleviation of cerebral ischemia.

**Keywords:** Recovery from cerebral ischemic injury; Baicalin; Gardenia; Microglia; Microglial cell polarization; 5-LOX.

**Abbreviations:** AA, arachidonic acid; BC, baicalin; BLT, leukotriene B4 receptor; CCA, common carotid artery; CD, cluster of differentiation; Con, control; CT, cycle threshold; CysLTs, cysteinyl leukotrienes; d, day; DMEM, Dulbecco's modified Eagle medium; GP, geniposide; IL, interleukin; 5-LOX, 5-lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; MG, microglia; pMCAO, permanent middle cerebral artery occlusion; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; TNF $\alpha$ , tumor necrosis factor alpha; iNOS, inducible nitric oxide synthase; WB, western blot.

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## Introduction

Cerebral ischemic injury, also known as stroke or cerebral stroke, is one of the most common cerebrovascular diseases in China and has a high incidence with high rates of disability and mortality globally. Stroke has been of increasing concern worldwide in recent years, since the sequelae of ischemic brain injury can greatly impact the quality of life of patients.<sup>1</sup> Consequently, research into the treatment and recovery from cerebral ischemia is now coming into the limelight.

Microglia (MG) are major immune effector cells within the central nervous system that play an important role in the early inflamma-

tory response.<sup>2</sup> Early microglial activation can promote neurological repair by the phagocytosis of necrotic nerve cells and the release of neurotrophic factors. However, excessive or persistent activation of MG can cause secondary brain injury by secreting substances such as pro-inflammatory factors and reactive oxygen species or reactive nitrogen species.<sup>3</sup> From the perspective of Chinese medicine, it is of theoretical significance and clinical value to investigate the mechanism of anti-inflammatory damage and the neuroprotective effects of Chinese herbal medicines and natural drugs in cerebral ischemia mediated by inhibition of excessive microglial activation.

Baicalin (BC) is a flavone glycoside extracted from the rhizome of *Scutellaria baicalensis*, a plant in the Labiateae family.<sup>4</sup> BC is the active ingredient of the purgative and detoxifying Chinese herb *Scutellaria baicalensis*, which has antibacterial, anti-inflammatory,<sup>5</sup> hypotensive, sedative, antimetamorphic, antithrombotic, and antitumor effects.<sup>6</sup> Geniposide (GP) is one of the active ingredients extracted from the dried mature fruit of *Gardenia jasminoides*, of the Rubiaceae family, and is a cyclic ether terpene glycoside. GP is the main active component of the Chinese herbal medicine *Gardenia jasminoides*, which has pharmacological properties that include antioxidant activity, melanogenesis promotion, nuclear factor kappa B/inhibitor of kappa B pathway inhibition, and cell adhesion molecule production.<sup>7</sup> Our research group predicted the molecular mechanism of BC/GP in combination in cerebral ischemic inflammatory injury based on network pharmacology. The prediction was verified at the cellular level by stimulating the immortalized mouse microglial cell line BV2 with lipopolysaccharide (LPS) and then treating the cells with BC/GP. The M1/M2 polarization ratio of the cells was reduced, suggesting that the combination may have a therapeutic effect on cerebral ischemic injury by reversing the polarization status of MG. The therapeutic effect of BC/GP in combination on cerebral ischemic injury was confirmed. In addition, our research group found that BC/GP in a ratio of 7:3 better protected against cerebral ischemia at an earlier stage<sup>8</sup> and that the mechanism involved the inflammatory pathway. However, the early dosing studies and the mechanistic studies in cellular experiments were based on short-term ischemia-reperfusion or inflammation models. There are limited literature reports on the combination of BC with GP in the recovery period after cerebral ischemic injury. Moreover, considering that early activation of MG has a protective effect on the brain but that overactivation is harmful, the discovery of drugs based on effective regulation of MG is of great clinical significance. Furthermore, there are currently no effective therapeutic drugs. Since our research group has found that the 7:3 BC/GP combination has a good regulatory effect on polarization, this study used the thread embolism method to establish a permanent middle cerebral artery occlusion (pMCAO) model<sup>9</sup> to investigate the effects of the combination and provide a reference for clinical application. The activation of MG and the expression of 5-lipoxygenase (5-LOX) in the brain tissue of rats with cerebral ischemia were evaluated during the recovery period.

## Materials and methods

### Experimental drug

BC ( $\geq 98\%$ , batch number: 20161107) and GP (*Gardenia jasminoides* lot number: 20160907) were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

### Experimental animals

Sprague Dawley male rats (150, body mass of 215–255 g) were

purchased from the Air Force Military Medical University Laboratory Animal Center (license number: SCXK (Army) 2016-0017). The animals were housed at the Pharmacology Experimental Center of Shaanxi University of Traditional Chinese Medicine. Ten animals per cage were provided free access to food and water. The control temperature was  $25 \pm 2^\circ\text{C}$ , the humidity was 55–75%, and normal day and night alternation was maintained. The experiment was conducted one week after adaptive feeding, and all studies were ethically compliant.

### Cells

Mouse microglial BV2 cells were purchased from the American Type Culture Collection, Shaanxi Zhong Collaborative Innovation Center for the Industrialization of Pharmaceutical Resources Cell Experiment Cryopreservation and Seed Preservation.

### Reagents and instruments for animal experiments

The following reagents and instruments were used for the animal experiments: Anti-CD11b (Abcam, Waltham, MA, USA), rabbit anti-mouse CD11b (fluorescently labeled) (Abcam), goat serum (Dr. Bio, USA), anti-5-LOX (Abcam), rabbit anti-mouse 5-LOX (fluorescent labeling) (Abcam), vortex mixer (Clinebell Equipment Co., Loveland, CO, USA), balance (Mettler Toledo, Inc., Columbus, OH, USA), cryogenic centrifuge (Eppendorf, Framingham, MA, USA), pipette (Eppendorf), water purifier (Millipore, Burlington, MA, USA), HM550 Frozen Slicer (Mecon, Kerpen, Germany).

### Reagents and instruments for cell experiments

The following reagents and instruments were used for the cell experiments: fetal bovine serum (Biological Industries, Beit Haemek, Israel), Dulbecco's modified Eagle medium (DMEM) High-Sugar Culture Solution (Biological Industries), penicillin mix (HyClone, Logan, UT, USA), phosphate-buffered saline (Biological Industries), trypsin (HyClone), BC (HPLC  $\geq 98\%$ , No. B20570) (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China), GP (HPLC  $\geq 98\%$ , No. B21661) (Shanghai Yuanye Biotechnology Co., Ltd.), interleukin 4 (IL4) (No. 500-M04) (PeproTech, Cranbury, NJ, USA), lipopolysaccharide (LPS, No. L8880) (Beijing Solabao Technology Co., Ltd., Beijing, China), zirconium (HPLC  $\geq 99\%$ , No. B33997) (Shanghai Yuanye Biotechnology Co., Ltd.),  $\beta$ -actin (HC201) (Beijing All-Formula Gold Biotechnology Co., Ltd., Beijing, China), 5-LOX (10021-1-Ig) (Proteintech, Rosemont, IL, USA), CysLT1 (ab95492) (Abcam), CysLT2 (A16556) (ABclonal, Woburn, MA, USA), leukotriene B4 receptor 1 (BLT1) (A15042) (ABclonal), BLT2 (A15479) (ABclonal), whole protein extraction kit (Beijing Solabao Technology Co., Ltd.), BCA protein concentration determination kit (Beijing Solabao Technology Co., Ltd.), total ribonucleic acid (RNA) extraction kit (Tiangen Biochemical Technology Beijing Co., Ltd., Beijing, China), reverse transcription kit (Beijing All-Style Gold Biotechnology Co., Ltd., Beijing, China), Tip Green quantitative polymerase chain reaction (qPCR) SuperMix (Beijing All-Style Gold Biotechnology Co., Ltd.), CO<sub>2</sub> incubator (Thermo Fisher Scientific, Waltham, MA, USA), EIX800 enzyme labeler (Bio-TEK, Winooski, VT, USA), vertical pressure steam sterilizer (Shanghai Shenan Medical Equipment Factory, Shanghai, China), benchtop centrifuge (Sigma, St. Louis, MO, USA), inverted microscope (Olympus, Tokyo, Japan), clean bench (Sujing Group Suzhou Antai Air Technology Co., Ltd., Suzhou, China), 25-cm<sup>2</sup> cell culture flask (Corning, Corning, NY, USA), fluorescence qPCR instrument (Xi'an Tianlong Technology Co., Ltd., Xi'an, China).

### **pMCAO model in rats**

The rats were fasted for 12 h before surgery and provided normal drinking water. The animals were anesthetized by an intraperitoneal injection of 3% pentobarbital sodium (1 mL/100 g) and subjected to abdominal upward fixation to the mouse plate. The middle line of the neck skin was cut, followed by blunt separation of the muscles to expose the common carotid artery (CCA), and the internal and external carotid arteries were separated. The external carotid artery and the CCA were ligated. The distal end of the internal carotid artery was clamped at approximately 1 cm above the intersection of the internal carotid artery and the external carotid artery in the opening above the CCA. Fishing line (diameter: 0.25 mm) was slowly inserted, and the insertion distance was calculated at the fork. After cutting the excess fishing line, the wound was sutured and the rat was kept at room temperature, waking after about 1.5 h. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

### **Neurological functional scores**

The animals were neurologically scored based on Bederson's 5-point scale:<sup>10</sup> grade 0, no obvious neurological signs, score 0; grade 1, ischemic contralateral forelimb rotates against the thorax after lifting the tail, score 1; grade 2, the animal tilts toward the ischemic contralateral side when placed on a horizontal plane, score 3; grade 3, the animal makes circular movements toward the ischemic contralateral side, score 6; grade 4, the animal is unconscious or paralyzed, score 10.

### **Animal groupings and drug administration**

The experimental animals with neurological function of grade 2 or 3 were randomly grouped. The animals in the control (Con) group were modeled separately, and only CCA ligation was performed, without threading; the model (pMCAO) group had permanent ischemia; treatment with BC/GP combination (7:3) was dosed as follows: 30, 45, and 60 mg/kg. Each of the treatment groups was dosed by gastric administration starting at 12 h after modeling, twice a day, for 35 days (d). The Con and pMCAO groups were dosed with equivalent amounts of purified water.

Five different time points were set to study the changes in cerebral ischemic injury during the recovery period: 7, 14, 21, 28, and 35 d. The start was taken as the time of awakening of the experimental animals after completion of the model. Drug was administered until the selected time point, and the corresponding batch of experimental animals was taken for experimentation.

### **Microglial activation assay**

The effect of activated MG on 5-LOX expression was observed using immunofluorescence dual standard technology, and the effect of activated MG on 5-LOX expression was selected as the detection object.

### **Cell groupings and treatments for western blot analysis**

Well-grown BV2 cells were made into a cell suspension, adjusted to a cell density of  $2 \times 10^5$  cells/mL, and inoculated into 6-well culture plates at 2 mL/well. After 24 h of normal cell culture, the cells were divided into the following groups: blank group, BV2 cells were cultured in complete medium without drug; M1 group, BV2 cells were cultured in 100 ng/mL LPS in DMEM for 24 h and then in drug-free DMEM for 24 h; M1-to-M2 transformation group, BV2 cells were cultured in DMEM containing 20 ng/mL IL4 for 24 h and then in 100 ng/mL LPS for 24 h; positive control

drug group, BV2 cells were cultured in 10  $\mu$ M zileuton in DMEM for 2 h and then in drug-free DMEM for 24 h; BC group, BV2 cells were cultured in DMEM containing 125  $\mu$ M GP for 2 h, followed by 100 ng/mL LPS for 24 h; BC/GP group, BV2 cells were cultured in DMEM medium containing 125  $\mu$ M BC/GP for 2 h and then in 100 ng/mL LPS for 24 h.

### **Cell groupings and treatments for qPCR determination**

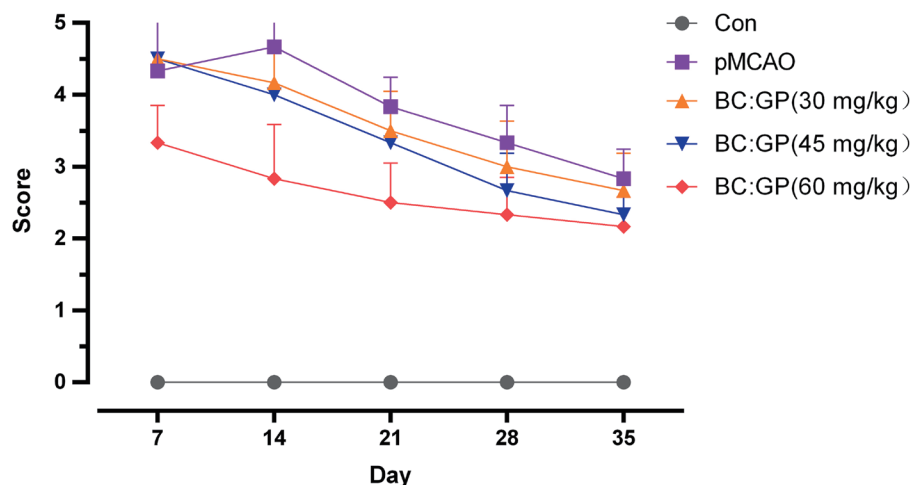
Well-grown BV2 cells were made into a cell suspension, adjusted to a cell density of  $2 \times 10^5$  cells/mL, and inoculated into 6-well culture plates at 2 mL/well. After 24 h of normal cell culture, the cells were divided into the following groups: M1-to-M2 transformation group, BV2 cells were incubated with 20 ng/mL IL4 in DMEM for 2 h before switching to 100 ng/mL LPS stimulation for 2 h; BC group, BV2 cells were incubated for 2 h in DMEM with or without 10  $\mu$ M zileuton and then switched to DMEM containing 125  $\mu$ M or 31.25  $\mu$ M BC; GP group, BV2 cells were incubated in DMEM with or without 10  $\mu$ M zileuton for 2 h, then DMEM with 125  $\mu$ M or 31.25  $\mu$ M GP for 2 h, and then DMEM containing 100 ng/mL LPS for 2 h; BC/GP group, BV2 cells were incubated in DMEM with or without 10  $\mu$ M zileuton for 2 h, and then DMEM containing 100 ng/mL LPS for 2 h; BC/GP group, BV2 cells were incubated in DMEM with or without 10  $\mu$ M zileuton for 2 h, then in DMEM medium containing 125  $\mu$ M or 31.25  $\mu$ M BC/GP for 2 h, and then in DMEM containing 100 ng/mL LPS for 2 h.

### **Total RNA extraction and qPCR**

Total RNA was extracted from the rat hippocampus using a total RNA extraction kit, according to the manufacturer's instructions. A reverse transcription kit was used to process the extracted RNA. cDNA was synthesized using mRNA as the template for reverse transcription in a 20- $\mu$ L reaction system: total RNA/mRNA (3  $\mu$ L); anchored Oligo(dT)18 Primer (0.5  $\mu$ g/ $\mu$ L, 1  $\mu$ L); 2 $\times$ TS Reaction Mix (10  $\mu$ L); TransScrip RT/RI Enzyme Mix (1  $\mu$ L); and GPNA Remover (1  $\mu$ L). RNase-free water (4  $\mu$ L) at 85°C for 15 min was sufficient for reverse transcription. The mRNA primer sequences were as follows: 5-LOX F: 5'-ACTACATCTACCTCAGCCTCATT-3', R: 5'-GGTGACATCGTAGGAGTCCAC-3'; IL10 F: 5'-GCTCTTACTGACTGGCATGAG-3', R: 5'-CGCAGCTCTAGGAGCATGTG-3'; cluster of differentiation 206 (CD206) F: 5'-CTCTGTTTCAGC-TATTGGACGC-3', R: 5'-CGGAATTTCTGGGATTCAGCTTC-3'; tumor necrosis factor alpha (TNF $\alpha$ ) F: 5'-CCCTCAGACTCAGATCATCTTCT-3', R: 5'-GCTACGACGTGGGCTACAG-3'; inducible nitric oxide synthase (iNOS) F: 5'-GTTCTCAGCCCAACAATA-CAAGA-3', R: 5'-GTGGACGGGTGCGATGTCAC-3';  $\beta$ -actin F: 5'-ACCGTAAAGATGACCCAGAT-3', R: 5'-CCAGAGGCATACAGGGACAA-3'.

Using Tip Green qPCR SuperMix, the reagents were added according to the requirements of the real-time fluorescence quantification kit to form a 20- $\mu$ L qPCR reaction system: template (3  $\mu$ L); forward primer (10  $\mu$ M, 0.5  $\mu$ L); reverse primer (10  $\mu$ M, 0.5  $\mu$ L); 2 $\times$  TransStart® Tip Green qPCR SuperMix (10  $\mu$ L); passive reference dye (50 $\times$ , 0.5  $\mu$ L); nuclease-free water (5  $\mu$ L). The reaction parameters were as follows: denaturation at 94°C for 30 s; denaturation at 94°C for 5 s, 55°C for 15 s, 72°C for 15 s, 45 cycles; extension at 72°C for 1 min. Three replicate wells per sample were used. For the qPCR result, the cycle threshold (CT) value represents the result of fluorescence real-time qPCR,  $\Delta$ CT = CT (target gene) – CT (internal reference gene),  $\Delta\Delta$ CT =  $\Delta$ CT (experimental group) –  $\Delta$ CT (control group). The relative expression amplification fold of target gene expression in each group of cells was calculated according to the  $2^{-\Delta\Delta$ CT method.





**Fig. 1.** Effect of the BC/GP combination (7:3) on the neurological functional score in pMCAO rats. BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion.

### Statistical analysis

All experimental data were subjected to the chi-squared and normality tests, and data were expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). SPSS24, GraphPad Prism 6, and other software were used for statistical analysis and data processing. The independent samples t-test was used for the comparison of the same time points, one-way analysis of variance was used for the comparison of multiple data, and the least significant difference test was used for the comparison of data between two groups. A value of  $p < 0.05$  was defined as a statistically significant difference.

## Results

### Effect of BC/GP on neurological function in rats recovering from pMCAO

The results revealed that pMCAO caused neurological deficits in rats compared with the Con group. The rats suffered from forelimb paralysis, leaning to the left side, and rotating around the left leg during movement. The animals had high neurological scores ( $p < 0.01$ ), which continued to deteriorate until 14 d. After 14 d, the rats showed a slow recovery until 21 d, when the pMCAO group began to show a slow recovery. In the pMCAO group, the neurological scores exhibited a decreasing trend. In contrast, the administration of BC/GP ameliorated the neurological impairment between 1 and 14 d, and the neurological scores decreased during the subsequent recovery period (Fig. 1).

### Effect of BC/GP in combination on microglial activation and 5-LOX expression by immunofluorescence double-labeling

MG does not always exert immune effects, but they can be activated to exert multiple immune effects by external stimuli. CD11b is a specific marker of activated MG, and the level of activation is directly correlated with the degree of the central inflammatory response.

After 7 d of recovery (Fig. 2), compared with the Con group, the degree of microglial activation was significantly enhanced after pMCAO, and the expression of 5-LOX increased. Compared with the pMCAO group, the BC/GP combination reduced 5-LOX expression and attenuated MG activation in all dose groups in a

dose-dependent manner.

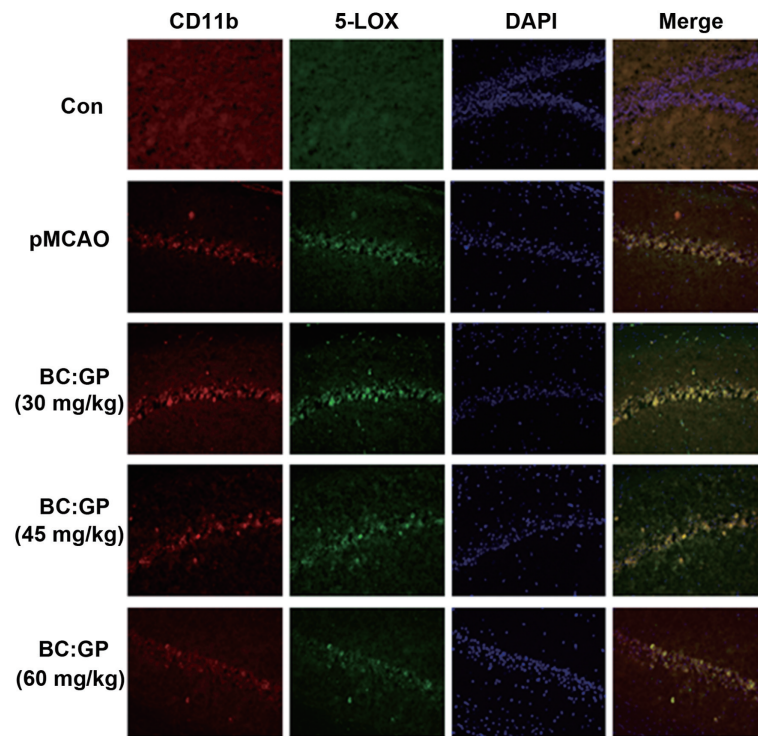
After 14 d of recovery (Fig. 3), compared with the Con group, the pMCAO group maintained a high level of microglial activation and significant 5-LOX expression. Compared with the pMCAO group, the BC/GP combination groups still exhibited reduced 5-LOX expression and diminished MG activation. The dose-dependent effects persisted.

After 21 d of recovery (Fig. 4), compared with the Con group, the degree of microglial activation and 5-LOX expression remained higher in the pMCAO group, but the positive expression area of CD11b and 5-LOX was reduced under a microscope. Compared with the pMCAO group, the BC/GP combination groups downregulated 5-LOX and reduced MG activation. The positive zone was weaker than that observed at 14 d under a microscope.

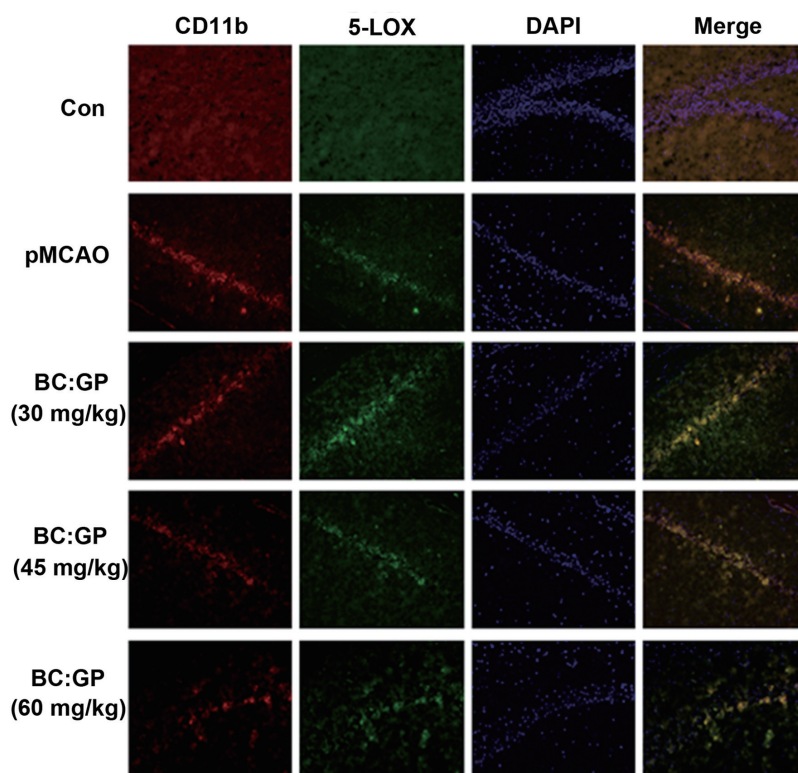
After 28 d of recovery (Fig. 5), compared with the Con group, CD11b- and 5-LOX-positive expression could be observed in some areas in the pMCAO group, but the positive expression area in the entire visual field was significantly reduced. Compared with the pMCAO group, a small amount of positive expression of CD11b and 5-LOX was observed in the low-dose BC/GP combination group. No positive areas could be found in the medium- and high-dose groups.

After 35 d of recovery (Fig. 6), no CD11b- or 5-LOX-positive expression regions were present in any group, and all were similar to the Con group.

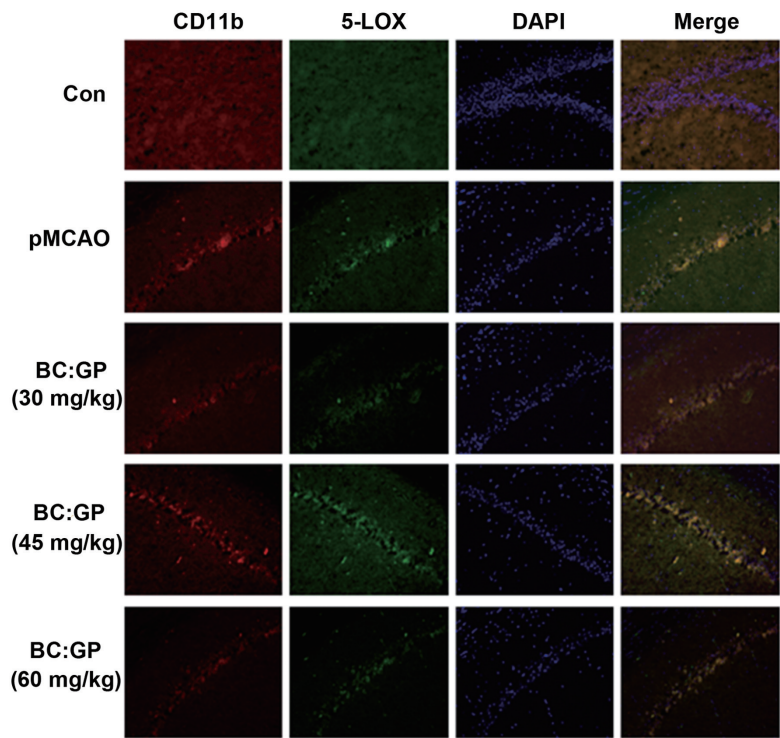
The pMCAO group maintained a high level of microglial activation and significant 5-LOX expression from 1 d to 14 d. The BC/GP combination groups showed downregulation of 5-LOX, reduced MG activation, and dose-dependent effects. From 14 d to 21 d, the pMCAO group still exhibited high levels of microglial activation and 5-LOX expression, but the positive zone of CD11b and 5-LOX expression was reduced under microscopy. The expression of 5-LOX was downregulated in the BC/GP combination groups, the degree of MG activation was attenuated, and the positive areas were reduced under a microscope. From 28 d to 35 d, the pMCAO group showed some areas of positive CD11b and 5-LOX expression, which gradually decreased to no positive expression areas, similar to the Con group. Activation of MG is considered to be key to triggering the inflammatory response and aggravating inflammatory damage in the later stages, so we observed microglial activation by immunofluorescence. After modeling, activated MG



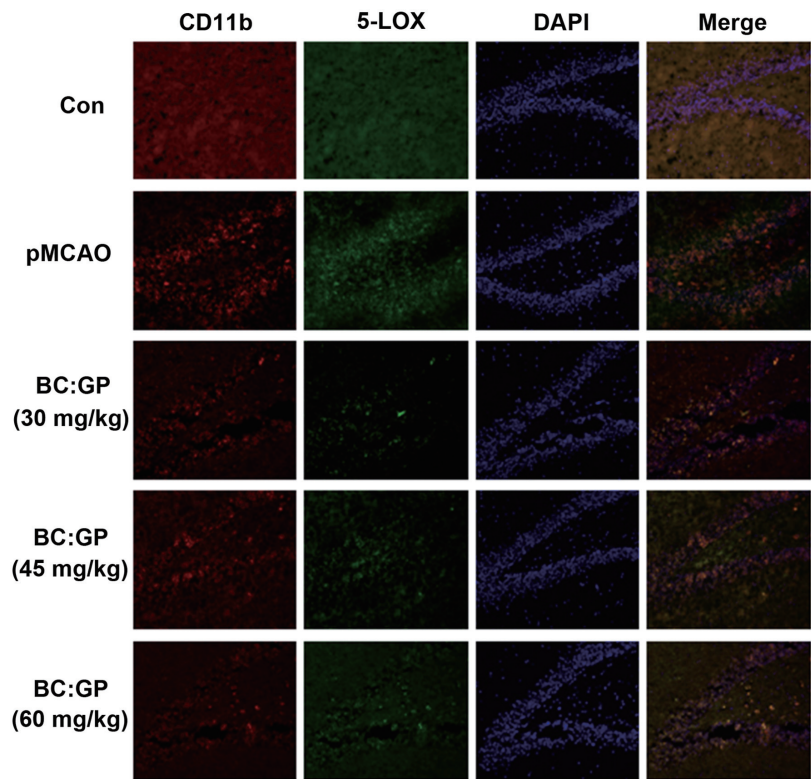
**Fig. 2.** Effect of the BC/GP combination (7:3) on microglia and 5-LOX expression in the brain tissue of pMCAO rats (7 d). BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion; 5-LOX, 5-lipoxygenase.



**Fig. 3.** Effect of the BC/GP combination (7:3) on microglia and 5-LOX expression in the brain tissue of pMCAO rats (14 d). BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion; 5-LOX, 5-lipoxygenase.

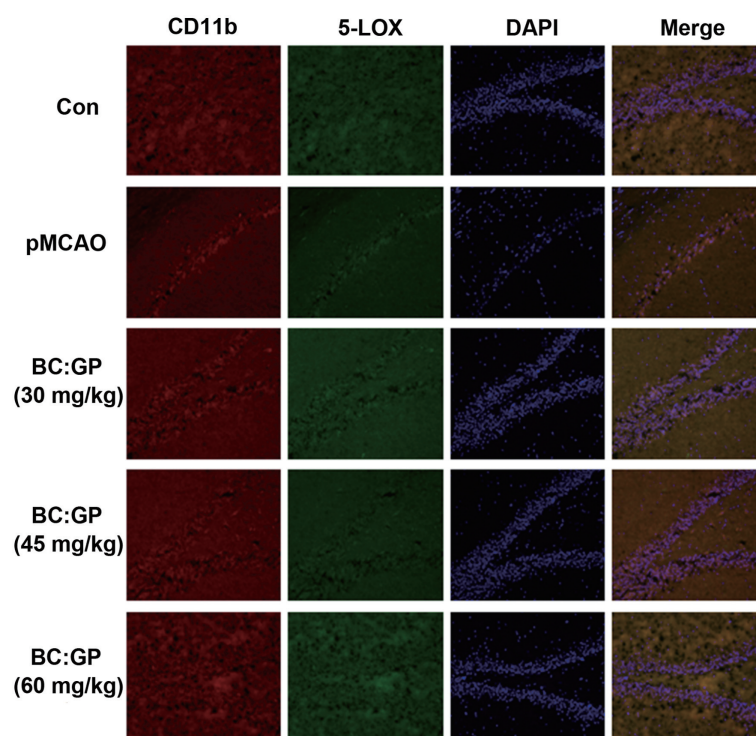


**Fig. 4.** Effect of the BC/GP combination (7:3) on microglia and 5-LOX expression in the brain tissue of pMCAO rats (21 d). BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion; 5-LOX, 5-lipoxygenase.



**Fig. 5.** Effect of the BC/GP combination (7:3) on microglia and 5-LOX expression in the brain tissue of pMCAO rats (28 d). BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion; 5-LOX, 5-lipoxygenase.





**Fig. 6.** Effect of the BC/GP combination (7:3) on microglia and 5-LOX expression in the brain tissue of pMCAO rats (35 d). BC, baicalin; GP, geniposide; pMCAO, permanent middle cerebral artery occlusion; 5-LOX, 5-lipoxygenase.

were significantly upregulated, accompanied by high expression of 5-LOX. After the administration of the BC/GP combination, activated MG and 5-LOX were significantly downregulated, indicating that the inhibitory effect of the drug on 5-LOX was related to inhibition of microglial activation. Therefore, the experiment showed that the combination of BC and GP reduced cerebral ischemic injury by inhibiting microglial activation and decreasing 5-LOX expression. The best effect was observed between 1 d and 14 d.

#### **Effects of BC, GP, and BC/GP on the 5-LOX pathway in MG**

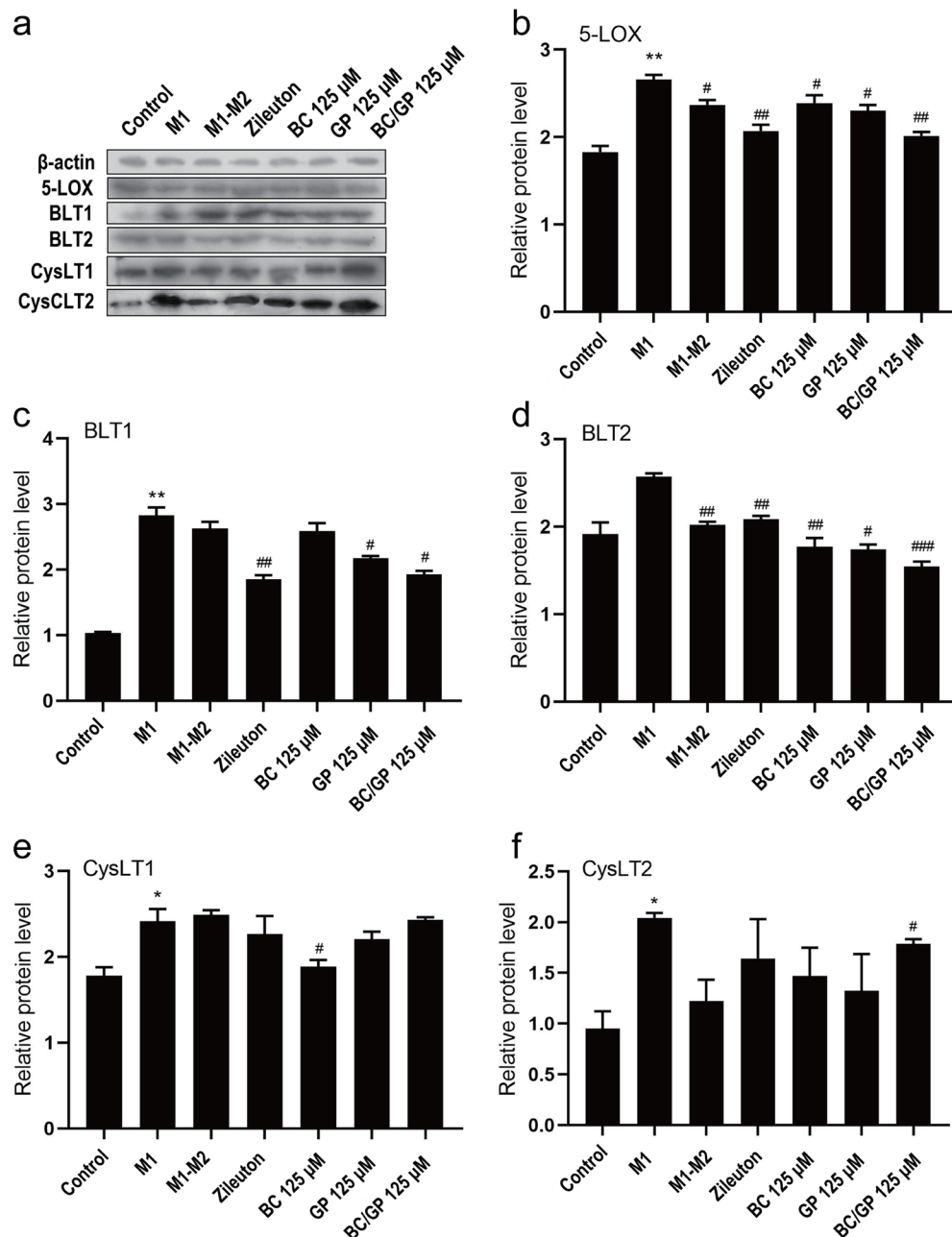
In this experiment, the levels of 5-LOX, CysLT1, CysLT2, BLT1, and BLT2 proteins were evaluated by western blotting. After the administration of LPS to MG, the levels of 5-LOX and its pathway proteins, CysLT1, CysLT2, BLT1, and BLT2, were higher than those in the blank group. In the M1-to-M2 transformation group, 5-LOX and BLT2 protein expression tended to decrease significantly compared to that in the M1 group. After the administration of the positive control drug zileuton, the expression of 5-LOX together with M1 and M2 characteristic polarization molecules in MG after LPS stimulation showed a decreasing trend. The protein expression of 5-LOX, BLT1, and BLT2 were all decreased compared to that in the M1 group, as evidenced by the best effect induced by the BC/GP combination. There was a decreasing trend for the expression of CysLT1 and CysLT2, but the effect was not significant (Fig. 7).

#### **Effects of BC, GP and BC/GP on the mRNA expression of 5-LOX, iNOS, TNF $\alpha$ , IL10, and CD206 in BV2 microglia**

In this experiment, BV2 cells were pretreated with the 5-LOX inhibitor zileuton, followed by high and low concentrations of BC, GP, and BC/GP before LPS induction. 5-LOX together with M1

and M2 characteristic polarization molecules were determined by qPCR, and the results are shown in Figure 8. After treatment of BV2 MG with zileuton followed by BC, GP, and BC/GP, the expression of 5-LOX mRNA was inhibited. In addition, the expression of CD206 and IL10 mRNA was significantly increased by high and low concentrations of GP and BC/GP, and the MG showed M2-type polarization. When BV2 cells were pretreated with high and low concentrations of BC, GP, and BC/GP, the mRNA expression of the M1-type marker iNOS was inhibited, and low doses of BC and BC/GP resulted in a significant decrease of TNF $\alpha$  mRNA expression compared with that of the M1 group. High doses of GP and BC/GP decreased TNF $\alpha$  mRNA expression, but the decreases were not significant.

Arachidonic acid (AA), one of the most important polyunsaturated fatty acids in mammalian cells, is not only required to maintain membrane integrity but also serves as a direct precursor to many bioactive mediators, such as leukotrienes (LTs), prostaglandins, thromboxane A<sub>2</sub>, epoxyeicosatrienoic acid, and endogenous cannabinoids.<sup>11</sup> We know that AA can be metabolized by lipoxygenases, of which 5-LOX is the rate-limiting enzyme responsible for the biosynthesis of LTs, the primary mediators of inflammation that ultimately lead to a variety of human diseases, including asthma, atherosclerosis, and diabetes.<sup>12–14</sup> The specific function of 5-LOX is to catalyze the conversion of AA into the unstable intermediate 5-hydroperoxyeicosatetraenoic acid. 5-LOX also catalyzes the conversion of 5-hydroperoxyeicosatetraenoic acid into LTA<sub>4</sub>, which can further produce pro-inflammatory factors, including LTB<sub>4</sub> and LTC<sub>4</sub>, via LTA<sub>4</sub> hydrolase and LTC<sub>4</sub> synthase, respectively.<sup>15</sup> Cysteinyl leukotrienes (CysLTs), represented by LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, are mainly produced by cells of the natural immune system during inflammation. These mediators are



**Fig. 7. Effects of BC, GP, and the BC/GP combination on 5-LOX pathways in BV2 microglia ( $\bar{x} \pm s$ , \* indicates vs. the control group, \* $p < 0.05$ , \*\* $p < 0.01$ ; # indicates vs. the M1 group, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ ). BC, baicalin; GP, geniposide; 5-LOX, 5-lipoxygenase.**

produced by the 5-LOX pathway after AA cleavage and interact with their receptors, CysLTR1, especially, or CysLTR2, on the outer membrane of many cells, including immune cells such as basophils, mast cells, dendritic cells, and eosinophils.<sup>16</sup> LTB<sub>4</sub> is a well-known pro-inflammatory lipid mediator that, by binding to its receptor BLT1, is involved in the regulation of leukocyte chemotaxis and pro-inflammatory cytokines.

This study investigated the effects of BC, GP, and BC/GP (7:3) in combination on microglial polarization, the changes in the 5-LOX pathway before and after drug administration, and whether inhibition of the 5-LOX pathway favors microglial polarization

toward M2. Western blot analysis after LPS stimulation of MG demonstrated that the protein expression of 5-LOX, BLT1, and BLT2 was decreased in the BC/GP group compared to the M1 group. The best effect was observed in the BC/GP group, showing that BC, GP, and the BC/GP combination inhibited the expression of 5-LOX as well as downstream proteins. qPCR showed that the expression of 5-LOX mRNA was suppressed in the BV2 cells after incubation with zileuton followed by treatment with BC, GP, and BC/GP, indicating that 5-LOX is not involved in microglial polarization. High and low concentrations of GP and BC/GP significantly increased the mRNA expression of the M2



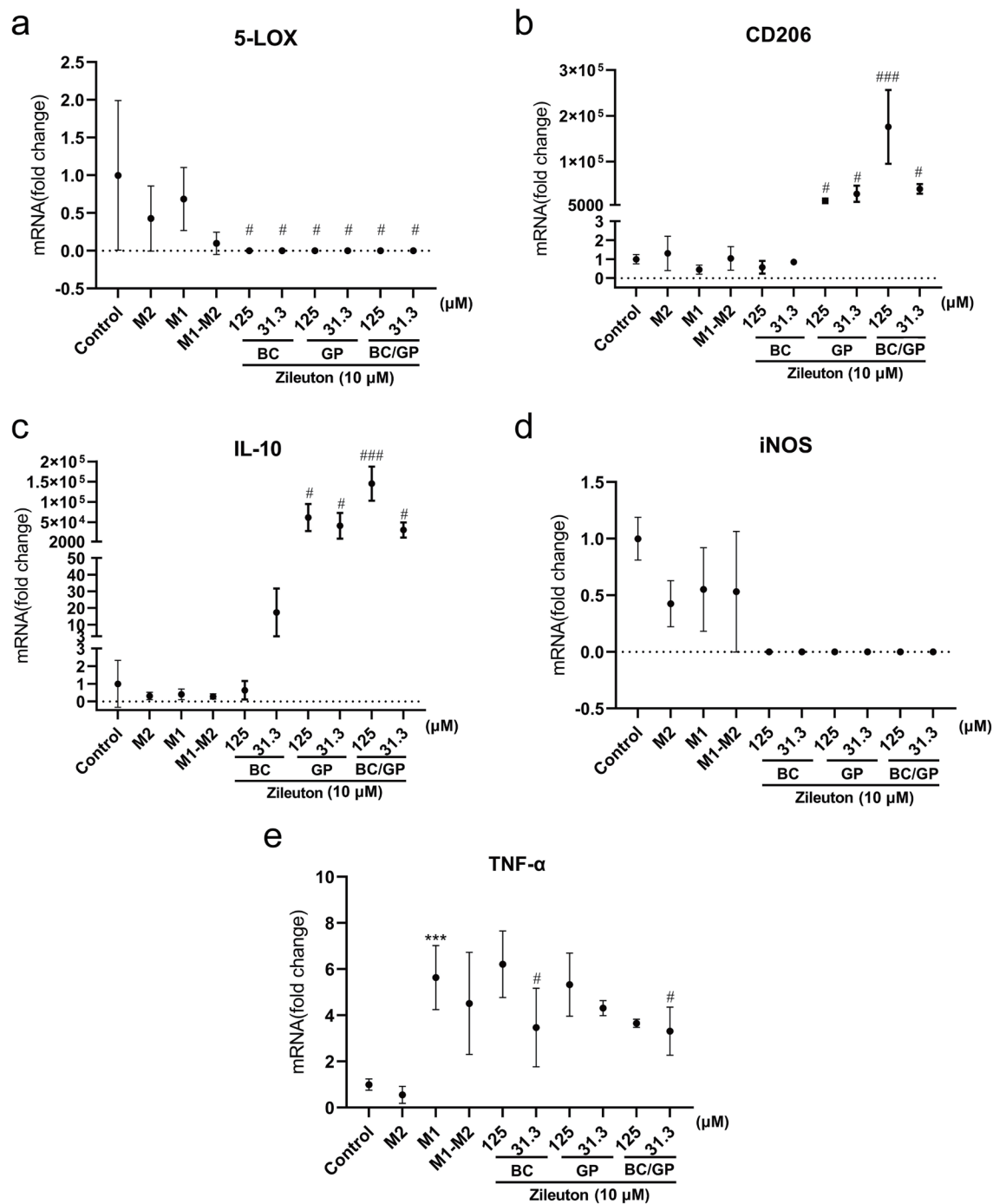


Fig. 8. Effects of BC, GP, and the BC/GP combination on 5-LOX, iNOS, TNF $\alpha$ , IL10, and CD206 mRNA expression in BV2 microglia ( $\bar{x} \pm s$ , \* indicates vs. the control group, \* $p < 0.05$ , \*\* $p < 0.01$ ; # indicates vs. the M1 group, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ ). BC, baicalin; GP, geniposide; 5-LOX, 5-lipoxygenase.

markers CD206 and IL10, indicating M2-type polarization. When BV2 cells were pretreated with high and low concentrations of BC, GP, and BC/GP, M1-type expression of iNOS mRNA was inhibited and low doses of BC and BC/GP resulted in significantly decreased expression of TNF $\alpha$  mRNA compared to the M1 group. This result suggests that inhibition of the 5-LOX pathway favors microglial polarization toward M2.

## Discussion

The BC/GP combination significantly improved neurological function during recovery over 35 d in pMCAO rats compared with the untreated model group. MG are widely distributed in the central nervous system, accounting for about 10–25% of the total number of glial cells, with an equal number of neurons. Disorders of the central nervous system usually lead to inflammation and ac-

tivation of MG, the surface membrane proteins of which are modified and heavily activated in a phenomenon known as “reactive gliosis.” In rapid neurodegenerative diseases (cerebral ischemia, traumatic brain injury, *etc.*),<sup>17</sup> MG are rapidly transformed into the M2 type, accompanied by the release of cytokines, chemokines, and other mediators.<sup>18,19</sup> It has been shown that microglial activation increases iNOS expression, upregulates nitric oxide levels via the iNOS pathway, and affects cyclooxygenase-2 activity. Inhibition or knockdown of cyclooxygenase-2 significantly reduces the area of ischemic necrosis in brain tissue.<sup>20</sup> Price *et al.* have studied patients with cerebral ischemia and observed three different time periods after ischemia ( $\leq 72$  h, 7–14 d, and 25–30 d) by evaluating the microglial activation time and changes in the location and phenotype.<sup>21</sup> Their results showed that MG were significantly activated in patients within 72 h of ischemia, the activation persisted for up to 30 d, and there were still large numbers of activated MG in the infarct area, the ischemic semi-dark zone area, and the contralateral hemisphere. These findings suggest an important role of MG in cerebral ischemic injury. We also found a similar phenomenon through continuous observation over 1–35 d, where monitoring of the microglial activation marker CD11b indicated that a large number of MG were present at 1–21 d after ischemia. The expression of 5-LOX also increased, suggesting that activation of the 5-LOX pathway is associated with microglial activation. The BC/GP combination significantly inhibited this phenomenon, indicating that the effect of treatment on the 5-LOX pathway is related to microglial activation. By 28 d, few MG and low expression of 5-LOX were observed in the high-dose BC/GP treatment group. After 35 d, due to death and compensatory effects in some animals, normal levels of MG and 5-LOX expression were found in all dose groups. Consequently, the present study remains deficient.

The pathological mechanisms of cerebral ischemic injury include impaired energy metabolism, excessive release of excitatory amino acids, mitochondrial damage, reactive oxygen species release, and inflammatory factor release in a waterfall cascade.<sup>22</sup> Numerous studies have shown that the neuroinflammatory response after ischemia is an important factor contributing to brain injury.<sup>23</sup> Activation of inflammatory cells intensifies the adhesion and infiltration of leukocytes and vascular endothelial cells, promoting the release of chemokines and inflammatory factors. These factors promote each other and constitute a signal cascade response that is amplified at each step.<sup>24</sup> This in turn aggravates neuronal injury, causes dysfunction of the blood–brain barrier, causes secondary damage after ischemia, and ultimately leads to neuronal injury or death. Counteracting the inflammatory response after cerebral ischemia is therefore an important way to mitigate neuronal damage.

In this study, we first investigated the effect of BC/GP on histopathological changes in the brain during ischemic recovery. It was found that BC/GP effectively increased the number of normal neurons and reduced the degree of tissue necrosis, indicating that the combination had certain therapeutic effects in the recovery period after cerebral ischemia. CysLTs are a class of inflammatory factors formed from AA by the action of 5-LOX. The increased release of CysLTs induces blood–brain barrier damage and brain edema, followed by activation of downstream CysLT1 and CysLT2 receptors.<sup>25</sup> The CysLT1 receptor is involved in various inflammatory injury diseases, including allergic rhinitis,<sup>26</sup> while activation of the CysLT2 receptor can increase vascular permeability and enhance ischemia-reperfusion injury.<sup>27</sup> In addition, activation of CysLT2 also has been found to induce activation of MG, exacerbating inflammatory injury during ischemia.<sup>28</sup> Activation of CysLTs/CysLT signaling pathways during cerebral ischemia is therefore a key aspect

of inflammatory injury. In this study, we observed that the BC/GP combination downregulated the expression of CysLTs. The expression of CysLT1 and CysLT2 was also found to be downregulated by western blotting. Therefore, inhibition of 5-LOX expression during the ischemic phase is the key to inhibiting pathway activation of CysLTs. We observed a significant downregulation of 5-LOX expression after the administration of BC/GP in combination. Activation of MG is considered to be crucial in triggering the inflammatory response and exacerbating late inflammatory damage due to its specific function.<sup>29,30</sup> We observed significant upregulation of the activated state of MG in the pMCAO model, accompanied by high expression of 5-LOX, indicating that the central inflammatory response was increased. After BC/GP administration, the activation state of MG and 5-LOX expression were significantly downregulated, suggesting that the inhibitory effect of the drug on 5-LOX may be related to inhibition of microglial activation.

## Conclusion

The combination of BC and GP can reduce activation of the 5-LOX inflammatory pathway by inhibiting microglial activation, thus promoting polarization of M1-type MG to M2-type MG. Injured neurons are protected by this M2-type polarization, alleviating cerebral ischemia.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

## Author contributions

YW: conceptualization, supervision, data curation, writing of the original draft, writing of the review, and critical revision of the manuscript. HL: acquisition and analysis of data. JW: acquisition and analysis of data. LZ: investigation and acquisition of the data. HHZ: investigation and acquisition of the data. QQS: assay performance and data analysis. ADZ: assay performance and data analysis. HFZ: conceptualization, supervision, validation, writing of the review, and editing. BW: conceptualization, supervision, validation, writing of the review, and editing. All authors read and approved the final manuscript.

## Data sharing statement

The data used to support the findings of the study are included in the article.

## Ethical statement

The animal handling procedures and experimental protocols were consistent with the guidelines for the management of laboratory animals, and approved by the Animal Ethics Committee of Shaanxi University of Chinese Medicine. Ethics approval no.: SUCMDL20220401004.

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