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Ginsenoside Rb1 attenuates mouse cerebral ischemia/reperfusion induced neurological impairments through modulation of microglial polarization

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Abstract

Cerebral ischemia/reperfusion causes high disability, recurrence, and mortality. Ischemic stroke is a powerful stimulus that triggers significant microglia activation. Ginsenoside Rb1 (GS-Rb1) has been demonstrated to have neuroprotective effects in the central nervous system. In this study, the effects of GS-Rb1 against cerebral ischemia/reperfusion were explored. A mouse model of middle cerebral artery occlusion (MCAO) was used to mimic the cerebral ischemia/reperfusion. Mice in MCAO + GS-Rb1 groups received 5, 10, or 20 mg/kg GS-Rb1 through intraperitoneal injection. Modified neurological severity scoring (mNSS) showed neurological function, while the open field test tested the anxiety-like behaviors. Cognitive impairment was evaluated by Morris water maze. Protein levels were evaluated by ELISA and Western blot and mRNA levels were analyzed by qRT-PCR. When compared to the MCAO mice, mice in the MCAO + GS-Rb1 group had significantly lower mNSS scores and less brain water content. GS-Rb1 alleviated both cognitive impairment and anxiety and inhibited microglial activation in the cerebral ischemia/reperfusion model. GS-Rb1 enhanced M2-type microglia polarization while inhibiting M1-type microglia polarization. In summary, we observed that GS-Rb1 had neuro-protective effects in a cerebral ischemia/reperfusion mouse model through regulating the microglia polarization.

Key words: *cerebral ischemia/reperfusion, MCAO, GS-Rb1, microglia.*

Introduction

In China, stroke has become the third most dangerous disease for human health and quality of life, behind only tumor and coronary heart disease [27]. Most cases are ischemic stroke [19]. Currently, the widely used effective therapeutic strategy of ischemic stroke is endovascular revascularization with ultra-early intravenous thrombolysis and mechanical thrombectomy [10]. However, the strict treatment time window restriction results in losing treatment opportunities and facing long-term disability for most of the patients.

Ginsenosides (GS) have been shown to be the most important active constituent of ginseng. GS are a group of triterpenoid saponins with similar structures [8]. GS all have similar structures and are divided into two groups according to their different glycosidic base structures, namely protopanaxadiol and protopanaxatriol [13]. Rb1 is the most abundant and major component in GS.

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There are numerous experiments demonstrating the anti-inflammatory and antioxidant effects of GS; thus GS-Rb1 is gaining more and more attention in the alleviation of inflammation and cell injuries.

Studies have shown that GS can produce therapeutic effects by enhancing brain-derived neurotrophic factor (BDNF) expression in neurons [31]. Subsequent studies have also shown that the GS-Rb1 homolog GS-Rd significantly alleviates neurological deficits in cerebral ischemic diseases [30]. This finding reveals that GS has neuroprotective effects. Previous research has demonstrated that GS-Rb1 has beneficial effects on learning and memory [21]. GS-Rb1 promotes synapsin phosphorylation and further stimulates synaptic vesicle release and neurite outgrowth [17,29]. GS-Rb1 also has potential in the prevention of ischemia and glutamate-induced neurodegeneration in hippocampal neurons [12,16]. GS-Rb1 inhibits the expression of oxidative stress factors NADPH oxidase (NOX) 1 (NOX1), NOX2, NOX4, NADPH, and the inflammatory factor interleukin (IL)-1β, while it upregulates ZO-1 and occludin expression in the cerebral ischemia-reperfusion model [2]. In the traumatic brain injury (TBI) model, GS-Rb1 could reduce the neurological damage and brain edema caused by TBI [1]. In this study, the effects of GS-Rb1 on neurological damage and cognitive behavior were explored.

Material and methods

Middle cerebral artery occlusion (MCAO) model

Briefly, male C57BL/6J mice (26-28 g) were anesthetized by inhaling isoflurane. Through midline neck incision, the carotid artery was revealed. The original site of the middle cerebral artery was inserted with a nylon suture. It was withdrawn after 30 min to establish reperfusion. The People's Hospital of Shanghai Pudong New Area approved the experimental protocols.

Treatment

Mice were randomized to five groups: the sham operation (Sham) group, the MCAO group, and three MCAO + GS-Rb1 groups. Mice in the MCAO group received vehicles *via* intraperitoneal injection. Mice in the MCAO + GS-Rb1 groups received 5, 10, or 20 mg/kg GS-Rb1 (Nanjing Dasf Biotechnology, Nanjing, China) through intraperitoneal injection. GS-Rb1 treatment was performed at 1 h, 12 h, 24 h, and each day once after MCAO.

NSS

The mNSS (modified neurological severity score) scoring system was used to identify the brain injury: normal function with a score of 0, mild damage with scores of 1-6, moderate damage with scores of 7-12, severe damage with scores of 13-18. mNSS scores were evaluated before MCAO and at indicated time points after MCAO.

Brain water content

Three days after MCAO, brain tissues were collected. Wet weight was determined by weighing the brain tissues. Dry weight was determined by weighing the tissues which were dried at 100°C for 24 h.

Morris water maze

The Morris water maze test was composed of cue, spatial, and probe tests and each test lasted for no more than 60 s. The cue test had a visible platform 2 cm below the water level where the mice could stay for 30 seconds after finding or being guided to the platform. The space test submerged the platform in water. The mice were released and searched for the platform by swimming. The latency in finding the platform was measured in the spatial test. Mice were placed in each of the 4 quadrants at the beginning. The time was then recorded. Tests were performed at 15 days after MCAO.

Open field test

There were 3 trials of training for this test. Mice were housed in an acrylic box. The total numbers of circuit breaks were counted as locomotion counts and locomotive behavior. Tests were performed at 8 days after MCAO. The time in the central area was recorded.

Immunofluorescence

Brain sections from the ischemic hippocampus were deparaffinized and blocked by goat serum. Iba-1 (ab178847, Abcam, Cambridge, MA) was used as the primary antibody. DAPI was used to show the nucleus. The numbers of Iba-1-positive cells were analyzed at 7 days after MCAO.

Western blot

The tissues of the ipsilateral injury hemispheres were collected and the total cell lysate was extracted by RIPA at 7 days after MCAO. Western blot was performed using a standard protocol. Anti-Iba-1 (ab178847, Abcam) and anti-β-actin (ab8226, Abcam) were used in this experiment.

ELISA

The levels of IL-10, IL-6, IL-1β, and tumor necrosis factor α (TNF-α) in the ipsilateral injury hemispheres

were measured by relative ELISA kits (Wuhan Boster Biological Technology LT, China) at 7 days after MCAO.

qRT-PCR

The tissues of the ipsilateral injury hemispheres were collected at 7 days after MCAO. Total RNA was isolated by TRIzol reagent (Invitrogen). Reverse transcription was conducted using a First Strand cDNA Synthesis Kit (Roche).

The following primers were used:

- Inducible nitric oxide synthase (iNOS) F: CAGGAGGAGAGATCCGATTTA;
- iNOS R: GCATTAGCATGGAAGCAAAGA.
- CD206 F: GTGGAGTGATGGAACCCCAG;
- CD206 R: CTGTCCGCCCAGTATCCATC.
- CD86 F: TAAGCAAGGTCACCCGAAAC;
- CD86 R: AGCAGCATCACAAGGAGGAG.
- IL-10 F: ACCAAGACCCAGACATCA;
- IL-10 R: TTCACAGGGAAGAAATCG.
- Arginase (Arg) 1 F: GAACACGGCAGTGGCTTTAAC;
- Arg 1 R: TGCTTAGCTCTGTCTGCTTTGC.
- GAPDH F: GGCACAGTCAAGGCTGAGAATG;
- GAPDH R: ATGGTGGTGAAGACGCCAGTA.

Statistics

The data, analyzed by SPSS software, were shown as mean and standard deviation (SD). ANOVA analysis followed by post hoc tests was used for the comparison.

Results

GS-Rb1 alleviates neurological deficit and brain edema

In the ipsilateral injury cortex, the brain water conent was significantly elevated 3 days after MCAO. Meanwhile, GS-Rb1 treatment at the doses of 5-20 mg/kg significantly reduced the content of brain water (Fig. 1A).

Furthermore, the scores of mNSS were evaluated to show the severity of neurological deficit. When compared to MCAO mice, the mice in the MCAO + GS-Rb1 groups had significantly lower mNSS scores (Fig. 1B).

GS-Rb1 alleviates cognitive impairment in the cerebral ischemia/reperfusion model

When compared to the mice in the MCAO + GS-Rb1 group, the escape latencies in the MCAO mice were significantly higher during the four trials. while the average swim speed was significantly lower (Fig. 2A, B). Similarly, the platform site crossing numbers and time in the target quadrant were significantly reduced by MCAO and elevated by the administration of GS-Rb1 in the probe test (Fig. 2C, D). It should be noted that GS-Rb1 treatment at the dose of 5 mg/kg had no obvious effects on the swimming speed and platform crossings in MCAO mice.

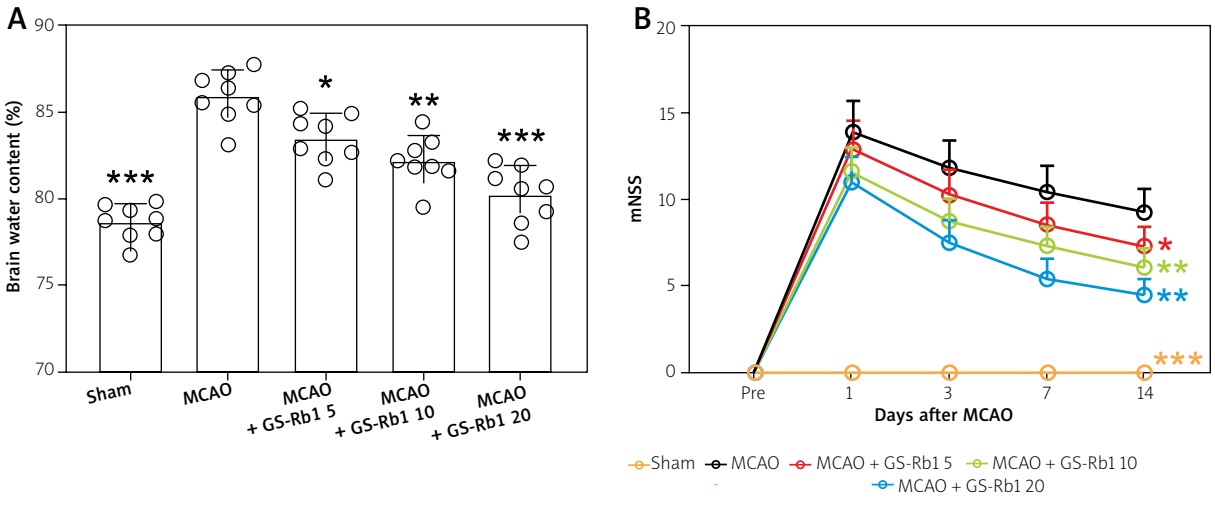

Fig. 1. Ginsenoside Rb1 (GS-Rb1) attenuated cerebral ischemia/reperfusion induced neurological deficit and brain edema in mice. Brain water content at ipsilateral injury cortex was compared 3 days after MCAO (**A**). Neurological deficit scores were measured before, 1, 3, 7 and 14 days after MCAO (**B**). $N = 8$ for each group. Mean ±SD, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to MCAO group. One-way ANOVA followed Dunn's multiple comparisons test and two-way ANOVA followed Tukey's multiple comparisons test.

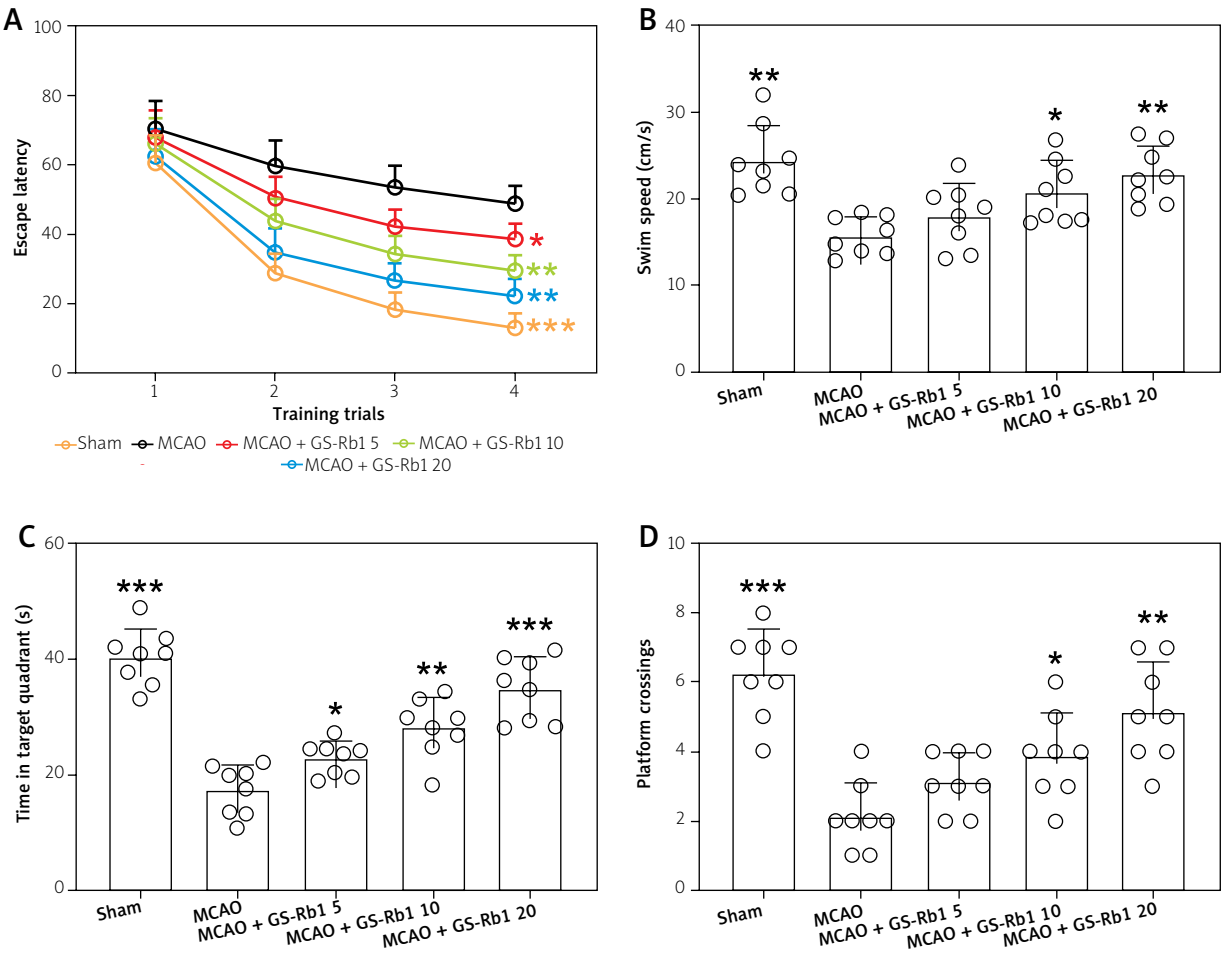

Fig. 2. Ginsenoside Rb1 (GS-Rb1) protected cerebral ischemia/reperfusion induced cognitive impairments in mice. In 4 trials of the Morris water maze test, the mice's escape latencies (**A**) and the average swim speed (**B**) were measured. In the probe trial, the time in the target quadrant in 60 s (**C**) and the number of platform site crossings (**D**) were recorded. $N = 8$ for each group. Mean ±SD, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to MCAO group. One-way ANOVA followed Dunn's multiple comparisons test and two-way ANOVA followed Tukey's multiple comparisons test.

GS-Rb1 alleviates anxiety-like behaviors in the cerebral ischemia/reperfusion model

As shown in Figure 3A, B, the locomotion counts within 10 min were significantly higher in the MCAO mice, while the duration in the center was remarkably lower compared to the MCAO + GS-Rb1 group. Considering that 20 mg/kg GS-Rb1 exhibited the most profound protective effects, this dose of GS-Rb1 was used in the following experiments.

GS-Rb1 alleviates the activation of microglia in the cerebral ischemia/reperfusion model

Based on the results of immunofluorescence, the Iba-1-positive cell ratio in the ischemic hippocampus was significantly elevated by MCAO and decreased by the administration of 20 mg/kg GS-Rb1 (Fig. 4A). As demonstrated in Figure 4B, C, it was found that Iba-1 protein expression was also significantly increased in MCAO mice, and decreased by the administration of GS-Rb1 (20 mg/kg).

GS-Rb1 modulates the polarization of microglia

Interleukin 6, IL-1β, and TNF-α in the ipsilateral injury hemispheres were significantly increased in MCAO mice, and decreased by the administration of 20 mg/kg GS-Rb1 (Fig. 5A-C). However, the protein level of IL-10 was not influenced by MCAO but was significantly increased by GS-Rb1 (Fig. 5D).

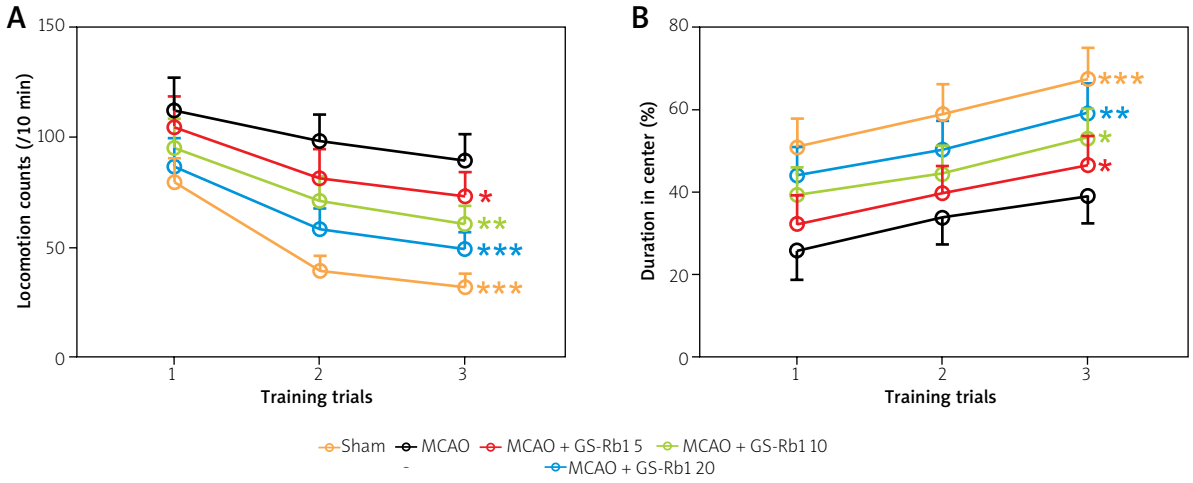

Fig. 3. Ginsenoside Rb1 (GS-Rb1) protected cerebral ischemia/reperfusion induced anxiety-like behaviors in mice. An open field test with 3 trials of training was carried out to test anxiety. The total number of circuit breaks was counted as a locomotive behavior and locomotion counts are shown in (**A**). The time spent in the central area is shown in (**B**). *N* = 8 for each group. Mean ±SD, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 compared to MCAO group. Two-way ANOVA followed Tukey's multiple comparisons test.

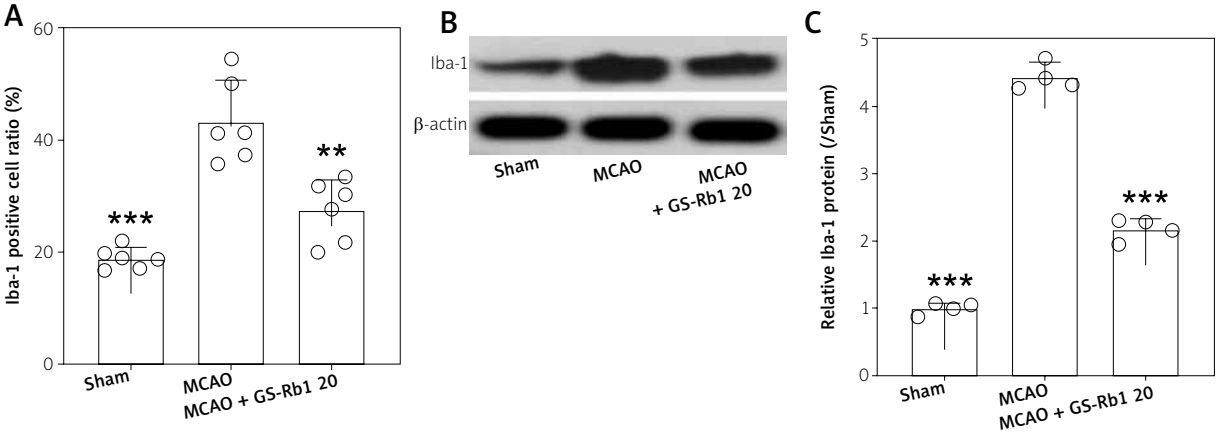

Fig. 4. Ginsenoside Rb1 (GS-Rb1) attenuated cerebral ischemia/reperfusion induced microglial activation in the ischemic hippocampus of mice. **A**) Quantification of Iba1-positive cell ratios in the ischemic hippocampus between each group. Western blotting was used to analyze the proteins levels of Iba-1 in the ipsilateral injury hemispheres at 7 days after injury (**B**) and the expression levels were normalized to the Sham group (**C**). 6 mice in each group were used for immunofluorescence staining. 8 mice in each group were used for Western blotting. The tissues were homogenized for 4 repeated experiments. Mean ±SD, ***p* < 0.01 and ****p* < 0.001 compared to MCAO group. One-way ANOVA followed Dunn's multiple comparisons test.

As shown in Figure 5E, F, CD86 and iNOS mRNA levels were obviously increased in the ipsilateral injury hemispheres of MCAO mice, and decreased by the administration of GS-Rb1. Arg-1, CD206, and IL-10 mRNA levels were not influenced by MCAO but significantly increased by GS-Rb1 (Fig. 5G-I).

Discussion

This research demonstrated that GS-Rb1 showed neuro-protective effects in the mouse model of cerebral ischemia/reperfusion. Mechanically, GS-Rb1 was found to promote M2-type polarization while inhibiting microglia M1-type polarization. Our findings highlight the potential of GS-Rb1 against the impairments caused by ischemic stroke.

Ischemic strokes may result in ischemia-reperfusion injury, which accounts for most of the impairments [26]. Ischemia-reperfusion injury initiates from blood supply restriction and is followed by perfusion restoration and reoxygenation [6,22]. Ischemia-reperfusion injury

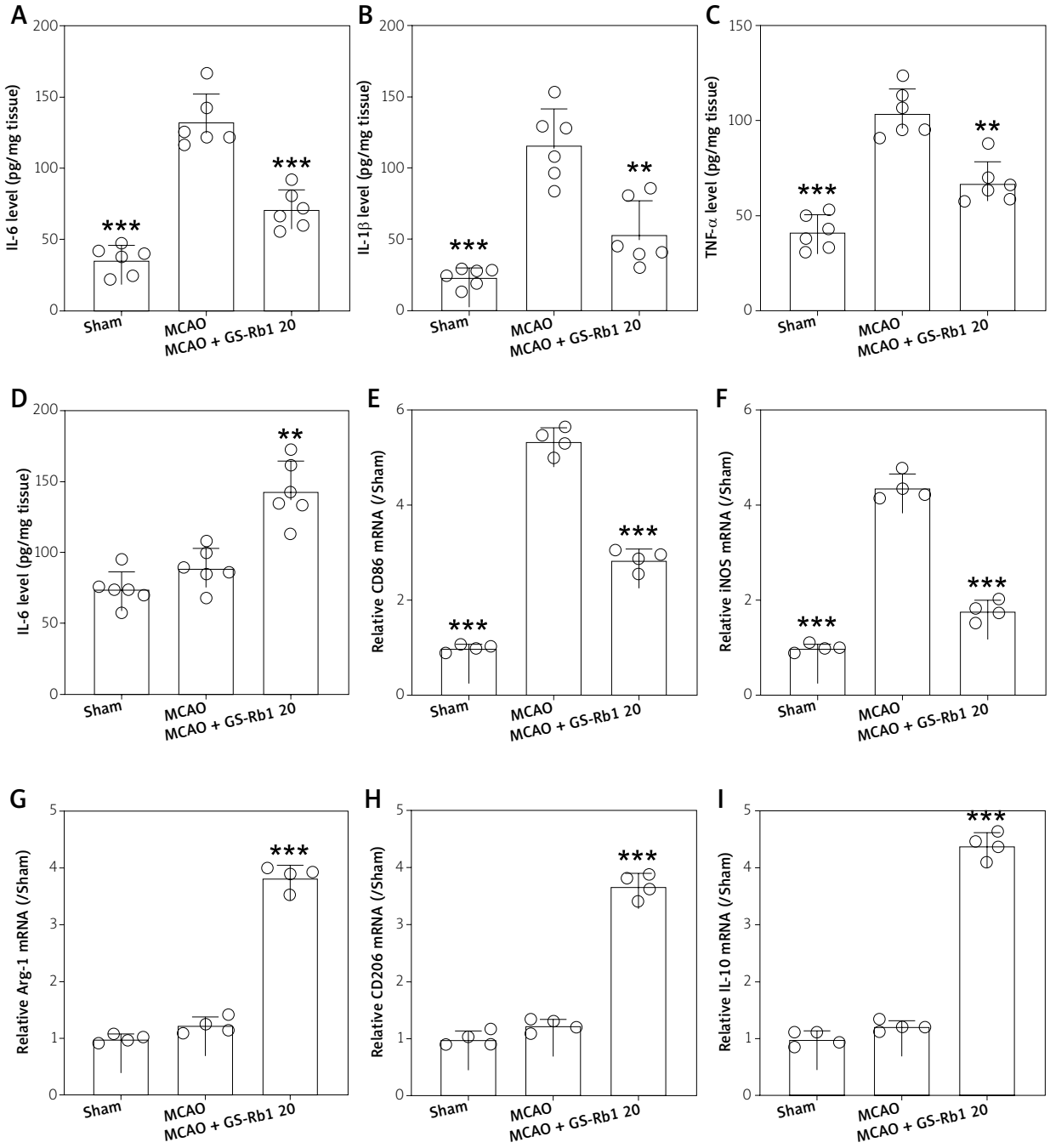

Fig. 5. Ginsenoside Rb1 (GS-Rb1) inhibited M1-type polarization but promoted M2-type polarization of microglia after cerebral ischemia/reperfusion injury in mice. Protein levels of IL-6 (**A**), IL-1β (**B**), TNF-α (**C**) and IL-10 (**D**) in the ipsilateral injury hemispheres were measured by ELISA at 7 days after injury. qRT-PCR was used to measure the mRNA levels of CD86 (**E**), iNOS (**F**), Arg-1 (**G**), CD206 (**H**) and IL-10 (**I**) in the ipsilateral injury hemispheres were measured by ELISA at 7 days after injury. 6 mice in each group were used for ELISA. 8 mice in each group were used for qRT-PCR. The tissues were homogenized for 4 repeated experiments. Mean ±SD, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to MCAO group. One-way ANOVA followed Dunn's multiple comparisons test.

leads to inflammatory stress, impaired energy metabolism, cytokine damage, oxidative stress, and apoptosis [5,23]. Considerable efforts have been made to explore therapeutic strategies for ischemic stroke to reduce stroke mortality [4].

Six GS, namely GS-Rb1, GS-Rh2, GS-Rg3, and GS-Rg5 as protopanaxadiol, and GS-Rg1 and GS-Re as protopanaxatriol, have been reported to exhibit beneficial effects to improve cerebral ischemia-reperfusion injury [28]. Among them, GS-Rg3 and GS-Rb1 show the strongest therapeutic activity [15]. For example, in a study using a rat MCAO model, GS-Rb1 treatment greatly reduced the infarct volume and promoted neurological recovery in MCAO rats [3].

In this research, mNSS scores were measured to show the neurological damage. After different concentrations of GS-Rb1 administration, GS-Rb1 was found to be effective in improving neurological dysfunction with dose efficiency. On the third day after MCAO, edema of the damaged ipsilateral cerebral cortex was detected. GS-Rb1 was found to improve the brain edema caused by ischemia-reperfusion.

The water maze experiment was performed at 15 days after cerebral ischemia-reperfusion injury, through which different concentrations of GS-Rb1 administration were found to improve the cognitive impairment caused by cerebral ischemia-reperfusion with a dose effect.

The anxiety of mice after ischemia-reperfusion injury was evaluated by the open field test at 8 days after MCAO. We found that GS-Rb1 could improve the anxiety of mice caused by ischemia-reperfusion with a dose-dependent effect.

Microglia are macrophages in the brain which are derived from erythromyeloid progenitor cells [9]. Microglia participate in synaptic formation, neuronal differentiation and proliferation, and neuro-inflammation [20]. Evidence has indicated that there are two states of microglia polarization, M1-type and M2-type [7]. M1-type microglia promote neuro-inflammation through secreting iNOS, TNF-α, and IL-6. M2-type microglia mainly reduce neuro-inflammation [14]. It has been demonstrated that the reduction of M1-type microglia protects the hemorrhagic brain [25].

As a pan-microglial cell marker [24], the expression of Iba-1 is associated with microglia activation and inflammation [11]. Thus, we also analyzed the influence of GS-Rb1 on the activation of microglia through Iba-1. We first performed Iba-1 staining of hippocampal tissue on the ischemic side by immunohistochemistry and analyzed the ratio of Iba-1-positive cells. The results showed that GS-Rb1 could inhibit microglia activation.

To explore GS-Rb1 function in microglia polarization in the cerebral ischemia/reperfusion mouse model, we explored the expression of relative polarization markers. M1 associated factors included major histocompatibility complex-II (MHC-II), CD86, CD40, NADPH oxidase, iNOS, and pro-inflammatory cytokines [18]. M2 associated factors included mannose receptor (MR), CD206, Arg-1, and anti-inflammatory factors [18]. Based on the detection of factors related to M1/M2 polarization, GS-Rb1 could inhibit M1 polarization and enhance M2 polarization. The molecular mechanism of GS-Rb1 in the modulation of M1/M2 polarization was not explored in this research, but should be investigated in the future.

Conclusions

In conclusion, GS-Rb1 showed neuro-protective effects in the cerebral ischemia/reperfusion mouse model, which may be due to the regulation of microglia polarization.

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Disclosures

The study has been approved by Bioethics Committee of the People's Hospital of Shanghai Pudong New Area (No. prylz2020-058).

The authors report no conflict of interest.

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