Original Article
Icariside II affects hippocampal neuron axon regeneration and improves learning and memory in a chronic cerebral hypoperfusion rat model

Tao Liu[2*], Fang He[1,5*], Jiahong Yan[3], Wei Kuang[4], Changyin Yu[4]

[1]Key Laboratory of Cell Engineering of Guizhou Province, Departments of [2]Cerebrovascular Disease, [3]Pediatric Medicine, [4]Neurology, The Affiliated Hospital of Zunyi Medical College, Zunyi 563003, Guizhou, China; [5]Department of Oral and Maxillofacial Surgery, University Hospital of Tübingen, 72076 Tübingen, Germany. *Co-first authors.

Abstract: Chronic cerebral hypoperfusion (CCH) is a basic pathological process that is comorbid with brain diseases, such as vascular Parkinsonism and Alzheimer's disease. Icariside II (ICS II), which is one of the main metabolites of icariin, has anti-inflammatory and antioxidant effects and protects against ischemic brain injury. This study aims to investigate the neuroprotective effects of ICS II on neuronal axon regeneration-related factors in a CCH rat model. Sprague-Dawley (SD) rats were divided into the following four groups: sham group, model group and 4 and 8 mg/kg/day ICS II administration groups. Learning and spatial memory functions were tested using a Morris water maze. Pathological changes were observed in the rat hippocampal tissue by hematoxylin and eosin (H&E) staining. Neuronal axon regeneration-related proteins (GAP-43, MAP-2 and Nogo-A) were observed by immunohistochemical staining and detected by the average optical density method. The results showed that 8 mg/kg/day of ICS II can effectively reduce the escape latency and prolong the target quadrant residence time at 12 weeks and that ICS II can improve the histopathological changes in the CA1 area of the rat hippocampus. Moreover, ICS II administration at 8 mg/kg/day significantly increased GAP-43 and MAP-2 expression and reduced Nogo-A expression in the CA1 area of the rat hippocampus at 12 weeks; however, significant differences were not observed at 4 and 8 weeks. Hence, ICS II at a dosage of 8 mg/kg/day could promote learning and memory abilities and improve histopathological changes in the rat hippocampus in a CCH rat model. These results may be related to the promotion of neuronal axon regeneration in the CA1 area of the hippocampus under increases in hippocampal GAP-43 and MAP-2 protein expression and decreased Nogo-A protein expression.

Keywords: Icariside II, axon regeneration, chronic cerebral hypoperfusion, GAP-43, MAP-2, Nogo-A

Introduction

Chronic cerebral hypoperfusion (CCH) is a common consequence of arthrosclerosis and a primary cause of vascular dementia [1, 2]. Alzheimer's disease, Binswanger disease, and other neurodegenerative diseases could worsen under the condition of CCH [3, 4]. Therefore, treatment for patients with CCH is important. However, compared with the obvious symptoms of acute stroke, the symptoms of the early stage of CCH are difficult to detect but become ingravescent [5]. Recently, researchers have realized the serious consequences of chronic and progressive hypoperfusion and have become interested in therapies for CHH.

CCH can cause a series of pathophysiological changes, such as neuroregeneration reduction, glial cell proliferation, cholinergic receptor loss, and neurotransmitter abnormalities, which eventually lead to impaired neurotransmission and cognitive impairment [6]. Interestingly, the remaining neuronal cytoplasm can produce new protuberances using budding from the side branches, reestablish synaptic connections, and promote the recovery of nerve function [7]. In recent years, studies have shown that stem cell regenerative medicine is important for the treatment of many diseases [8, 9]. Thus, treatment strategies for promoting or inhibiting axon regeneration factors may provide breakthrough

improvements in the cognitive dysfunction caused by CCH.

Icariin and icariside II (ICS II) are the two effective active flavonoids in Herba Epimedii. Of these flavonoids, icariin, which is the most commonly studied, is the main component of Herba Epimedii, and it has been found to be efficacious in preventing osteoporosis, cancer depression, and neurological dysfunctions [10]. When taken orally, intestinal bacteria can decompose icariin, resulting in the production of a series of metabolites. Many studies have found that ICS II is the key metabolite of icariin. Furthermore, ICS II possesses anti-inflammatory and antioxidant activities and prevents ischemic brain injury [11]. The neuroprotective properties of ICS II are attracting increasing interest in the search for promising agents to treat neurodegenerative diseases. Therefore, this study aimed to explore the influence of ICS II on axon regeneration by testing rat learning and memory abilities and axon regeneration by related molecules in a CCH rat model to provide a basis for the treatment of CCH-induced cognitive dysfunction.

Materials and methods

Reagents

ICS II (purity ≥ 98%) was obtained from Zelang Medical Technology (Nanjing, China), and it was dissolved in double distilled water and ultrasonicated for 8 min.

Animals

Specific pathogen-free (SPF) adult male Sprague-Dawley (SD) rats (260-300 g) were purchased from the Experimental Animal Center of Daping Hospital (license no. SCXK 2012-0005) and raised in a SPF-grade environment (certificate no. SYXK 2011-003). Animal experiments were executed according to the Animal Management Rules of the State Committee of Science and Technology of China (no. 2 on November 14, 1988, revised in 2011) and approved by the Animal Experimental Ethics Committee of Zunyi Medical University (no. 201612A001).

Experimental design

Sixty SD rats were randomly divided into the following four groups according to a random num-

ber table: sham, model, ICS II 4 mg/kg and ICS II 8 mg/kg. The CCH model was generated as described in the literature [12]. Briefly, a rat was fixed on an experimental table and anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and then the neck area was shaved and disinfected. The spontaneous breathing of the rat was monitored. The skin at the center of the neck was cut, and the bilateral common carotid artery was separated. The common carotid artery was ligated with a medical suture. The ICS II 4 mg/kg group and the ICS II 8 mg/kg group received intragastric administration of 4 and 8 mg/kg ICS II (dissolved in double distilled water) every day. The model group received the same volume of double distilled water intragastrically. The sham group was not treated. The Morris water maze test, tissue preparation and immunohistochemistry analyses were performed at 4, 8, and 12 weeks after intragastric administration.

Morris water maze test

The spatial memory of the rats was assessed using the Morris water maze (MWM). The water maze test equipment consists of a platform, black circular pool (60 cm in radius and 30 cm deep) and a camera. The black circular pool was divided into four quadrants labeled I, II, III, and VI with four start positions. The circular platform was hidden in quadrant III and submerged 10 cm below the water. At the beginning of the test, the rats were placed on the platform, where they remained for 20 s. The rats were allowed to swim in the water until they found the hidden platform (maximum swim time of 120 s). The swimming paths and the escape latencies of the rats were recorded by a video camera. If a rat could not find the platform within 120 s, the training session ended and the rat was guided to the hidden platform, where it remained for 20 s before the next trial. Each rat received two trials per day, with an interval between the trials of 2 min. The time taken to find the platform (escape latency) was averaged over the two trials. After the last learning trial on day 5, the rats were placed into the pool and swam freely for 60 s. The percentage of the time in the target quadrant was recorded as an assessment of spatial memory. The escape latencies and target quadrant time were recorded at 4, 8, and 12 weeks.
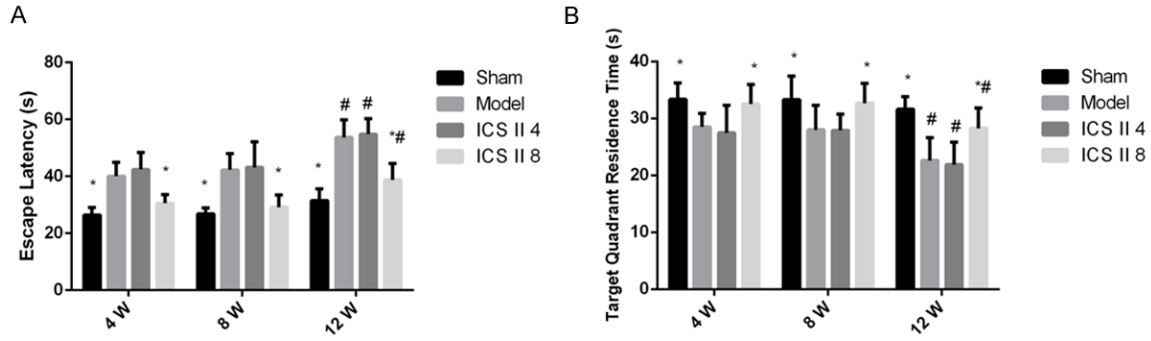
Figure 1. Effect of icariside II on learning and memory abilities in CCH rats. A. Escape latency times at 4, 8 and 12 weeks. B. Target quadrant residence time at 4, 8 and 12 weeks (mean ± SD, n = 5, *$P < 0.05$ vs sham group, #$P < 0.05$ vs 4 weeks).
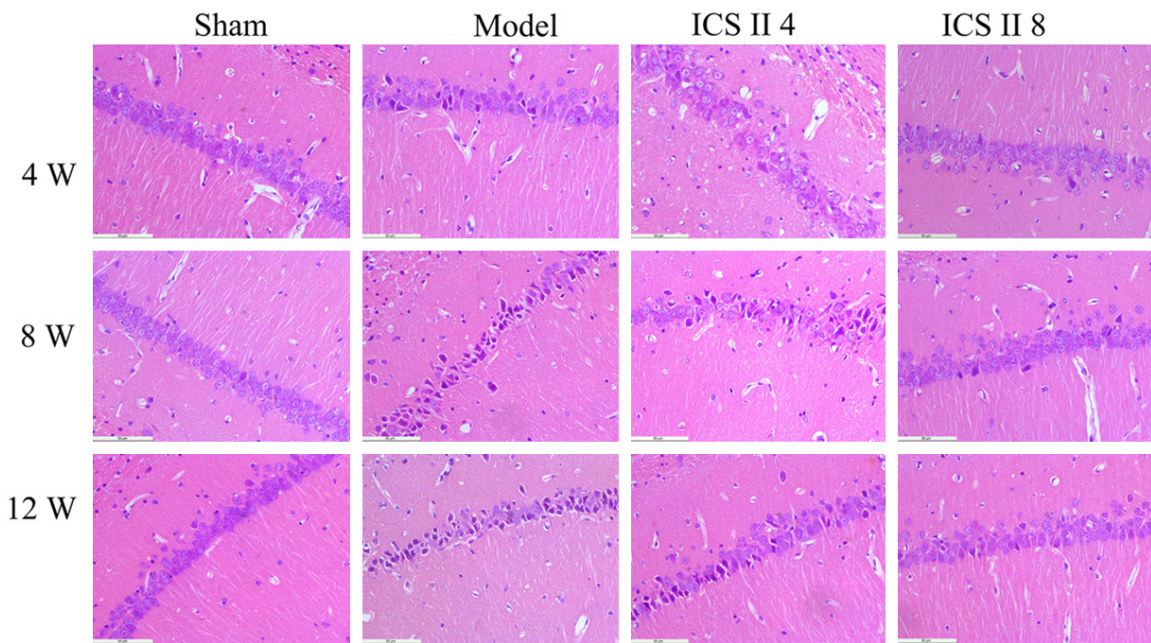

Figure 2. Effect of icariside II on histopathologic changes in the rat hippocampal CA1 region. Representative sections of HE staining (magnification 400×).

Tissue preparation

After the MWM tests, the animals were deeply anesthetized using an overdose of sodium pentobarbital. Five of the rats from each group were perfused with 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) at 4, 8, and 12 weeks. The brains were removed, fixed in 4% paraformaldehyde for 48 h, subjected to graded alcohol dehydration, embedded in paraffin, and then cut into 5-μm thick sections for hematoxylin-eosin (H&E) staining and immunohistochemistry.

Immunohistochemical staining

Immunohistochemistry was performed according to the avidin-biotin-peroxidase complex method using polyclonal anti-GAP43, MAP-2 and Nogo-A antibodies. Briefly, the brain sections were treated with 3% H_2O_2 to block endogenous peroxidase and then incubated in blocking buffer. After incubation with the anti-GAP43 (1:2000, Abcam, USA), MAP-2 (1:200, Abcam, USA) or Nogo-A (1:200, Wuhan Boster Biotechnology, China) primary antibodies from Abcam at 4°C for 24 h, the sections were treat-
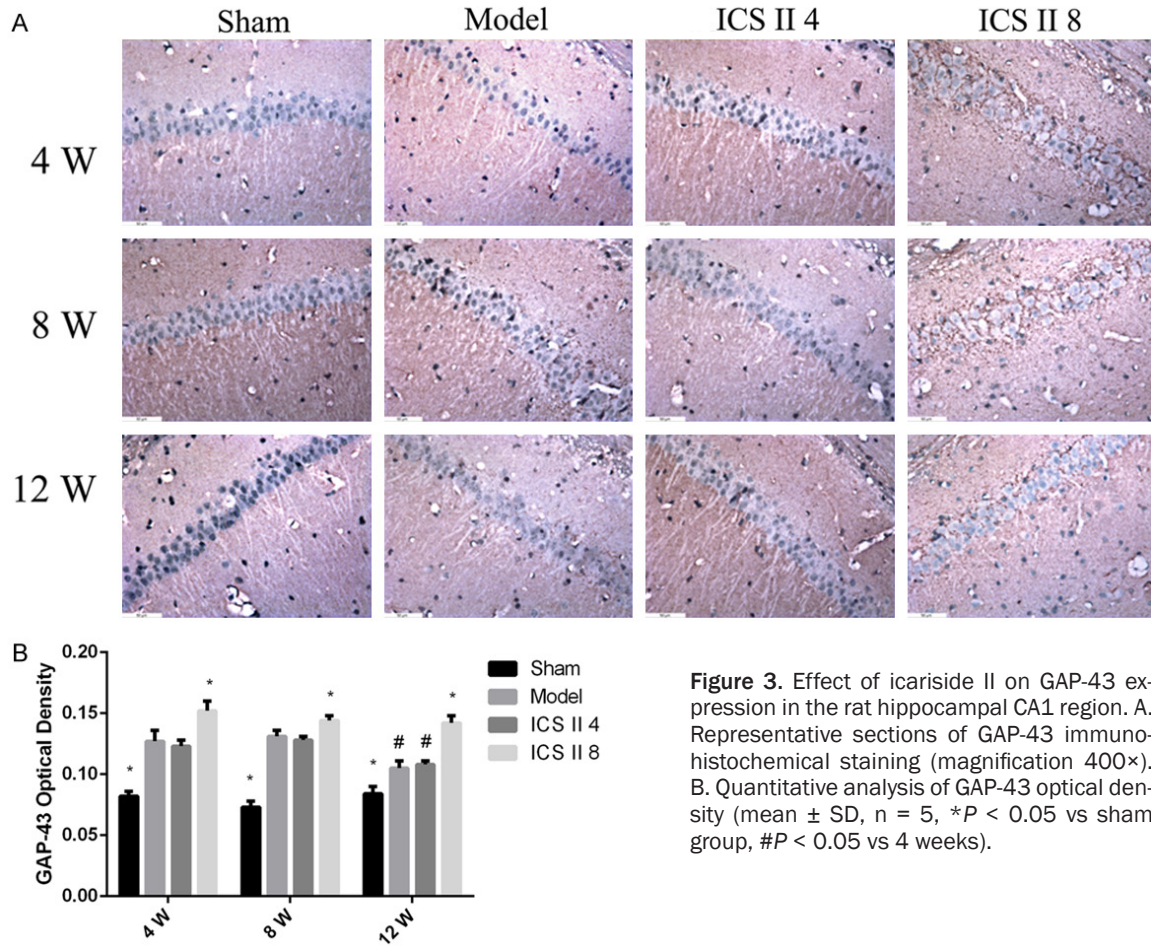
Figure 3. Effect of icariside II on GAP-43 expression in the rat hippocampal CA1 region. A. Representative sections of GAP-43 immunohistochemical staining (magnification 400×). B. Quantitative analysis of GAP-43 optical density (mean ± SD, n = 5, *P < 0.05 vs sham group, #P < 0.05 vs 4 weeks).

ed with biotinylated secondary antibody and reagents from the ABC kit (Shanghai Gene Technology Inc., Shanghai, China). Immunoreactivity was detected using 3,3'-diaminobenzidine. The Image Pro Plus analysis system was used to quantify the immunohistochemical staining. The hippocampal CA1 area of interest was delineated at 400× magnification under an Olympus microscope after the background subtraction and grayscale threshold determination. The area covered by the antibody was computed as the percentage of the total area delineated.

Statistical analysis

The data were analyzed with SPSS 17.0 software and expressed as the mean ± SE. Group differences in escape latency and target quadrant residence time during the MWM test were analyzed using ANOVA for repeated measurement. Other data collected in this study were analyzed using multifactor ANOVA. The Bonferroni method was used for pairwise compari-

son of mean values at each time point within the group. P < 0.05 represented statistically significant differences.

Results

ICS II significantly improved learning and memory impairments induced by CCH

Learning and memory retention were demonstrated in the MWM and used to evaluate spatial memory in rats. As shown in **Figure 1A** and **1B**, compared to the sham group, the model group had a significantly prolonged escape latency (time to find the hidden platform) (P < 0.05) and a significantly decreased target quadrant residence time (P < 0.05) at 4, 8 and 12 weeks. No significant differences were found between the model group and ICS II 4 mg/kg group in escape latency at any time point (P > 0.05). The escape latency was significantly reduced in the ICS II 8 mg/kg group compared with that in the model group (P < 0.05).
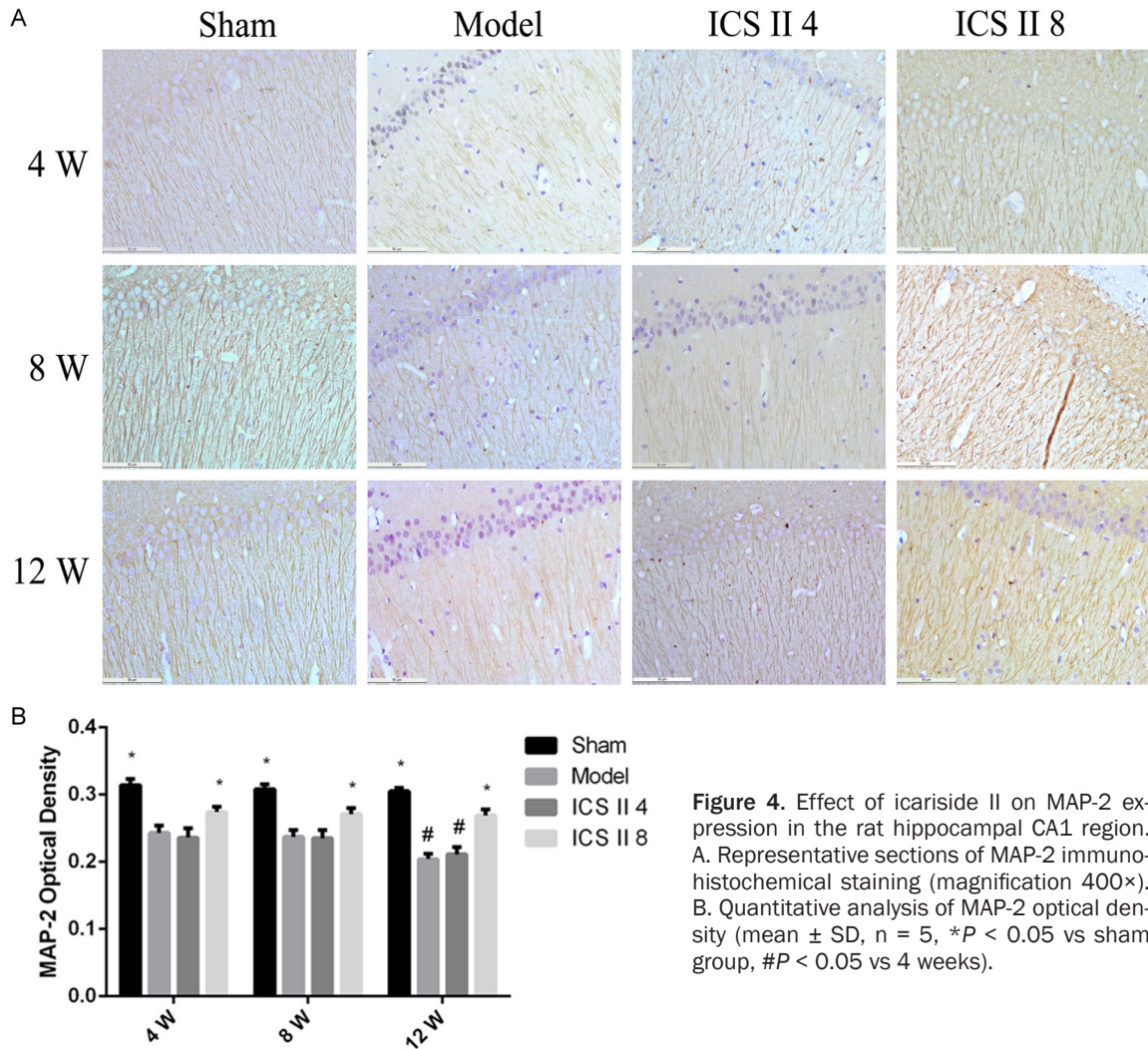
A

| | Sham | Model | ICS II 4 | ICS II 8 |

Figure 4. Effect of icariside II on MAP-2 expression in the rat hippocampal CA1 region. A. Representative sections of MAP-2 immunohistochemical staining (magnification 400×). B. Quantitative analysis of MAP-2 optical density (mean ± SD, n = 5, *$P < 0.05$ vs sham group, #$P < 0.05$ vs 4 weeks).

No significant differences were found in escape latency or target quadrant time within the same group between 4 weeks and 8 weeks ($P > 0.05$). Compared with the times at 4 and 8 weeks, at 12 weeks, the escape latency was prolonged and the target quadrant time was reduced in the model group, ICS II 4 mg/kg group and ICS II 8 mg/kg group ($P < 0.05$).

ICS II protected against neuronal damage of the CA1 area in the hippocampus induced by CCH

A representative H&E organizational structure diagram of the CA1 area in the six groups is shown in **Figure 2**. The neurons in the CA1 area in the hippocampus were closely and regularly packed and showed a clear structure and form in the sham group at all time points. Extensive neuronal changes, such as neuronal cell loss, degeneration, dark staining and structures with loosely arranged vacuoles, were visualized in the CA1 area of the hippocampus in the model group. Administration of ICS II 8 mg/kg reduced the pathologic changes noted above in the CA1 area of the hippocampus of rats.

ICS II promoted the expression of axon-regeneration-related molecules

Representative images of GAP-43 and MAP-2 immunohistochemical staining of the CA1 area in the four groups are shown in **Figures 3A** and **4A**. GAP-43 was faintly expressed at the cytomembrane in the hippocampal CA1 region in the sham group. MAP-2 was normally expressed in the cytoplasm and neurites in the sham group. Compared with the sham group, the
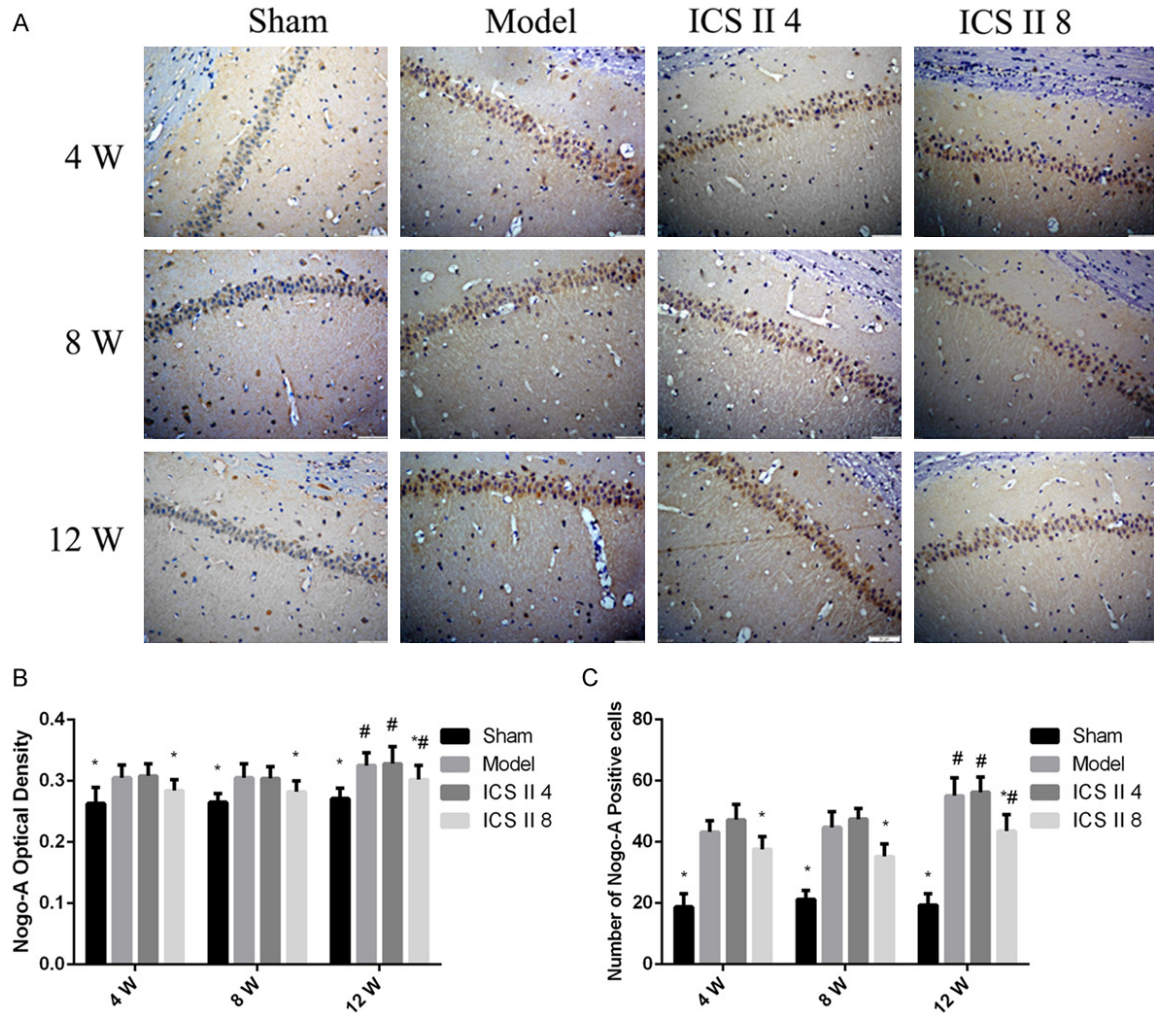
Figure 5. Effect of icariside II on Nogo-A expression in the rat hippocampal CA1 region. A. Representative sections of Nogo-A immunohistochemical staining (magnification 400×). B. Quantitative analysis of Nogo-A optical density. C. Number of Nogo-A-positive cells (mean ± SD, n = 5, *$P < 0.05$ vs sham group, #$P < 0.05$ vs 4 weeks).

model group had clearly increased GAP-43 expression and decreased MAP-2 expression at all time points ($P < 0.05$). No significant difference was found between the model group and the ICS II 4 mg/kg group ($P > 0.05$). Compared with the model group, the ICS II 8 mg/kg and 4 mg/kg groups had increased levels of GAP-43 and MAP-2 at all time points ($P < 0.05$). No significant differences were found in the levels of GAP-43 and MAP-2 within the same group between 4 weeks and 8 weeks. The levels of GAP-43 and MAP-2 were significantly reduced in the model and ICS II 8 mg/kg groups at 12 weeks compared with the levels at 4 weeks ($P < 0.05$), whereas no significant differences were observed in the ICS II 4 mg/kg group (**Figures 3B** and **4B**).

Representative Nogo-A immunohistochemical figures of the CA1 area in the six groups are shown in **Figure 5A**. Nogo-A was expressed at the proximal end of endochylema and in neurites and formed ovals or spindles. Part of the neurites in the normal and sham groups expressed Nogo-A. Compared with those in the normal and sham groups at the same time point, the number of cells expressing Nogo-A and the mean optical density of Nogo-A were significantly increased in the model group ($P < 0.05$) but not in the ICS II 4 mg/kg group ($P > 0.05$). The number of cells expressing Nogo-A and the mean optical density of Nogo-A were significantly increased in the 4 and 8 mg/kg groups compared with those in the model group at the same time point ($P < 0.05$). No significant

difference was found in the expression of Nogo-A in any group between 4 weeks and 8 weeks. Nogo-A was significantly increased in the model, ICS II 4 mg/kg and ICS II 8 mg/kg groups at 12 weeks compared with the levels at 4 and 8 weeks (P < 0.05) (**Figure 5B** and **5C**).

Discussion

CCH plays a key role in the occurrence and development of cognitive dysfunction, and it is an important cause of brain neuron degeneration [13]. Thus, developing a suitable animal model is particularly important for clinical research and new drug development for CCH. In this study, a CCH rat model was generated by permanent ligation of the bilateral common carotid arteries. The cerebral blood flow is sustainably reduced for 4 to 12 weeks, which reproduces similar pathological physiological changes that occur during human CCH [14]. In this experiment, clear spatial learning and memory function injuries appeared at 4, 8, and 12 weeks after ligation of the bilateral common carotid artery in rats, which replicated the clinical manifestations of CCH-induced cognitive dysfunction.

Icariin can improve spatial learning and memory in CCH model rats, and this improvement is related to the upregulation of the expression of PPAR-alpha, PGC-1-alpha, and insulin-degrading enzymes and a disintegrin and metalloproteinase domain 10 protein; the downregulation of the expression of amyloid precursor protein and beta-secretase 1; or the antioxidant effects on the circulatory and cholinergic systems [15-17]. In addition, icariin and ICS II are flavonoid constituents with similar structures, and pharmacokinetic studies have reported that approximately 91.2% of icariin is metabolized to ICS II after oral administration. In addition, ICS II may be absorbed faster and metabolized slower than icariin *in vivo* [18, 19]. A recent study showed that ICS II ameliorates cognitive impairment and neuronal loss in ibotenic acid-induced rats by a mechanism related to the regulation of calbindin expression and the inhibition of the apoptotic response [20]. In this study, the model group exhibited a clear spatial learning memory disorder characterized by extended escape latency periods and decreased target quadrant residence times. The histopathological results showed that the disordered arrange-

ment of hippocampal neurons and the degeneration and edema of neurons occurred in the model group. However, improved learning and memory abilities and reduced pathological damage of hippocampal neurons were observed in the ICS II 8 mg/kg group. Therefore, we concluded that ICS II at a dose of 8 mg/kg may play a functional role in cognitive competence in CCH model rats.

GAP-43 is a general early marker of postmitotic neurons, and it is expressed at high levels during axonal growth and synapse formation. Moreover, it accumulates in axonal growth cones and presynaptic nerve terminals, where it associates with the cortical cytoskeleton [21]. GAP-43 overexpression is sufficient to induce neurite formation and axonal sprouting in different regions of the adult CNS [22]. MAP-2 is the main element in microtubules, and it is closely related to the extension of axons and sensitive to ischemic anoxic injury [23]. The dynamic changes in MAP-2 can indirectly reveal the level of recovery from axonal injury. Nogo-A is a potent inhibitor of neurite growth and a monoclonal antibody antigen produced by oligodendrocytes [24]. In this study, compared with the sham group, the model group in the early stage (4 weeks) after permanent ligation of the bilateral common carotid arteries presented increased protein levels of Nogo-A and GAP-43 and decreased protein levels of MAP-2 in the hippocampus, which is indicative of the nerve axonal injury that occurred in the CCH rats, which led a reduced ability to self-repair in the early stage. The immunohistochemical results at 12 weeks showed that the increased GAP-43 levels fell and that the decreased MAP-2 levels rose in the model and ICS II 4 mg/kg groups over a longer period of time. These results show that axonal injuries increased over time, which is consistent with damage to the learning and memory ability demonstrated in the water maze experiment. In addition, CCH is an important cause of cell senescence and apoptosis, which leads to neurologic dysfunction in the hippocampus [25]. After 12 weeks, the hypoperfusion injury gradually accumulated and the number of aging cells in the hippocampal region increased, which may be the reason for the changes that led to decreased GAP-43 and MAP-2 or increased Nogo-A expression in the sham and ICS II 4 mg/kg groups at 12 weeks. After the ICS II 8 mg/kg treatment, the

GAP-43 and MAP-2 protein levels were increased, the Nogo-A protein levels were decreased, and the learning and memory abilities of CCH rats were improved. This finding suggests that adequate doses of ICS II can promote axonal regeneration and repair and cell axon skeleton formation by adjusting GAP-43 expression and simultaneously reducing the overexpression of Nogo-A protein, which lowers the level of resistance to axon regeneration. In summary, ICS II may promote axon regeneration by regulating GAP-43, MAP-2 and Nogo-A protein expression levels and improve cognitive ability in CCH rats. However, the specific signaling pathways that regulate axon regeneration require further verification. In addition, whether changes in other factors, such as the mitochondrial function of neurons [26] and the release level of neurotransmitters [27], jointly participate in the regulation of CCH cognitive impairment is also worth further exploration.

Conclusions

ICSII at a dosage of 8 mg/kg/d can promote learning and memory abilities and improve the histopathologic changes in the rat hippocampus in a CCH rat model. These results may be related to increased hippocampal GAP-43 and MAP-2 protein expression and decreased Nogo-A protein expression in the neurons in the hippocampal CA1 area. ICSII may modulate axon regeneration factors to exert neuroprotective effects in a rat CCH model, and these findings lay a theoretical foundation for the use of ICSII for the treatment of vascular Parkinsonism and Alzheimer's disease.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Changyin Yu, Department of Neurology, The Affiliated Hospital of Zunyi Medical College, Zunyi 563003, Guizhou, China. E-mail: ycynerve@163.com

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