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Effects of astaxanthin on axonal regeneration *via* cAMP/PKA signaling pathway in mice with focal cerebral infarction

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Abstract. – OBJECTIVE: To investigate the effect of astaxanthin on the neurological function of the middle cerebral artery occlusion (MCAO) mice and its possible mechanism.

MATERIALS AND METHODS: The male C57BL/6 mice were selected to establish the model of MCAO *via* electrocoagulation, and they were randomly divided into 4 groups: the sham operation group (Sham group), the cerebral ischemia model group (MCAO group), the astaxanthin intervention group (gavage with 30 mg/kg astaxanthin for 28 days, twice a day; Ast group), and astaxanthin + H89 group (Ast + H89 group). At 3, 7, 14, and 28 d after the operation, the Rotarod test and the balance beam footstep error test were performed. The brain tissues were taken for immunofluorescence to observe the expression of the growth-associated protein 43 (GAP43) in the cortex around the infarction. The GAP43 protein and mRNA levels in the cortex around the infarction were detected *via* Western blotting, and the Reverse Transcription-Polymerase Chain Reaction (RT-PCR), the levels of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) in the bilateral cerebral cortex were detected *via* enzyme-linked immunosorbent assay (ELISA), and the PKAc and phosphorylated-cAMP-response element-binding protein (p-CREB) levels in the bilateral cerebral cortex were detected *via* Western blotting. Biotin dextran amine (BDA) was injected at 14 d after the operation, and the brain was taken at 28 d. The BDA-labeled neurons or axons were observed in the bilateral cortex *via* immunohistochemistry and immunofluorescence, and the colocalization of BDA and GAP43 in the cortex around the infarction was observed using double immunofluorescence staining.

RESULTS: Compared with those in the MCAO group, the mean residence time in the Rotarod test was significantly increased, and the times of the footstep error on the balance beam were significantly reduced in the Ast group. In the Ast group, the expression of GAP43 in the cortex around the infarction, the GAP43 protein, and the mRNA levels were all significantly elevated. Immunofluorescence showed that in the Ast group, the number of the labeled neurons and axons in the bilateral cortex was slightly larger than that in the other groups, and the number of labeled axonal fibers in the ischemic cortex was significantly increased. The colocalization area of BDA and GAP43 was observed, and it was found that the positive area in the Ast group was significantly larger than that in the MCAO group. The cAMP level was higher in the Ast group and Ast + H89 group at 7, 14, and 28 d after operation, while the PKA level was lower in the Ast + H89 group at 7 and 14 d after operation and higher in the Ast group at 7, 14, and 28 d after operation. The results of the Western blotting manifested that the PKAc and p-CREB levels were upregulated in the Ast group at 7, 14, and 28 d after the operation, and downregulated in the Ast + H89 group at 7, 14, and 28 d after the operation.

CONCLUSIONS: Astaxanthin activates the cAMP/PKA/CREB signaling pathway by increasing the cAMP concentration in brain tissues, ultimately promoting the axonal regeneration in the cerebral cortex and improving the motor function.

Key Words:
Astaxanthin, Cerebral Infarction, Axonal Regeneration, CAMP/PKA.

Introduction

Stroke is one of the most serious diseases threatening human health currently, whose fatality rate ranks first in all the diseases in China[1]. Ischemic stroke is characterized by high morbidity, mortality, disability, and recurrence rates[2].

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The health and quality of life of stroke patients survived, especially disabled patients, are seriously affected. Studies[3-5] have found that after ischemic stroke, the neurological function recovers to a certain degree, indicating that the brain tissues have certain self-repairing capability. It has been found in the animal experiments that the intervention measures, such as appropriate drugs or exercise after cerebral infarction can stimulate endogenous axonal regeneration in brain tissues, which can benefit the reconnection of neural network and compensate for neurological functions of some denervated regions[6].

There are various influencing factors for axonal regeneration, and many complex intracellular and extracellular signal transduction mechanisms are involved. Currently, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway is considered to be an important pathway affecting axonal regeneration[7,8]. The cAMP keeps the vigorous neuronal growth by activating the PKA-mediated signaling pathway and can relieve the damage of the neuronal growth inhibitory factor to axonal growth cone by affecting the molecular effect caused by the downstream gene transcription, thereby promoting the axonal regeneration[9,10].

Astaxanthin, widely distributed in nature, is the pigment of crustaceans, which possesses the broad-pharmacological activities, and its neuroprotective effect has attracted much attention of the researchers[11,12]. According to pharmacokinetic study, astaxanthin can be localized on the surface of the lipid membrane or passes through the lipid membrane, and it can also pass through the blood-brain barrier of rodents, thus achieving a better efficacy on the nervous system diseases. Some reports[13,14] have found that astaxanthin can significantly inhibit the expression of interleukin-1α (IL-1α), IL-6, and tumor necrosis factor-α (TNF-α) in the brain and improve the lipopolysaccharide-induced neuroinflammatory response in the brain of mice. Moreover, astaxanthin, through simulating the neurotrophic factors and promoting synaptic survival, can alleviate the cortical damage volume, neuronal loss, and neural degeneration[15,16]. However, there are few investigations on the effect of astaxanthin on cerebral infarction. In this study, C57BL/6 mice were used as an object of study, the focal middle cerebral artery occlusion (fMCAO) model was established *via* electrocoagulation, the restorative effect of astaxanthin on the neurological function of MCAO mice was observed, and its possible mechanism and signaling pathway were investigated, so as to provide new ideas for the mechanisms of cerebral infarction and nerve repair.

Materials and Methods

Laboratory Animals and Models

A total of 100 male C57BL/6 mice aged 8-12 weeks old weighing 25-30 g were purchased from the Shanghai SLAC Laboratory Animal Company (Shanghai, China). The mouse model of MCAO was established *via* electrocoagulation[17]: the mice were fasted for solids and liquids before the operation, and they were anesthetized *via* intraperitoneal injection of tribromoethanol (0.4 g/kg). After successful anesthesia, the mice were fixed in a supine position, a median incision was made on the neck, and the right common carotid artery (CCA) was exposed, separated, and permanently ligated. Then, the mice were fixed in a left lateral position, an incision was made along the line between external auditory canal and medial canthus, the skin was separated, the temporalis muscle was fixed on the right under a stereoscopic microscope, and the MCA was positioned under the skull. The skull was worn using the dental drill right above the MCA until the vessels were exposed, and the MCA was carefully burned using the single-pole electrocoagulator till coagulation. In the Sham operation group, the operations were the same as those in the operation group, but the CCA was not ligated, and the MCA was not coagulated. This study was approved by the Animal Ethics Committee of Tengzhou Central People's Hospital.

Animal Grouping and Drug Administration

The mice were randomly divided into 4 groups: the sham operation group (Sham group, n=20), cerebral ischemia model group (MCAO group, n=20), astaxanthin intervention group (Ast group, n=20), and astaxanthin + H89 group (Ast + H89 group, n=20). Astaxanthin was prepared with olive oil for postoperative gavage (30 mg/kg), twice a day for 28 d. H89, a PKA inhibitor, was dissolved in ultrapure water (4 μg/μL) and injected into the ventricle (2 μL) using the stereotaxic apparatus before modeling. Biotin dextran amine (BDA) was injected into the cortex at 14 d after the operation as follows: the mice were anesthe-

tized with 10% chloral hydrate and fixed on the stereotaxic apparatus in a prone position. Then, the anterior fontanel was exposed, and BDA was injected into the left motor-sensory cortex in two points (1 μL/point).

Rotarod Test

The Rotarod test was performed at 3, 7, 14, and 28 d after the operation. The mice were placed on the rotarod rotating at 4 rpm in a quiet environment, the speed was gradually increased from 4 rpm to 40 rpm, and the total test time was not more than 300 s. The test was terminated when the mice fell off the rotarod. The test was repeated for 3 times, and an adaptive training was given for mice at 1 d before modeling.

Balance Beam Footstep Error Test

The test was performed at 3, 7, 14, and 28 d after the operation. The mice were placed on a balance beam (L×W×H: 120 cm × 0.6 cm × 60 cm) with a platform set at one end. The mice walked through the balance beam, and they usually turned 180°C and continued to walk to the other end once reaching the end of the balance beam. If the left hind limb or forelimb slid down from the balance beam, the footstep error was recorded once. The total times of footstep errors within 50 steps were recorded. The test was repeated 3 times, and an adaptive training was needed for mice before modeling.

Immunofluorescence Staining

At 7, 14, and 28 d after the operation, the mice were anesthetized with chloral hydrate and infused with 4% paraformaldehyde into the heart. The brain was taken, and the brain tissues were stored in 4% paraformaldehyde solution at 4°C for 48 h, and then placed in 30% sucrose solution until they completely sank to the bottom. Then, the brain tissues were taken, sliced into 30 μm-thick sections using a freezing microtome, sealed with 10% donkey serum at room temperature for 1 h, and incubated with the primary antibody on a shaking table at 4°C overnight. After that, the sections were re-warmed for 1 h and incubated with the secondary antibody in a dark place at 37°C for 2 h. After the anti-fluorescence attenuating agent was added, the sections were sealed, and the images were acquired under a fluorescence microscope.

Western Blotting

At 7, 14, and 28 d after the operation, the mice were decollated, and the brain was taken. The total protein was extracted according to the total protein extraction kit (Beyotime, Shanghai, China), and the protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Abcam, Cambridge, MA, USA). The discontinuous sodium dodecyl sulfate-polyacrylamide gel was prepared, and 50 μg total proteins in each group were loaded into the loading well for electrophoresis. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, sealed with 5% skim milk for 1 h, and incubated with the primary antibody diluted with Tris-Buffered Saline and Tween-20 (TBST) on a shaking table at 4°C overnight. After the protein was re-warmed on the next day, it was incubated again with the secondary antibody at room temperature for 2 h. After chemiluminescence image development, the protein was scanned using the far-infrared fluorescence scanning imaging system.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from 100 mg brain tissues on the infarct side, and the purity and content of RNA were detected. After the total RNA was reversely transcribed at 42°C for 50 min, and the reverse transcriptase was inactivated at 95°C for 5 min, the PCR amplification was performed. GAP43: Forward: 5'-AGAAGGAGGGAGATGGCT-3', Reverse: 5'-CTTGGAGGACGGGGAGTT-3'. glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: 5'-CCTTCCGTGTTCCTACCC-3', Reverse: 5'-CCCAAGATGCCCTTCAGT-3'. After amplification, the data were quantitatively analyzed using the $2^{-\Delta\Delta CT}$ method with GAPDH as an internal reference gene.

Diaminobenzidine (DAB) Immunohistochemistry

DAB was injected into the mice at 14 d after the operation, the mice were decollated, and the brain was taken at 28 d. Then, the brain was fixed and sliced into sections in the same way as above. The sections were incubated with the avidin-HRP (Horse Reddish Peroxidase) working solution for 4 h, followed by color development with DAB working solution for 15 min, dehydration with gradient alcohol, transparentization with xylene, and sealing. Finally, the images were acquired under an optical microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

The mice were decollated, and the brain was taken at 3, 7, 14, and 28 d after the operation. The brain was immediately weighed and homogenized in a pre-cooled 2 mL homogenate tube. The homogenate was collected and centrifuged using the low-temperature ultra-speed centrifuge at 4°C and 2000 rpm for 15 min, and the supernatant was taken. The concentrations of cAMP and PKA were measured according to the instructions of the ELISA kit (R&D Systems, Minneapolis, MN, USA) taken from a refrigerator at 4°C.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The detection results were expressed as Mean ± SEM (Standard Error of Mean). The analysis of variance (ANOVA) was performed for the data comparison among groups, and the homogeneity test of variance was used. In the case of a significant difference in ANOVA, the Student-Newman-Keuls (SNK) test was further used for pairwise comparison. The nonparametric rank test was adopted in the case of heterogeneity of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Astaxanthin Promoted the Recovery of Motor Function of Mice After Ischemic Infarction

It was found in the Rotarod test that the mean residence time in the Sham group was longer than 300 s at each time point after the operation. Compared with that in the MCAO group, the mean residence time of mice in the Ast group was markedly increased at 7, 14, and 28 d after the operation. The mean residence time of mice in the Ast + H89 group was shorter than that in the MCAO group at 14 and 28 d after the operation, and there were statistically significant differences (Figure 1A). The results of the balance beam footstep error test revealed that there were almost no footstep errors at each time point after the operation in the Sham group. The times of footstep errors were fewer in the Ast group than that in MCAO group at 7, 14, and 28 d after the operation, showing statistically significant differences, and it was larger in the Ast + H89 group than that in the MCAO group at 7, 14, and 28 d after operation (Figure 1B).

Astaxanthin Stimulated the Axonal Regeneration of Cortex Around the Infarction After Ischemic Infarction

In terms of GAP43 fluorescence expression in the cortex around the infarction, the GAP43 expression was significantly increased in the MCAO group compared with that in the Sham group at 7, 14, and 28 d after the operation, and it was further increased in the Ast group compared with that in the MCAO group, displaying a statistically significant difference. The GAP43 expression in the Ast + H89 group significantly declined compared with that in the Ast group (Figure 2A). The GAP43 protein and mRNA expressions in the cortex around the infarction showed consistent trends. At 7, 14, and 28 d after operation, the GAP43 protein and mRNA expressions were always lower in the Sham group than those in the MCAO group, they were overtly increased in the

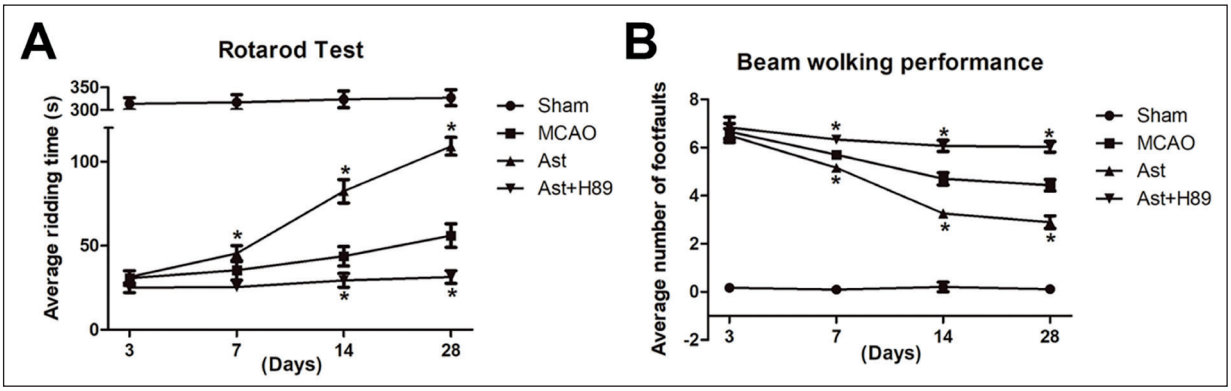

Figure 1. Astaxanthin promoted the recovery of motor function of mice after ischemic infarction. ***A,*** Analysis of average ridding time in different groups. ***B,*** Analysis of average times of footstep error in different groups. *$p<0.05$ *vs.* MCAO group.

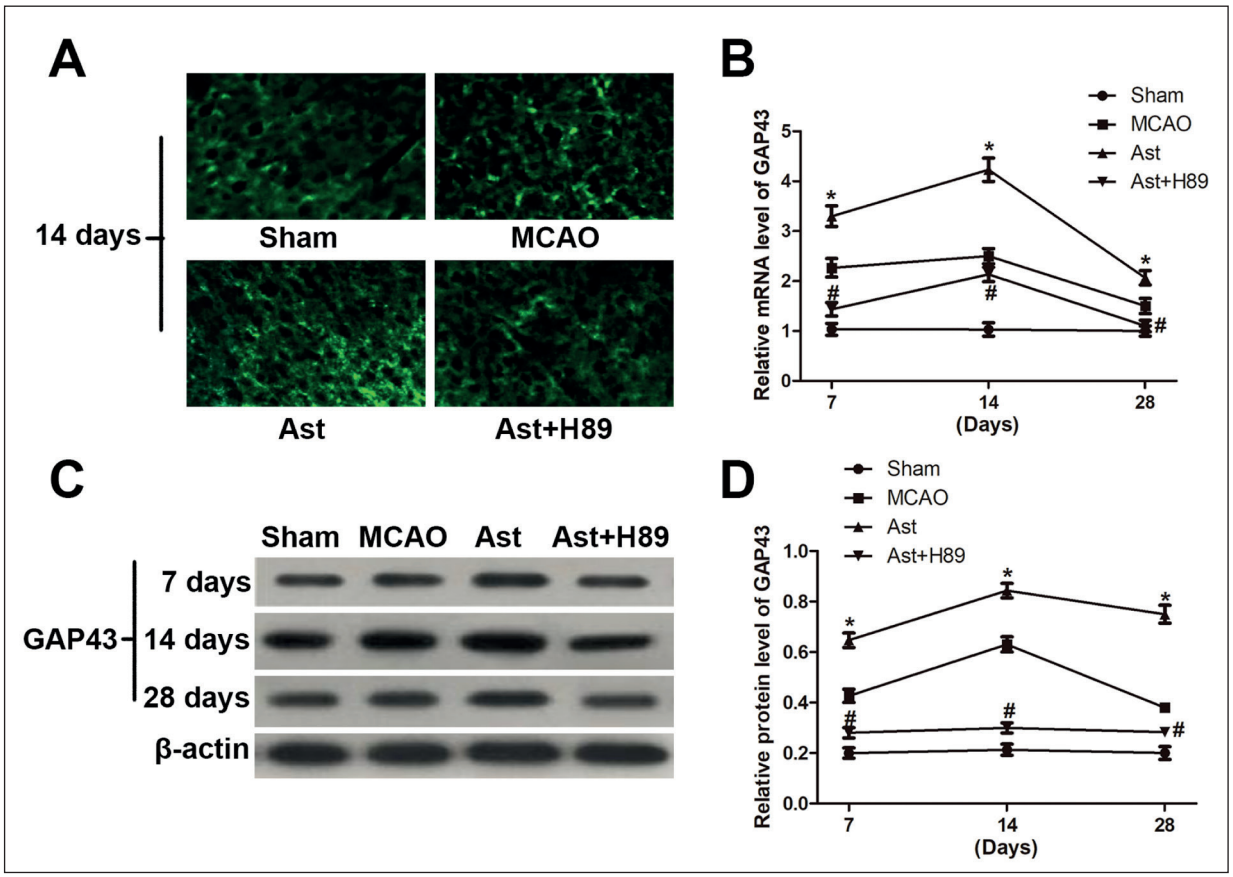

Figure 2. Astaxanthin stimulated the axonal regeneration of cortex around the infarction after ischemic infarction. ***A,*** Representative images of GAP43 fluorescence expression in the cortex around the infarction (magnification 400×). ***B,*** Analysis of relative mRNA level of GAP43 in different groups. ***C,*** Western Blotting showed the protein level of GAP43 in different groups. ***D,*** Analysis of the relative protein level of GAP43 in different groups. *$p<0.05$ *vs.* MCAO group, #$p<0.05$ *vs.* Ast group.

Ast group compared with those in the MCAO group, and they were significantly decreased in the Ast + H89 group compared with those in the Ast group (Figure 2A-2D).

Astaxanthin Promoted the Reconnection of New Axons of Cortex Around the Infarction

According to immunohistochemistry, the neurons and new axons taking in BDA were labeled brown yellow. In each group, there were a certain number of labeled neurons and their axons in the cortex on the unaffected side, as well as a certain number of axonal fibers in the cortex around the infarction without labeled neurons. There were very few BDA-labeled neurons and axons in the Sham group (Figure 3A). The immunofluorescence staining revealed that the neurons and new axons taking in BDA were labeled red. It was found in the semi-quantitative analysis that the number of labeled neurons and axons in the contralateral cortex in the Ast group was slightly larger than that in the other groups, and there were no significant differences among groups except for the Sham group. Compared with that in the MCAO group, the labeled axonal fibers in the cortex on the ischemic side was remarkably increased in the Ast group, and declined in the Ast + H89 group, displaying statistically significant differences (Figure 3B). The colocalization area of BDA (red) and GAP43 (green) was observed in the cortex around the infarction, and it was found that the positive area in the MCAO group was remarkably smaller than that in the Ast group, but larger than that in the Ast + H89 group (Figure 3C).

Astaxanthin Activated the CAMP/PKA Signaling Pathway to Promote Axonal Regeneration

The cAMP level in the other groups was lower than that in the Sham group at 3 d after operation. Compared with that in the MCAO group,

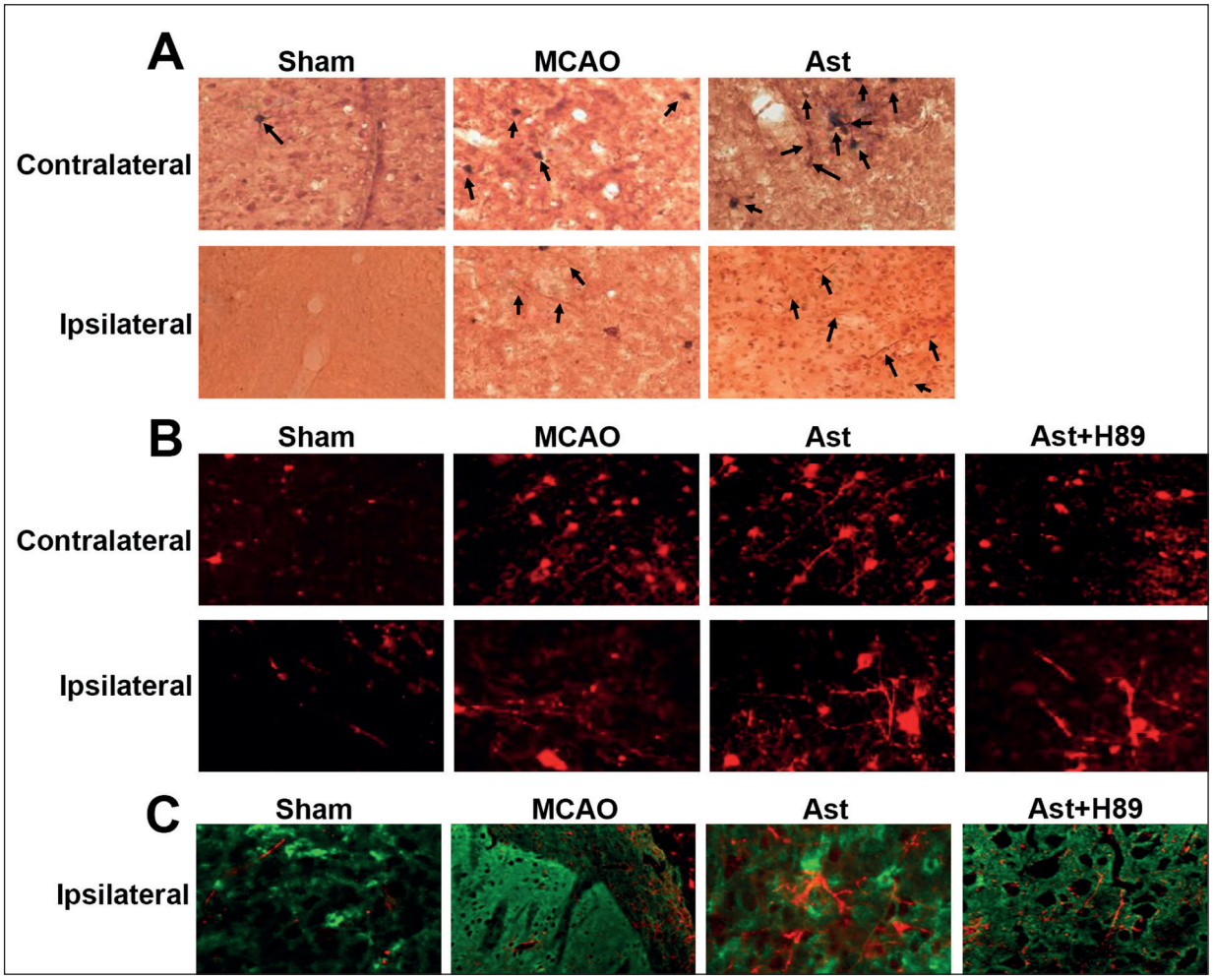

Figure 3. Astaxanthin promoted the reconnection of new axons of cortex around the infarction. ***A,*** Representative images of immunohistochemistry showed new axons in the cortex on the unaffected side and around the infarction (magnification 400×). ***B,*** Representative images of immunofluorescence staining showed new axons in the cortex on the unaffected side and around the infarction (magnification 400×). ***C,*** Colocalization area of BDA (red) and GAP43 (green) in the cortex around the infarction.

the cAMP level was lower in the Sham group at 7 and 14 d after the operation, and higher in the Ast group and Ast + H89 group at 7, 14, and 28 d after the operation. Compared with that in the Ast + H89 group, the cAMP level was higher in the Ast group at 14 d after the operation, and there was a statistically significant difference (Figure 4A). The PKA level in the other groups was lower than that in the Sham group at 3 d after the operation. Compared with that in the MCAO group, the PKA level was lower in the Sham group and Ast + H89 group at 7 and 14 d after the operation, and higher in the Ast group at 7, 14, and 28 d after the operation (Figure 4B). The Western blotting results manifested that the PKAc (PKA catalytic subunit) and p-CREB (CREB active molecule) protein expressions in the cortex displayed consistent trends. Compared with those in the MCAO group, the PKAc and p-CREB levels were upregulated in the Ast group at 7, 14, and 28 d after the operation, and downregulated in the Ast + H89 group at 7, 14, and 28 d after the operation (Figures 4C, 4D).

Discussion

Ischemic stroke leads to acute and severe neurological dysfunction, which is the leading cause of disability in patients[18,19]. The nervous system possesses a certain self-repairing capability. Therefore, exploring appropriate intervention measures

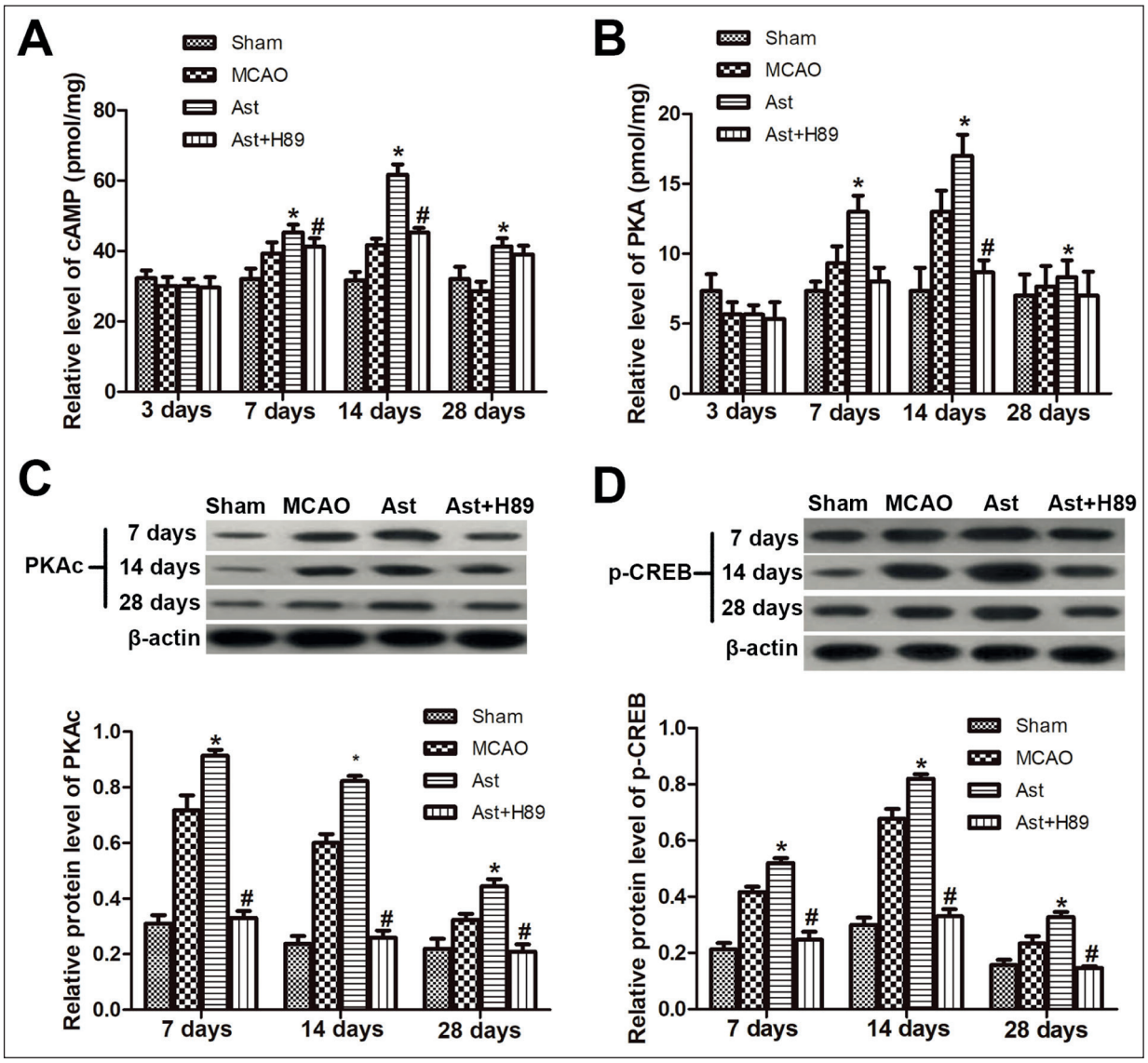

Figure 4. Astaxanthin activated the cAMP/PKA signaling pathway to promote axonal regeneration. ***A,*** Analysis of the cAMP level in different groups. ***B,*** Analysis of the PKA level in different groups. ***C,*** Western Blotting showed protein level of PKAc in different groups. ***D,*** Western Blotting showed protein level of p-CREB in different groups. **p*<0.05 *vs.* MCAO group, #*p*<0.05 *vs.* Ast group.

to stimulate the endogenous nerve repair has been a hot spot in medical research currently. Limb dyskinesia is one of the major clinical manifestations after stroke, and the partial recovery of limb motor function is an evident feature of nerve repair. The Rotarod test and the balance beam footstep error test are commonly-used behavioral tests to evaluate the motor function after brain injury[20,21]. The results of the two tests revealed that after astaxanthin treatment, the time of footstep errors and the residence time on rotarod of mice with cerebral ischemic infarction were superior to those of mice under spontaneous recovery. The motor function of mice had spontaneous recovery after cerebral infarction, the recovery effect after astaxanthin treatment was better than that under spontaneous recovery, and the recovery of balance and fine motor of mice was earlier than that of coordination and muscle strength.

The neural plasticity after ischemic infarction is a process of building a new structural connection between the tissues around the cerebral lesion and the lesion region. The axonal regeneration is the anatomical basis and the key link

of neural remodeling. Studies[22-24] have found that the surviving pyramidal bundle axons after cerebral infarction of rats can sprout at the distal end of lesion region. Moreover, they found that the sprouting rate of contralateral pyramidal bundle axons is increased, and a large number of axonal fibers can be reorganized along the infarction edge. GAP43 is highly expressed during the axonal growth and differentiation and at the growth cone end of the new axons, and it plays a guiding role in the axonal growth, so it is a reliable marker for axonal growth[25,26]. In this experiment, the results of immunofluorescence, Western blotting, and RT-PCR showed that the GAP43 expression in the ischemic cortex of mice under spontaneous recovery also began to rise at 7 d after ischemia, indicating that the axonal regeneration has begun after injury. The GAP43 expression in the ischemic cortex of mice treated with astaxanthin was higher than that in mice under spontaneous recovery at 7, 14, and 28 d after ischemia, suggesting that the astaxanthin treatment can stimulate the axonal regeneration in the cortex around infarction, and facilitate the recovery of motor function.

After cerebral infarction, the cortical reorganization occurs around the infarction, and the cortex in the contralateral hemisphere to the injury is also involved in the nerve repair[27]. BDA is a specific neuronal anterograde tracer, which can be used to observe the growth track of the contralateral new axons on the affected side and axonal density. The cortex of the bilateral cerebral hemispheres in mice injected with BDA was observed *via* immunohistochemistry. BDA-labeled axonal fibers could be observed in the ischemic cortex, but there were no labeled neurons, indicating that these fibers come from the contralateral cortical neurons near the region around the infarction. Furthermore, the BDA-labeled neurons and axonal fibers were counted *via* immunofluorescence, and the results showed that the number of labeled neurons and axons in the cortex on the unaffected side had no significant difference among groups except the Sham group, and it was slightly larger in mice treated with astaxanthin. However, there was a significant difference in the number in the ischemic cortex, and the number of labeled axonal fibers in mice treated with astaxanthin was significantly larger than that in the other groups, indicating that astaxanthin can stimulate the cortical neurons on the unaffected side to produce more axonal branches extending smoothly to the cortex around the infarction and thereby supporting the neural circuit reconstruction after injury. The colocalization staining of GAP43 and BDA in the cortex around the infarction was basically consistent with the above results, indicating that astaxanthin treatment can promote the reconnection of new axons in the cortex around the infarction.

The cAMP-PKA signaling system has an important influence on regulating neuronal survival and axonal growth[9,10]. In this experiment, the effect of astaxanthin on promoting axonal regeneration was inhibited after H89 intervention, indicating that such an effect is related to the PKA signaling pathway. The detection of the cerebral cortex tissues after infarction manifested that the expressions of cAMP and PKA were slightly decreased at 3 d after ischemic infarction compared with those in the Sham group. After astaxanthin treatment, the levels of cAMP and PKA in the cortex of mice with cerebral infarction were increased at 7 d after the operation and reached the peak at 14 d. The results of the Western blotting also demonstrated that the changing trend of PKAc and p-CREB was consistent with that of PKA, indicating that the cAMP/PKA/CREB signaling pathway is activated, and astaxanthin can further stimulate the activation and effect of this pathway, thereby promoting axonal regeneration.

Conclusions

We demonstrated that astaxanthin can promote the recovery of motor function in the chronic phase after ischemic infarction and stimulate the axonal regeneration in the cerebral cortex. Its mechanism may be explained as follows: astaxanthin activates the cAMP/PKA/CREB signaling pathway by increasing the cAMP concentration in brain tissues, ultimately promoting the axonal regeneration in the cerebral cortex and improving the motor function.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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