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Apelin-12 exerts neuroprotective effect against ischemia-reperfusion injury by inhibiting JNK and P38MAPK signaling pathway in mouse

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Abstract. – OBJECTIVE: Cerebral ischemia is a common neurological disease, and its pathological process remains elusive. This study focused on the protective mechanism of Apelin-12 protein on the nervous system of mice during cerebral ischemia-reperfusion injury through JNK and P38MAPK signaling pathway.

MATERIALS AND METHODS: The mouse model with an ischemia-reperfusion injury in middle cerebral artery was prepared by the modified thread-occlusion method and divided into 4 groups randomly. Before implantation of the mice, we assessed the neurological function and evaluated the cerebral edema by the wet-dry weight method. Lactate dehydrogenase (LDH) kit was used to assess the degree of cell injury. Malondialdehyde (MDA) kit was used to measure the level of neuron MDA. Immunohistochemistry was performed to evaluate the neuronal cell in the ischemic brain. Protein expressions of JNK and P38MAPK and apoptosis-related molecules, including Bax, Bcl-2, caspase-3, and cleaved caspase-3, were measured by Western blot assay.

RESULTS: After focal cerebral ischemia-reperfusion, a significant decrease in neurobehavioral score, brain edema and neuron injury in mice occurred. Apelin-12 significantly improved the neurobehavioral score of the mice with ischemia-reperfusion injury, alleviated brain edema and the damage to neurons. In addition, Apelin-12 inhibited the morphological changes and apoptosis of neuronal cells in the ischemic penumbra of mice. Apelin-12 could downregulate the expression of Bax and caspase-3, inhibit the activity of caspase-3 and upregulate the expression of Bcl-2, an anti-apoptotic protein. A significant reduction in the protein expression of p-JNK and p-p38 was observed in the Apelin-12 group compared with that in the I/R or Vehicle group ($p<0.05$).

CONCLUSIONS: When an ischemia-reperfusion injury occurred, Apelin-12 can inhibit the JNK and P38MAPK signaling pathway of the apoptosis-related MAPKs family, thus offering protection to neurons.

Key Words
Apelin-12, Ischemia-reperfusion, Neuroprotection, JNK, P38.

Introduction

Cerebral infarction (Cerebral Ischemic, CI) is a major health threat to human[1]. With the great improvement in the prognosis of patients with cerebral ischemia, the occurrence of regional ischemia and reperfusion is still inevitable[2] due to thrombus dissolution. Ischemia-reperfusion injury is a cascade reaction and a complex network, the pathophysiologic mechanisms of which remain poorly understood. A variety of apoptosis-related proteins are involved, such as Bax, Bcl-2, and caspase-3[3-5]. In the event of ischemic damage, many protective proteins were produced, including Apelin-12[6,7]. The precise mechanism by which Apelin-12 exerts its anti-injury and anti-apoptosis effects is not fully understood. Now Apelin-12 has become a research hotspot in recent years[8,9].

Angiotensin receptor AT1 related receptor protein (putative receptor related to be the angiotensin receptor AT1, APJ) is a G-protein-coupled receptor and Apelin is its endogenous ligand. Apelin, including Apelin10, Apelin12, Apelin13, Apelin36, and other short peptide substances, is concentrated in the surface of the heart, pulmonary vascular endothelial cells, and nerve cells, among which APJ/Apelin has been widely found in the central nervous system and peripheral nervous system. Studies[10-12] have shown that APJ/Apelin13 system plays a protective role in inhibiting myocardial reperfusion by inhibiting the expression of myocardial apoptosis-related proteins. However, the research of APJ/ Apelin-12 on cerebral ischemia-reperfusion has received little attention.

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Apelin-12 is widely distributed in the nervous system[13]. We studied whether it protects the nervous system through the JNK and P38MAPK signaling pathway of the MAPKs family during cerebral ischemia-reperfusion injury to clarify its specific protective mechanism and provide a theoretical basis for the study of the clinical development of drugs in ischemic reperfusion machines.

Materials and Methods

Mice and Grouping

Healthy adult male CD-1 mice with weight 20-30 g were used in our investigation. They were randomly divided into 4 groups with 5 mice in each group: Sham, MCAO, ischemia-reperfusion (I/R), Apelin-12 group. Sham group included the mice which experienced all the operations such as ischemia-reperfusion group except inserting nylon fishing line. In the mice of the Apelin-12 group, 5 μL 2% dimethyl sulfoxide (DMSO) was intracerebroventricularly injected 15 min before cerebral ischemia, and 15 μL Apelin-12 was intracerebroventricularly injected 15 min before reperfusion. In the mice of Vehicle group, 5 μL 2% DMSO was intracerebroventricularly injected 15 min before cerebral ischemia and 15 min before reperfusion. This study was approved by the Animal Ethics Committee of Shiyan Maternity and Child Health-Care Hospital Animal Center.

Neurological Function Evaluation

Neurological function evaluations of each mouse were performed at 1, 3, 7, and 14 days after reperfusion of MCAO. The neurological defect was graded on a scale of 3-18 (3 = maximal deficit score; 18 = normal score). Each mouse score was evaluated by six individual test scores. The lower the score, the more severe the injury.

Determination of LDH and MDA

Detection of leakage rate of lactate dehydrogenase (LDH) was performed as follows. According to the instructions, the blank wells, standard wells, measuring wells, and control wells were prepared as required and left at room temperature for 3 min. The supernatant was collected, and then, the absorbance was read at 450 nm. The experiment was repeated 3 times. Malondialdehyde (MDA) was detected by the thiobarbituric acid method. In accordance with the instructions, blank, standard, testing, and control holes were prepared. Absorbance was read at 532 nm (1 cm diameter, distilled water zero).

Immunohistochemistry

The brain tissue removed from paraformaldehyde was sectioned into 2 mm slices and marked well, then soaked in alcohol with a different concentration in turn, which was 75%, 85%, 95% I, 95% II, 100% I, and 100% II. After dehydration, paraffin-embedded sections were prepared. We then removed the prepared glass slide from the oven, put it in xylene, soaked it in alcohol and washed with water for the (H&E) staining. After the sections were labeled, 40 μL serum from normal animal without being vaccinated was added in the sections, which were incubated at room temperature for about 1 hour. Subsequently, the serum was removed and horseradish peroxidase-labeled mAb, the mouse anti-neuronal nuclei (1/100, Millipore, Billerica, MA, USA) (diluted according to the instructions) was added. Then, the sections were incubated at 4°C overnight. After that, corresponding secondary antibodies with a biotin marker were used and incubated at room temperature. After being washed with phosphate-buffered saline (PBS), the corresponding third antibody was added to incubate the sections for 1 hour at room temperature. At last, the sections of ischemic brains were visualized by newly prepared diaminobenzidine (DAB) and observed under a light microscope.

Western Blot

The total tissue protein was extracted from the ischemic brain tissue of each group by radioimmunoprecipitation assay (RIPA) lysate (Roche, Basel, Switzerland), and 10 μL target protein sample was separated for electrophoresis with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The primary antibodies, anti-Bax, Bcl-2, Caspase-3, cleaved Caspase-3, p-JNK, and p-p38 (purchased from CST company, 1:1000, Danvers, MA, USA), were added and incubated overnight at 4°C. After washing the membrane incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 2 hours at room temperature (Cell Signaling Co., 1/5000 dilution of goat anti-rabbit IgG, Danvers, MA, USA), the binding was visualized with enhanced chemiluminescence (ECL) method (Shanghai Beyotime Biotechnology Co., Ltd. Shanghai, China). The determination of the

integral optical density (IOD) value of each band was performed by the gel imaging analysis system. β-actin was used as an internal control. Relative protein expression was determined based on the IOD ratio of the target protein to internal control.

Statistical Analysis

Statistical analysis was performed using statistical product and service solutions (SPSS11.0, SPSS Inc., Chicago, IL, USA) statistical software. All the test results were analyzed using mean±SD. Statistical data of multi group were compared using ANOVA and variance analysis was performed. When there was a significant difference, paired comparisons were made by Student-Newman-Keuls (SNK) test. $p<0.05$ was considered statistically significant.

Results

Neuroprotective Effect of Apelin-12 on Focal Cerebral Ischemia-Reperfusion Mouse Model

The neurological function score in I/R group was significantly higher than that in Sham group ($p<0.05$), indicating that the cerebral ischemia-reperfusion animal model (MCAO model) was successfully established and suitable for follow-up detection. The score in Apelin-12 group was significantly lower than that in I/R group ($p<0.05$, Figure 1A), suggesting that Apelin-12 could reduce the loss of nerve function in mice causing by focal cerebral ischemia-reperfusion. The water content of Sham and Apelin-12 groups was 78.40 + 0.77% and 79.02 + 0.62%, respec-

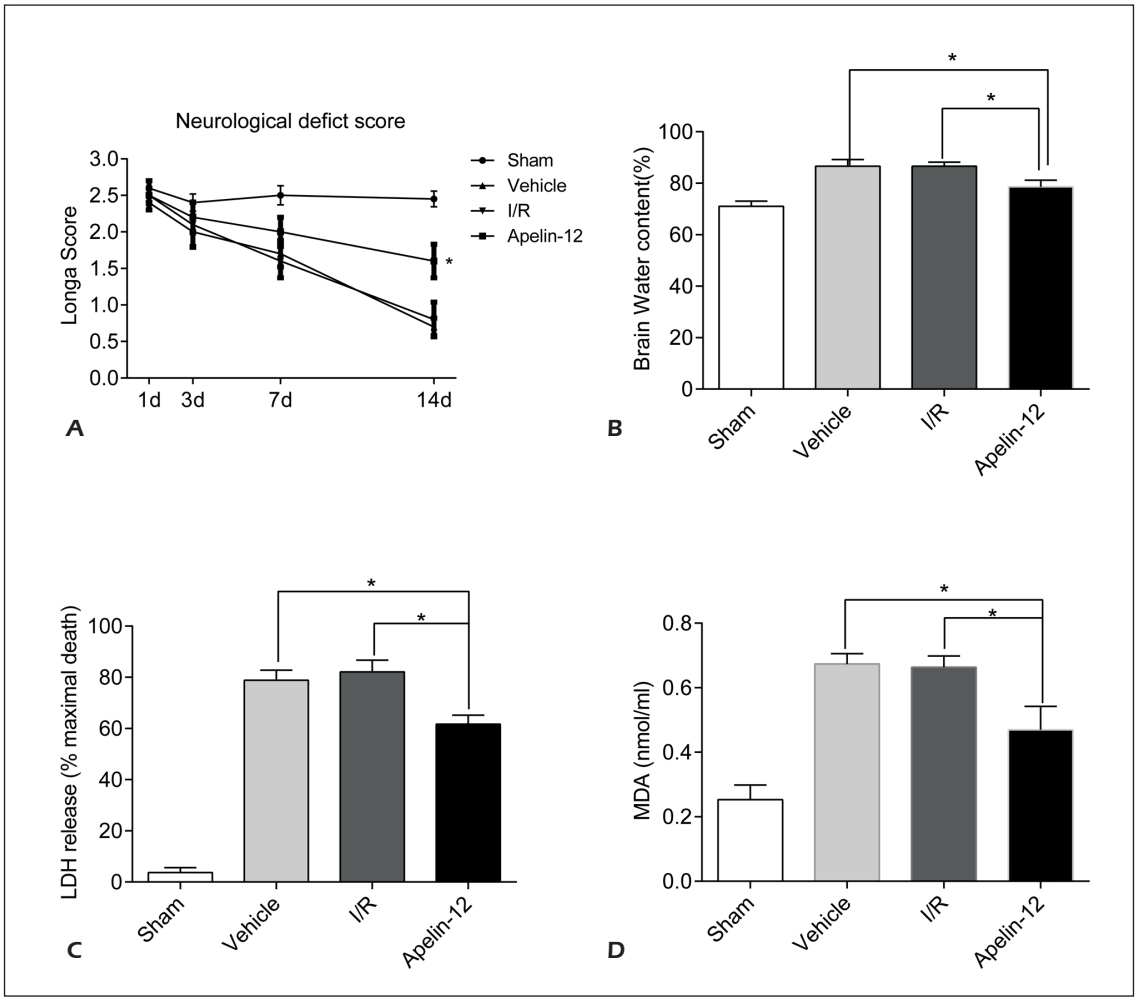

Figure 1. The neuroprotective effect of Apelin-12 on focal cerebral ischemia-reperfusion in mice. **A**, Evaluate the neurological deficit of mice in each group (n = 6) using the Longa scoring system *$p<0.05$. **B**, Effect of Apelin-12 on brain water content in mice with focal cerebral ischemia-reperfusion (n=6). *$p<0.05$. **C**, Detect the level of nerve cell injury in each group with LDH Kit (n=6) *$p<0.05$. **D**, Detected oxidative stress injury after cerebral ischemia-reperfusion with MDA kit (n=6) *$p<0.05$.

tively, which was significantly less than that in Vehicle group (82.92% + 0.62%) and I/R group (82.48 + 0.38%) (Figure 1B). The results of LDH and MDA detection showed that, compared with I/R group, the LDH and MDA values of MCAO in Apelin-12 group significantly decreased (Figure 1C and 1D).

Effect of Apelin-12 on Ischemic Damages in Neuronal Cells

To evaluate the neuroprotective effect of Apelin-12 on ischemic neuronal damages, we investigated the morphological changes of neuronal cells in the tissue of MCAO-induced ischemic mice. The results showed that the brain tissue of Sham group had no significant pathological changes, with complete cell morphology, while edema, necrosis, hyperchromatic and shrink infarction were visible in surrounding tissue of the I/R group (Figure 2A). In the I/R group, a large number of Nissl-stained apoptotic neurons were found around the infarct tissues. However, the Nissl-stained apoptotic neuronal cells in Apelin-12 group was significantly lower than that in the I/R group ($p<0.05$, Figure 2B).

Apelin-12 Protects the Nerve Cells by Regulating Bcl-2, Bax, and Caspase-3

It was found by Western blot detection that Bcl-2, Bax, and caspase-3 protein were expressed in each group. I/R group got higher protein expressions of Bax, Bcl-2, caspase-3, and cleaved caspase-3 than Sham group ($p<0.05$). The protein expression of Bax, caspase-3, cleaved caspase-3 in Apelin-12 group exhibited lower level than that in Vehicle and I/R groups, while the expression of Bcl-2 in Apelin-12 group was more than that in I/R group ($p<0.05$, Figure 3A-3E). The above results indicated that the anti-apoptotic effect of Apelin-12 was achieved through reducing the expression of pro-apoptotic protein Bax and Caspase-3, inhibiting caspase-3 activity and increasing the expression of anti-apoptotic protein Bcl-2.

Apelin-12 Participates in the Neuroprotective Effect of Cerebral Ischemia-Reperfusion in Mice by Inhibiting JNK and P38MAPK Signaling Pathway

Compared with Sham group, the expression level of p-JNK and p-P38 in Vehicle and I/R groups increased significantly ($p<0.05$), but the protein expression level of JNK and P38 did not change significantly. The expression level of p-JNK and p-P38 in Apelin-12 group decreased significantly compared with Vehicle and I/R groups ($p<0.05$), while the protein expression of JNK and P38 remained static (Figure 4). These results indicated that Apelin-12 could inhibit the JNK and P38MAPK signaling pathway of the apoptosis-related MAPKs family in ischemic brain tissue, so as to protect neurons.

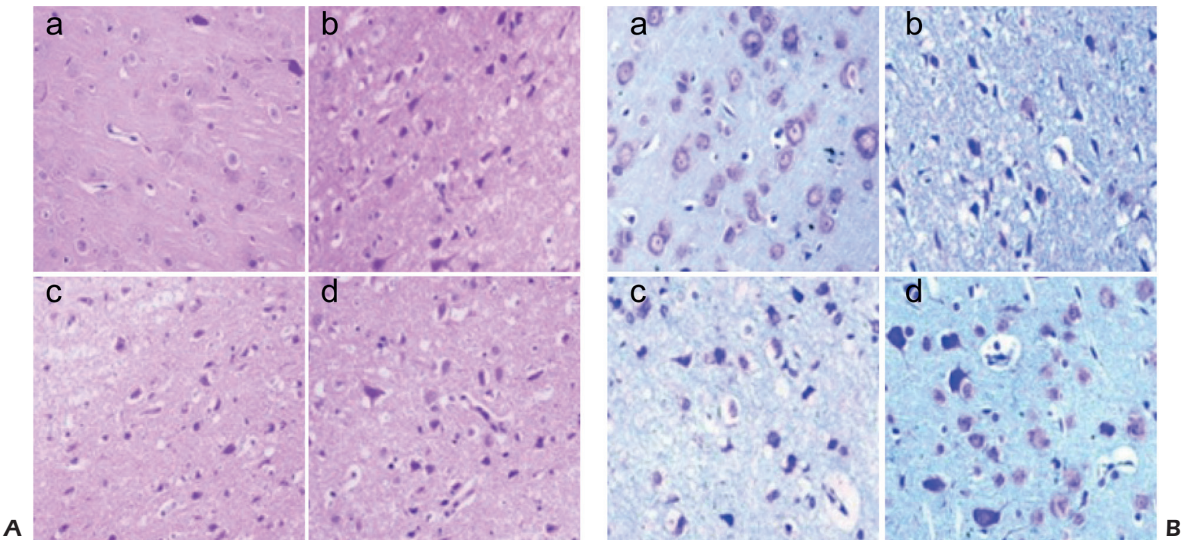

Figure 2. Effect of Apelin-12 on ischemic damages in neuronal cells. Stained by H&E (**A**) and Niss (**B**) of brain tissues after MCAO/reperfusion treatment. **A**, sham; **B**, I/R; **C**, vehicle: MCAO-induced ischemic group; and d, Apelin-12. (Magnification ×400).

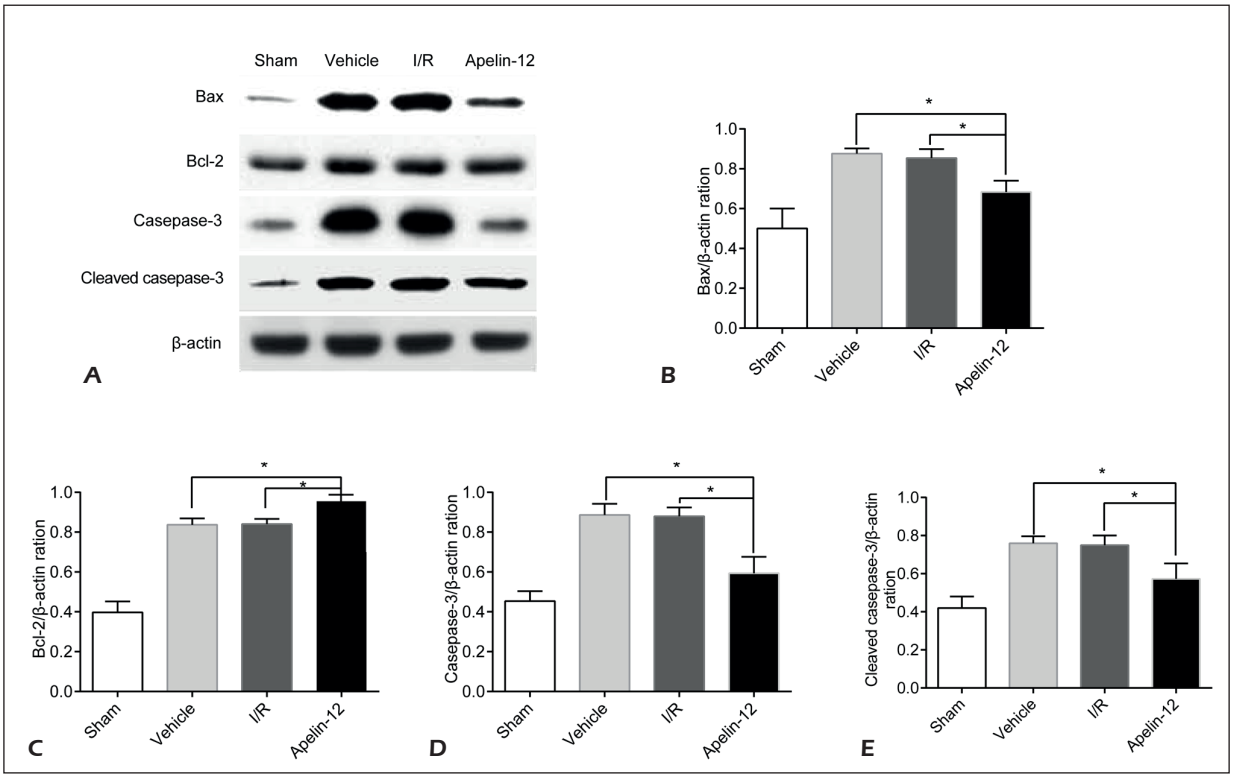

Figure 3. Apelin-12 protects the nerve cells by regulating Bcl-2, Bax, and caspase-3. **A**, Western blotting detected the effect of Apelin-12 on the protein expression of Bax, Bcl-2, caspase-3 and cleaved caspase-3 in each treatment group. **B**-**E**, Quantitative analysis of protein expression of Bcl-2, Bax, caspase-3, and cleaved caspase-3 in the group treated by Apelin-12

Discussion

Neuronal death means necrosis or apoptosis. After ischemia-reperfusion, the surrounding neurons will undergo an orderly apoptosis[14,15] due to various stimuli. Therefore, how to inhibit this apoptosis during ischemia-reperfusion is of great significance for the rescue of cerebral ischemic cells.

Apelin is a short peptide composed of 77 amino acids. Its carboxyl terminus is a functional area containing APJ receptor specific region and an enzyme cutting site of proteolytic enzymes. During transcription, proteolytic enzymes cut Apelin into a different length of fragments to participate in the endocrine system, circulatory system, immune system, and nervous system[16,17]. Shorter the length of the fragment, more the activity is. Some studies[18,19] have shown that Apelin13 protects against cardiac ischemia reperfusion injury by inhibiting apoptotic pathway.

During cerebral ischemia-reperfusion, apoptosis mediates the death of a large number of neurons in the central ischemic region. In addition, cerebral ischemia can affect the stability of the internal environment and induce the activation of protease, including mitogen-activated protein (MAPKs) and calcium regulating protein-dependent kinase (CaMKs)[20]. The MAPKs signal transduction pathway is vital for cell apoptosis and survival, and the role of MAPKs signaling pathway in cerebral ischemia-reperfusion is becoming always more important. It is considered that the main signaling pathways of MAPK transduction pathway are extracellular regulated protein kinases (ERK) pathway, c-Jun N-terminal kinase (c-Jun N-terminal kinase, JNK) pathway, and P38MAPKs pathway. In the process of cerebral ischemia-reperfusion, JNK mediated the occurrence of neuron cells apoptosis[21]. JNK plays a relatively extensive role in the phosphorylation of transcription factors such as ATF2, P53, and proteins, including BCL-Xl, Bad, Bcl-2, and Bim[22,23].

In the 1990s, Lennmyr et al[24] found that P38MAPK is a phosphorylated protein kinase containing tyrosine of 360 amino acids, which is homologous to mitogen-activated protein kinases in yeast. In the process of pathological changes, P38MAPKs mediate the occurrence of

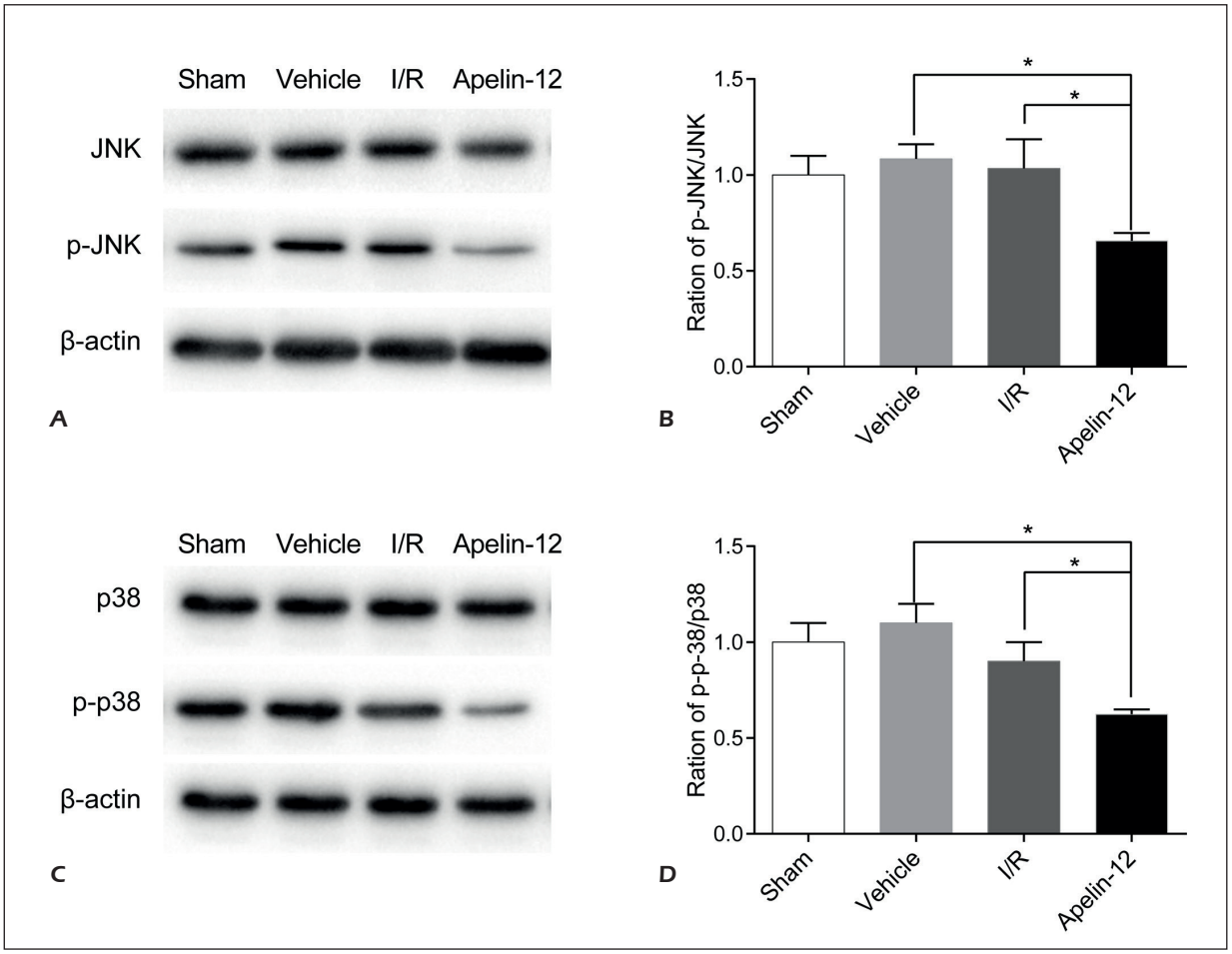

Figure 4. Apelin-12 inhibits the activation of JNK and P38MAPK signaling pathways. **A-B**, Western blot was used to detect the changes in the expression of p-JNK and JNK of each group. Data were presented as a mean + SD, n=3. **C-D**,Western blot was used to detect the expression of p-p38 and p38 during cerebral ischemia and reperfusion. Data were presented as a mean ± SD, n=3.

inflammation and apoptosis of cells[25]. In particular, P38MAPKs are involved in the occurrence of apoptosis and play an important role in the loss of growth factors or the fragmentation of Fas fragments in cells[26-28]. After local ischemia, the activation of P38MAPK[29-31] can also be found in brain tissue of mouse or gerbils.

The results of this work showed that apelin-12 could significantly improve the neurological score of the model mice, alleviate brain edema and the damage to neurons. Meanwhile, Apelin-12 was found to have the neuroprotective effect on ischemic neuronal damages in the MCAO-induced ischemic mouse by immunohistochemistry. Subsequently, we found that Apelin12 reduced the expression of Bax and Caspase-3 while increased the expression of Bcl-2 after focal ischemia-reperfusion injury. It was proved that apelin-12 had a protective effect on the cerebral tissue after ischemia-reperfusion, and anti-apoptosis was one of the targets of its action. JNK or P38MAPK plays an important role in cell apoptosis. Therefore, we studied the changes in the JNK and P38 signaling pathways after cerebral ischemia-reperfusion. When treated with Apelin-12, the phosphorylation level of p-JNK and p-P38 in brain tissue was downregulated, suggesting that the series of the protective role of Apelin-12 was related to the inhibition of JNK and P38MAPK signal pathway.

In summary, we found that Apelin-12 can regulate the apoptosis-related proteins by inhibiting the activation of JNK and P38MAPK signaling pathway in cerebral ischemia-reperfusion tissue, so as to protect the damaged neurons. It provides a theoretical basis for studying the protective mechanism of cerebral ischemia-reperfusion.

Conclusions

We showed that apelin-12 can inhibit the JNK and P38MAPK signaling pathways of apoptosis-related MAPKs family during cerebral ischemia and reperfusion, thereby protecting neuronal cells.

Conflict of interest

The authors declared no conflict of interest.

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