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The effects of calcitonin gene-related peptide on bFGF and AQP4 expression after focal cerebral ischemia reperfusion in rats

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The aim of this study was to investigate whether calcitonin gene-related peptide (CGRP) administration could produce neuroprotective effects after brain ischemia reperfusion in rats. Brain ischemia reperfusion injury was induced by a 2-hour left middle cerebral artery occlusion (MCAO) using an intraluminal filament, followed by 46 hours of reperfusion. CGRP (1 μg/ml) at the dose of 3 μg/kg, i.p., was administered at the beginning of reperfusion. Saline (3 ml/kg body weight) treated animals were used as control. Sham-operated animals were also used. Subsequently, 48 hours after MCAO, infarct volume, histological alterations, based fibroblast growth factor (bFGF) and aquaporin-4 (AQP4) expression were examined. The results showed that CGRP could significantly decrease infarct volume, improve brain tissue histological damage, promote bFGF expression and inhibit AQP4 expression after brain ischemia reperfusion injury. The results suggested that the neuroprotective effects of CGRP may be mediated by promoting bFGF expression and inhibiting AQP4 expression. The spatial and temporal distribution of molecules involved in the ischemic cascade by CGRP administration should be further studied.

1. Introduction

Ischemic stroke is a frequent cause of death and the most common cause of neurological disability (Arumugam et al. 2007; Sallustio et al. 2007). Stroke causes heterogeneous changes in tissue oxygenation, with a region of decreased blood flow, the penumbra, surrounding a severely damaged ischemic core (Liu et al. 2004). Early reperfusion following stroke results in reduced tissue injury. Paradoxically, restoration of blood flow under certain conditions may also cause delayed neuronal damage (reperfusion injury) (Barber et al. 2005). Understanding the mechanisms of ischemic brain damage is necessary for the development of innovative treatment strategies (Janardhan and Qureshi 2004). Neuroprotection in acute stroke remains a significant challenge but has not been clearly shown to be effective (Ford 2008). However, there are few effective and largely available therapies for this devastating disease. In addition to advancing acute reperfusion therapies, there is a need to develop treatments aimed to promote repair and regeneration of brain tissue damaged by ischemia (Arenillas et al. 2007). To develop innovative therapeutic modalities or strategies is necessary for effective treatment of ischemic stroke.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide, primarily released from sensory nerves (Brain and Grant 2004). CGRP is a potent neuropeptide vasodilator (Bulloch et al. 1998; Tam and Brain 2004). Applying CGRP could decreased the permeability of brain venules (Hu et al. 2005). Interestingly, 10 days after ischemia, CGRP-like immunoreactivity was decreased in the ischemic tissue but increased in the surrounding tissue in experimentally induced ischemia in rat (Bucinskaite et al. 1998). It is suggest that endogenous CGRP may be involved in the adaptive response to ischemia (Gherardini et al. 1996). Patients with neurological deficits after intracranial aneurysm surgery for subarachnoid haemorrhage were given CGRP at progressively increased concentrations showed improvement with no adverse effects. It is implicated that CGRP can reverse cerebral ischemia after early intracranial aneurysm surgery (Johnston et al. 1990). Interestingly, a recent report suggested that endogenous CGRP release in remote mesenteric ischemic preconditioning probably has a neuroprotective effect on cerebral ischemia reperfusion in mice (Rehni et al. 2007). Whether exogenous CGRP application could protect the brain against focal cerebral ischemia reperfusion injury is still unclear. To determine what about the effects of CGRP on brain ischemia reperfusion injury progression, we generated a rat model of middle cerebral artery occlusion (MCAO) to evaluate the effects of CGRP administration on brain infarct volume, histological alterations, basic fibroblast growth factor (bFGF) and

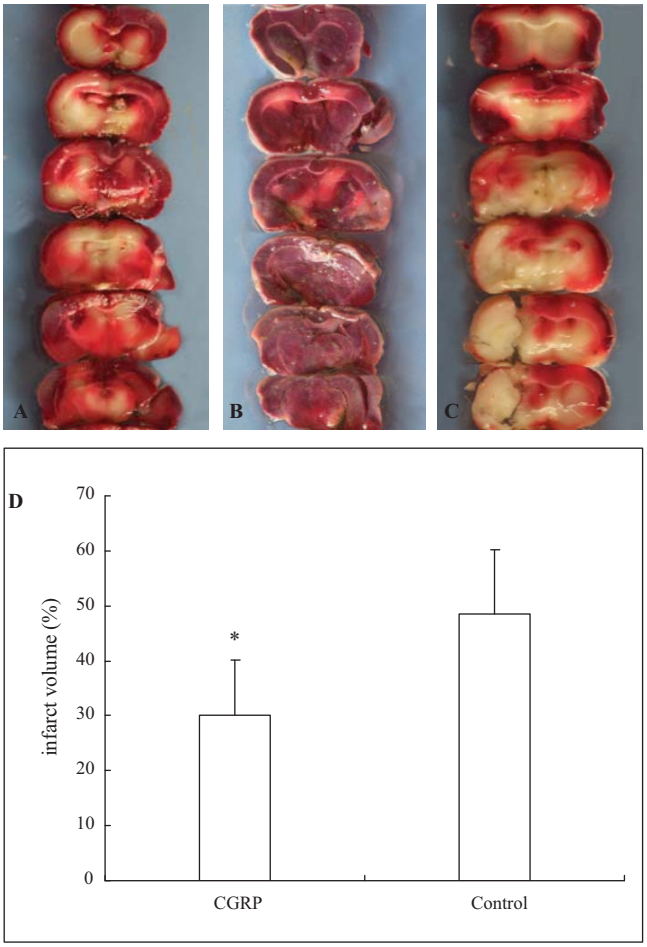

Fig. 1: Cerebral infarct volume using TTC staining after MCAO and reperfusion. Panel A: CGRP-treated MCAO group; Panel B: sham-operated group; Panel C: Control; Panel D: The cerebral infarct volume of CGRP-treated and control rats. The cerebral infarction (infarct volume/total brain volume) was 30.18% ± 10.04% in CGRP-treated rats and 48.53% ± 11.59% in control. Bar graphs with error bars represent mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* control

aquaporin-4 (AQP4) expression after cerebral ischemia reperfusion injury in rats.

2. Investigations and results

2.1. Cerebral infarct volume

To determine the cerebral infarct volume, TTC staining was used in the present study. The certain parts of parietal lobes, temporal lobes and striatum in left hemisphere of MCAO rats appeared white, whereas the normal non-infarcted tissue appear red. The infarct volume was reduced in CGRP-treated rats as compared with control (30.18% ± 10.04% *vs.* 48.53% ± 11.59%, $P < 0.05$) (Fig. 1).

2.2. HE staining

HE staining was used to determine the histopathological alterations induced by MCAO and reperfusion. The structure of brain tissues in sham-operated groups was normal. Lesions were observed in the frontal and parietal lobe cortex in the MCAO rats. There were numerous red neurons (eosinophilic degeneration) with neuron death, nuclei shrinkage, and Nissl bodies loss in widespread infarcted areas. The intermediate region between understained infarcted areas and normal brain tissues was ischemic penumbral zone. Brain edema, neutrophil infiltration, and increased peri-vascular space were also seen. CGRP treatment markedly attenuated these pathological

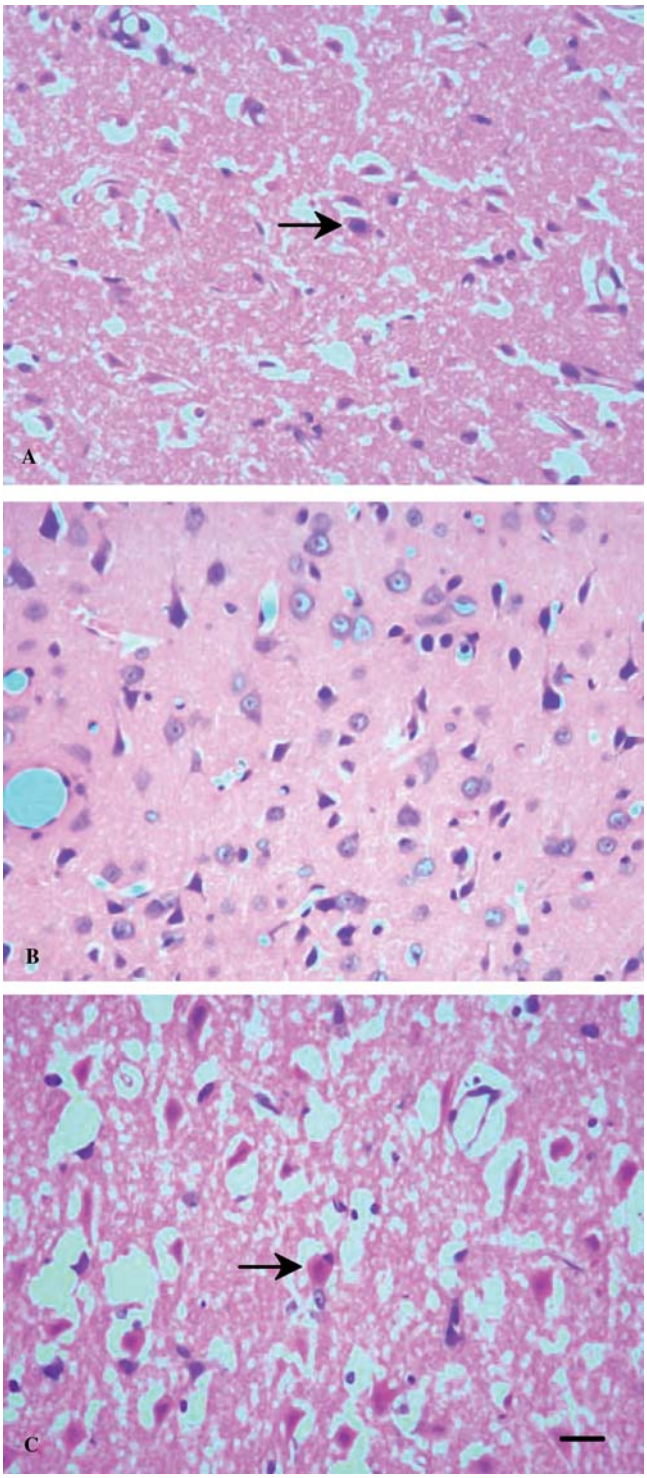

Fig. 2: The histopathological alterations after MCAO and reperfusion by HE staining. Panel A: Brain tissue damage improved after CGRP treatment compared with control. Panel B: Sham-operated group showed normal brain tissue. Panel C: Saline-treated control MCAO rats. Numerous red neurons (arrow) with nuclei shrinkage were observed in infarcted region. Brain edema, neutrophil infiltration, and increased peri-vascular space were also seen. Scale bar = 12.5 μm

changes with decreased ischemic penumbral zone and less red neurons. The conditions of edema and neutrophil infiltration were also much better (Fig. 2).

2.3. bFGF and AQP4 immunostaining

The levels of bFGF and AQP4 expression were analyzed by immunohistochemistry. bFGF immunoreactivity was present in

the nucleus of neuroglial cells in the cerebral cortex, and AQP4 immunoreactivity were mainly distributed in the cell membrane of neuroglial cells in the cerebral cortex. The rats with MCAO and reperfusion exhibited a remarkable increase in bFGF and AQP4 reactivity in ischemic penumbral zone of cortex (bFGF: 163.69 ± 8.01 *vs.* 148.73 ± 8.84, $P < 0.01$; AQP4: 148.26 ± 5.03 *vs.* 138.28 ± 4.80, $P < 0.01$). CGRP treatment at 3 μg/kg at the beginning of reperfusion significantly attenuated the increase of AQP4 expression (138.54 ± 4.82 *vs.* 148.26 ± 5.03, $P < 0.01$), while bFGF expression was increased with CGRP treatment compared to MCAO rats (170.32 ± 8.91 *vs.* 163.69 ± 8.01, $P < 0.05$) (Figs. 3, 4).

3. Discussion

Acute ischemic stroke is a major cause of both death and disability (Cimarosti et al. 2008; Green 2008). Reperfusion injury is a complication of recanalization therapies after focal cerebral ischemia. Damage to the brain will continue even after the blood flow is restored after cerebral ischemia (Xu et al. 2008). In the present study, we found that CGRP could significantly decrease infarct volume, improve brain tissue histological damage, promote bFGF expression and inhibit AQP4 expression after brain ischemia reperfusion injury.

CGRP not only decreases infarct volume but also improves brain tissue histological damage, decreases the number of red neurons, improves peri-vascular space enlargement, and reduces brain edema. The action of vasodilation of CGRP may directly help to rescue the restoration of blood flow in the penumbra region to protect neurons from ischemia damage. The indirect neuroprotective effects of CGRP may be through promoting endogenous bFGF expression to help damaged neuron recovery and inhibiting AQP4 over-expression to reduce brain edema.

It is demonstrated that both the striatum and the frontoparietal cortex showed increases of bFGF-like immunoreactivity after brain ischemia reperfusion injury. The expression of bFGF increased after focal cerebral ischemia in rats suggesting that over-expression of bFGF may play an important role in the protection of neurons (Wei et al. 2000). bFGF alleviates brain injury following global ischemia and reperfusion by down-regulating expression of inflammatory factors and inhibiting their activities (Zhang et al. 2005). In the present study, CGRP could promote endogenous bFGF expression after brain ischemia reperfusion injury. This might be one of the mechanisms of the neuroprotective effects of CGRP.

Cerebral edema is a major cause of morbidity and mortality in stroke (Frydenlund et al. 2006). AQP4 is the primary cellular water channel in the brain (Bloch and Manley 2007) and is involved in cerebral water balance (Papadopoulos and Verkman 2007). AQP4 is localized to astrocytic foot processes along the blood-brain barrier (BBB) and brain-cerebrospinal fluid interface (Bloch and Manley 2007). AQP4 has been shown to decrease osmotically-induced swelling (Kleindienst et al. 2006). It is suggested that AQP4 manipulation may serve as a novel therapeutic strategy during different periods of hypoxic-ischemic brain edema (Fu et al. 2007). In the present study, we observed that the alteration of AQP4 expression is consistent with the alterations of brain edema after brain ischemia reperfusion injury. This result is in agreement with a previous report showing that the cortical border zone displays a increase of perivascular AQP4 after reperfusion and AQP4 is critically involved in the formation and dissolution of brain edema (Frydenlund et al. 2006). We also observed that CGRP could inhibit AQP4 over-expression. The results of the present study suggest that treatment with CGRP reduces post-ischemic

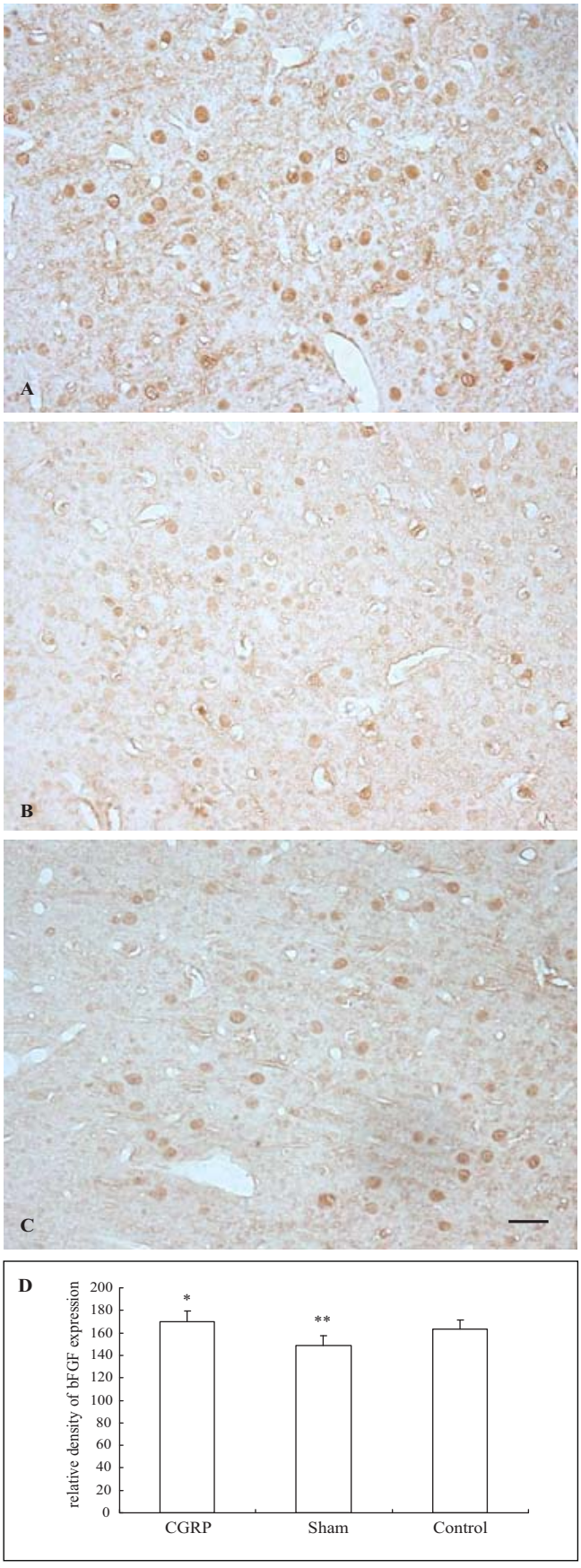

Fig. 3: Immunohistochemistry graphs show bFGF expression in ischemic penumbral zone of cortex after 2 h MCAO and 46 h reperfusion. Panel A: CGRP-treated MCAO group; Panel B: sham-operated group; Panel C: Control. Arrows represent bFGF positive cells. Scale bar = 12.5 μm. Panel D: the relatvie density of bFGF expression. Bar graphs with error bars represent mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* control, $^{**}P < 0.01$ *vs.* control

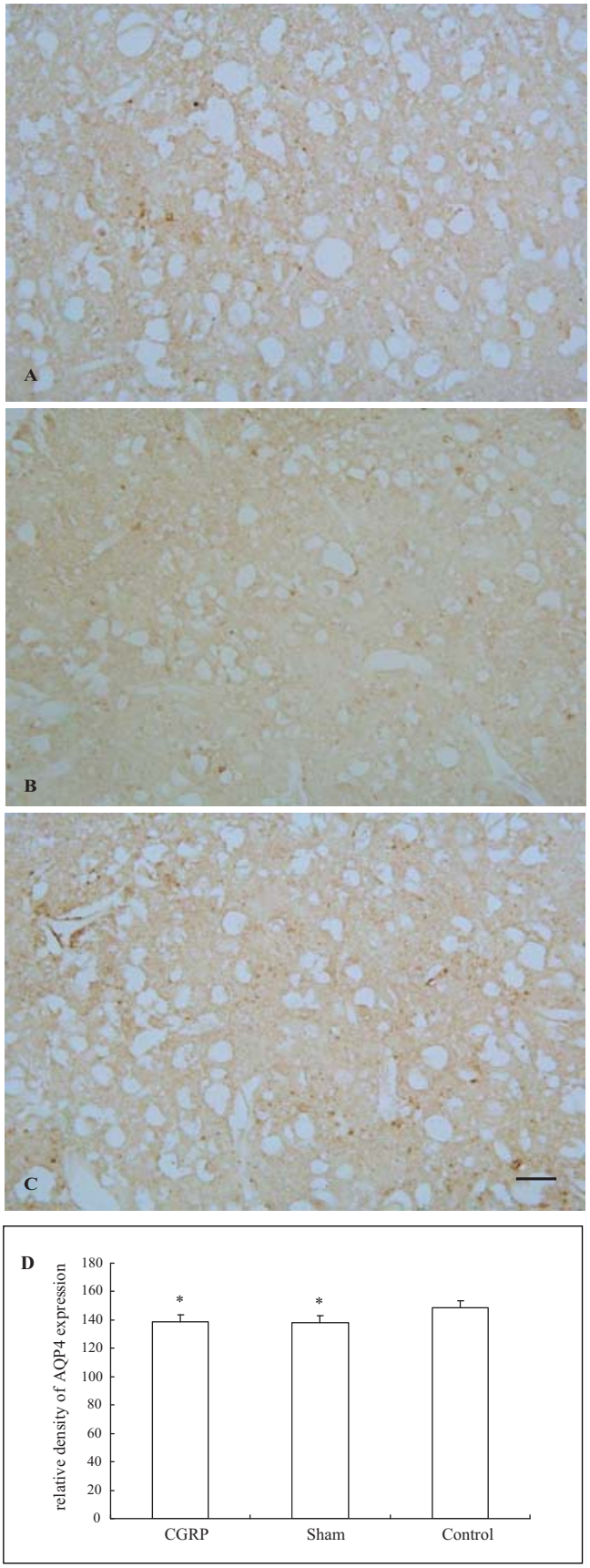

Fig. 4: Immunohistochemistry graphs show AQP4 expression in ischemic penumbral zone of cortex after 2 h MCAO and 46 h reperfusion. Panel A: CGRP-treated MCAO group; Panel B: sham-operated group; Panel C: Control. Arrows represent AQP4 positive cells. Scale bar = 12.5 μm. Panel D: the relatvie density of AQP4 expression. Bar graphs with error bars represent mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* control

cerebral edema in rats, possibly through inhibiting AQP4 over-expression in the boundary zone of ischemia.

The interesting results observed in our study may provide new insights about the therapy of brain ischemia reperfusion injury with the potent vasodialator CGRP, at least at certain dosage of CGRP and the experimental time point observed in the present study. Many factors contribute to the cellular damage resulting from ischemia reperfusion (Ishibashi et al. 2002). It is implicated that brain damage after cerebral ischemia and reperfusion is mediated by different cellular and molecular mechanisms (Reddy and Labhasetwar 2009). Experimental strategy investigation involving different neuroprotective agents has been developed to evaluate what mechanisms could be considered as a potential target in neuroprotection. Pre-clinical studies suggest that selection of agents for clinical development may not have been optimal, although the demonstration of the ischemic penumbra in animal models and the effectiveness of reperfusion therapy in humans led to considerable optimism for neuroprotection in acute stroke (Ford 2008). CGRP administration might represent only a minor narrow temporal and spacial therapeutic window of acute ischemia stroke. Yet its potential role observed in the present study needs to be further investigated.

In summary, CGRP decreases cerebral infarct volume, improves brain tissue histological damage, promotes bFGF expression, inhibits AQP4 over-expression. These results suggest that CGRP may play a neuroprotective role in cerebral ischemia reperfusion. The neuroprotective effects of CGRP may be through promoting bFGF expression and inhibiting AQP4 expression. The results may provide new directions in the development of treatment approaches for reducing the extent of brain ischemia reperfusion injury. The spatial and temporal distribution of molecules involved in the ischemic cascade by CGRP administration should be further studied.

4. Experimental

4.1. Cerebral ischemia reperfusion model of rat

Adult male Wistar rats weighing 280–320 g obtained from the Experimental Animal Center of Shandong University of China were used for this study. Animals were randomly assigned to the different treatment groups. The animals were anesthetized with 3.5% chloral hydrate solution (1 ml/100 g, i.p.). MCAO was induced by intraluminal filament method as described previously. Briefly, the left common carotid artery (CCA) and the external carotid artery (ECA) were exposed. Then, a 3-0 surgical monofilament nylon suture was carefully inserted from the external carotid artery into the internal carotid artery (ICA) and was advanced towards to occlude the origin of the left middle cerebral artery (MCA) until a light resistance was felt (18–20 mm from CCA bifurcation). Two hours after MCAO, the nylon suture was withdrawn, followed by 46 h of reperfusion. CGRP (1 μg/ml) at the dose of 3 μg/kg, i.p., was administered at the beginning of reperfusion. Saline (3 ml/kg body weight) treated animals were used as control. Sham-operated animals were also used.

4.2. Cerebral infarct volume determining

To determine the cerebral infarct volume, the rats were killed 48 h after MCAO and the brain tissue was removed and sliced coronally into 2 mm-thick sections. Brain slices were incubated in a 2% triphenyltetrazolium chloride (TTC) solution for 30 min at 37 °C and then transferred into a 4% formaldehyde solution for 48 h. The infarcted region appears white, whereas the normal non-infarcted tissue appears red. Infarct areas of the slices were measured using Image-Pro Plus software after photographed using a digital camera. Infarct volumes of each slice were calculated by multiplying the infarct area of the slice by its thickness and the total infarct volumes of each brain were calculated as the sum of the infarct volumes of each slices. The cerebral infarction was expressed as the ratio of the infarct volume to total brain volume.

4.3. HE staining

Rats were deep anesthetized and perfused intracardiovascularly by 2.5% glutaraldehyde-2% paraformaldehyde 48 h after MCAO. Then the brain

tissue was removed and hemispheres were separated. Then the samples were post-fixed in 2.5% glutaraldehyde-2% paraformaldehyde for 12 h and rinsed by PBS. After dehydrated in gradient alcohol and paraffin-embedded, the samples were cut into 5 μm-thick slices and stained with hematoxylin-eosin (HE).

4.4. bFGF and AQP4 immunostaining

To determine the expression of bFGF and AQP4 after MCAO, immunohistochemical staining was established. Briefly, the sections were deparaffinized in xylene, passed through graded alcohols and rehydrated in PBS. The sections were incubated in 3% hydrogen peroxide in methanol for 10 min at room temperature and antigen-retrieved by microwave. After being washed 3 times for 5 min with 0.01 mol/L PBS buffer, the sections were blocked with 10% goat serum for 10 min at room temperature. Then the sections were incubated with rabbit anti-bFGF IgG (1:200, Chemicon) or rabbit anti-AQP4 polyclonal IgG (1:100, Sigma) overnight at 4 °C. After being washed three times for 10 min with PBS, the sections were inbubated with biotinylated goat anti-rabbit secondary antibody (1:100) for 10 min at room temperature. Then the sections were washed with PBS and incubated with the streptavidin-HRP for 10 min at room temperature. according to SABC kit protocol. Reaction product was detected using 3,3′-diaminobenzidinetetrahydrochloride (DAB). Negative control was performed by replacing the primary antibody with PBS. Sections were dehydrated through graded alcohols, cleared in xylene and coverslipped in neutral gum. The sections were observed under light microscope. Five different visual fields were randomly selected in each slice. bFGF and AQP4 positive cells were analyzed by Image-Pro Plus software, respectively. The levels of bFGF and AQP4 expression were expressed as gray-scale value.

4.5. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student-Newman-Keuls test for significance to compare the differences among various groups. Significance was accepted at $P < 0.05$.

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