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Hypoxic preconditioning stimulates angiogenesis in ischemic penumbra after acute cerebral infarction

Sijie Li[1], Yanbo Zhang[2], Guo Shao[3], Mingfeng Yang[2], Jingzhong Niu[2], Guowei Lv[1], Xunming Ji[1]

1 *Institute of Hypoxic Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100069, China*
2 *Department of Neurology, Affiliated Hospital of Taishan Medical University, Taian 27100, Shandong Province, China*
3 *Research Center of Biology and Medicine, Baotou Medical College, Baotou 014060, Inner Mongolia Autonomous Region, China*

Research Highlights

(1) Previous studies have shown that hypoxic preconditioning had protective effects against acute cerebral infarction up to 24 hours after insult. However, 24 hours post acute cerebral infarction is not the peak period for damage following stroke. This study observed conditions at 72 hours after ischemia, the peak period for damage following stroke, to assess the protective effects of hypoxic preconditioning.
(2) Results showed that hypoxic preconditioning reduced infarct volume and attenuated the impairment of neurological function. These neuroprotective effects were related to increased vascular endothelial growth factor and CD31 expression, which promoted angiogenesis.
(3) This study lays a preliminary foundation for hypoxic preconditioning translational medicine.

Abstract

Previous studies have demonstrated the protective effect of hypoxic preconditioning on acute cerebral infarction, but the mechanisms underlying this protection remain unclear. To investigate the protective mechanisms of hypoxic preconditioning in relation to its effects on angiogenesis, we induced a photochemical model of cerebral infarction in an inbred line of mice (BALB/c). Mice were then exposed to hypoxic preconditioning 30 minutes prior to model establishment. Results showed significantly increased vascular endothelial growth factor and CD31 expression in the ischemic penumbra at 24 and 72 hours post infarction, mainly in neurons and vascular endothelial cells. Hy-Hypoxic preconditioning increased vascular endothelial growth factor and CD31 expression in the ischemic penumbra and the expression of vascular endothelial growth factor was positively related to that of CD31. Moreover, hypoxic preconditioning reduced the infarct volume and improved rological function in mice. These findings indicate that the protective role of hypoxic preconditioning in acute cerebral infarction may possibly be due to an increase in expression of vascular endothelial growth factor and CD31 in the ischemic penumbra, which promoted angiogenesis.

Key Words

neural regeneration; brain injury; hypoxic preconditioning; acute cerebral infarction; ischemic penumbra; vascular endothelial growth factor; CD31; angiogenesis; neuroprotection; grants-supported paper; neuroregeneration

Sijie Li, Master, Attending physician.

Sijie Li and Yanbo Zhang contributed equally to this work.

Corresponding author: Xunming Ji, M.D., Doctoral supervisor, Professor, Chief physician, Institute of Hypoxic Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100069, China, jixm70@hotmail.com.

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Conflicts of interest: None declared.

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INTRODUCTION

Hypoxic preconditioning is a condition where tissues and cells become tolerant to long periods of fatal ischemic/hypoxic injury following a short period of adaptation of non-fatal repetitive ischemia/hypoxia, mainly in the heart, brain, kidney, liver and intestine[1-10]. Previous studies from our group investigated the protective effects of hypoxic preconditioning and related mechanisms using behavior, physiology, morphology, neurochemistry and molecular biology. We found that the mechanism was complex, and involved animal behavior, metabolism, functional systems, neuromorphology, spinal cord activity, neural chemical composition, and molecular neurobiology[7, 11-20]. In addition, hypoxic preconditioning has been demonstrated to protect against brain ischemic injury induced by acute cerebral infarction through detection of infarct volume, neurological function assessment and cell apoptosis[17]. However, the underlying mechanism still remains uncertain.

Angiogenesis is a focus in recent studies of acute cerebral infarction, as it can significantly increase blood and nutrition supply to ischemic neurons in the ischemic penumbra. This increased flow can promote neural regeneration and synapse formation, improve neurological function, and ameliorate ischemic brain injury[21-25]. The molecular mechanisms of vascular endothelial cell mitogen, lytic enzymes of the extracellular matrix and endothelial cell migration-related signaling have been shown to participate in angiogenesis[21]. Increasing the positive effects of angiogenesis while decreasing adverse reactions is significant for developing drugs that can promote angiogenesis to treat ischemic brain injury[22].

Vascular endothelial growth factor, also known as vascular permeability factor, is a specific mitogen of endothelial cells and has the strongest specificity, playing a major regulatory role in angiogenesis and formation[26-30]. Platelet endothelial cell adhesion molecule-1, *i.e.,* CD31, expressed in blood vessel endothelium, is a commonly used index for angiogenesis[31-35]. Hu *et al* [31] reported that rehabilitation training of limbs significantly increased CD31 expression in the ischemic penumbra and improved neurological function following cerebral infarction, indicating that angiogenesis plays a critical role in recovery following cerebral infarction.

Based on previous studies, we observed vascular endothelial growth factor and CD31 changes in the ischemic penumbra following hypoxic preconditioning to investigate the protective mechanisms underlying this process during acute cerebral infarction and their relationship to angiogenesis.

RESULTS

Quantitative analysis of experimental animals

Ninety-six mice were randomly assigned to four groups: normal control (no treatment), sham-surgery (cold light irradiation with no rose Bengal), acute cerebral infarction (photochemical induction), and hypoxic preconditioning (hypoxic preconditioning + acute cerebral infarction) groups. All 96 mice were included in the final analysis.

Influence of hypoxic preconditioning on infarct volume in mice with acute cerebral infarction

Ischemic infarction foci were not observed in normal control and sham-surgery groups, but observed in acute cerebral infarction and hypoxic preconditioning groups 72 hours following cold light irradiation. The infarct volume was significantly smaller in the hypoxic preconditioning group (185.7 ± 11.7 mm^3) compared with the acute cerebral infarction group (287.7 ± 12.6 mm^3; t = –13.264, P = 0.000; Figure 1).

Influence of hypoxic preconditioning on neurological function in mice with acute cerebral infarction

Seventy-two hours after cold light irradiation, neurological function scores showed that control mice had no neurological impairment.

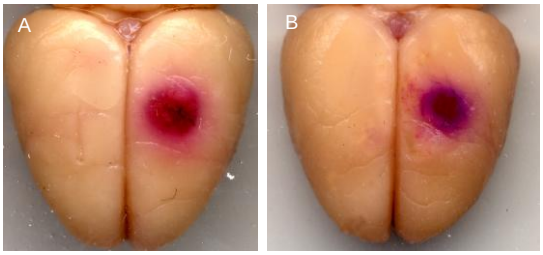

Figure 1 Influence of hypoxic preconditioning on infarct volume in mice with acute cerebral infarction.

Acute cerebral infarction group (A) revealed obvious infarction and a small ischemic penumbra; while the hypoxic preconditioning group (B) displayed a small infarction focus, and an increased ischemic penumbra. Red: Infarction focus; blue: ischemic penumbra.

No obvious neurological impairment was observed in the sham-surgery group as similar neurological function scores were obtained between this group and controls ($P > 0.05$). Symptoms of neurological impairment were found in the acute cerebral infarction and hypoxic preconditioning groups, and manifested as reduced spontaneous activity, asymmetric limb motion and creeping of the forelimbs, weakened grasping strength of the cage, and slowed reaction times. Neurological function scores were significantly reduced in the acute cerebral infarction and hypoxic preconditioning groups compared with normal control and sham-surgery groups ($P < 0.01$). Scores obtained for the hypoxic preconditioning group were higher than those for the acute cerebral infarction group ($P < 0.05$; Table 1).

Table 1 Influence of hypoxic preconditioning on neurological function scores in mice with acute cerebral infarction

Group	Neurological function scores
Normal control	17.25±1.02
Sham-surgery	17.58±1.18
Acute cerebral infarction	9.50±1.29[a]
Hypoxic preconditioning	11.75±0.96[ab]

Results are expressed as mean ± SD of six mice from each group. Neurological function was assessed according to the Garcia test[7], and higher scores indicate better neurological function. [a]$P < 0.01$ *vs.* normal control and sham-surgery groups; [b]$P < 0.05$ *vs.* acute cerebral infarction group (one-way analysis of variance, least significant difference test for paired comparison). Higher scores represent better neurological function.

Influence of hypoxic preconditioning on vascular endothelial growth factor and CD31 expression in the ischemic penumbra in mice with acute cerebral infarction

Immunofluorescent staining showed that only a small amount of vascular endothelial growth factorand CD31 expression was observed in the cortex of control and sham-surgery groups 24 and 72 hours following cold light irradiation. Vascular endothelial growth factor expression was mainly distributed in neurons of the ischemic penumbra 24 hours after cold light irradiation and distributed in vascular endothelial cells of the ischemic penumbra 72 hours after cold light irradiation in the acute cerebral infarction and hypoxic preconditioning groups (Figure 2). CD31 expression was found in vascular endothelial cells of the ischemic penumbra 24 and 72 hours after cold light irradiation in the acute cerebral infarction and hypoxic preconditioning groups (Figure 3).

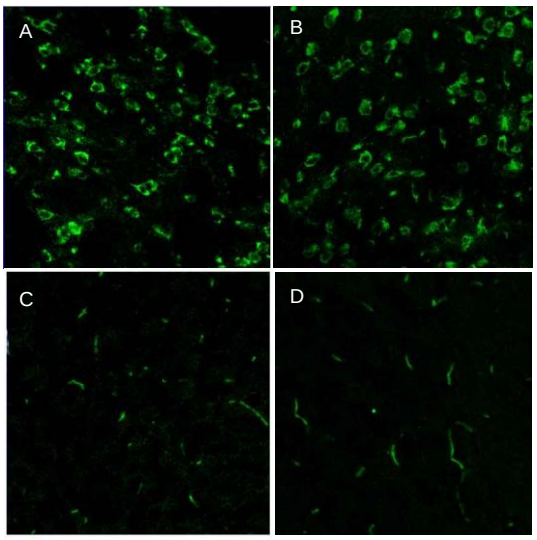

Figure 2 Influence of hypoxic preconditioning on vascular endothelial growth factor (VEGF) expression in the ischemic penumbra of mice with acute cerebral infarction (immunofluorescent staining, × 40).

Laser confocal microscopy showed that VEGF was mainly expressed in neurons of the ischemic penumbra in acute cerebral infarction (A) and hypoxic preconditioning (B) groups 24 hours following cold light irradiation. Seventy-two hours after cold light irradiation, the green fluorescence distribution migrated along blood vessels, mainly in vascular endothelial cells of the ischemic penumbra. VEGF fluorescence intensity was weaker in the acute cerebral infarction group (C) compared with hypoxic preconditioning group (D). Green: Fluorescein isothiocyanate (FITC)-labeled VEGF expression.

Influence of hypoxic preconditioning on vascular endothelial growth factor and CD31 fluorescence intensity in the ischemic penumbra of mice with acute cerebral infarction

Vascular endothelial growth factor fluorescence intensity

Statistical analysis showed that vascular endothelial growth factor fluorescence intensity in the ischemic penumbra was significantly stronger in acute cerebral infarction and hypoxic preconditioning groups compared

with normal control and sham-surgery groups ($P < 0.01$) 24 and 72 hours after cold light irradiation. Vascular endothelial growth factor fluorescence intensity in the ischemic penumbra was significantly increased in the hypoxic preconditioning group compared with the acute cerebral infarction group 24 and 72 hours after cold light irradiation ($P < 0.01$).

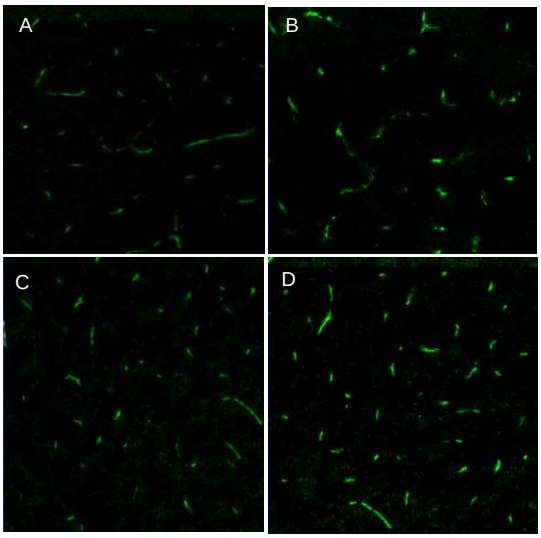

Figure 3 Influence of hypoxic preconditioning on CD31 expression in the ischemic penumbra of mice with acute cerebral infarction (immunofluorescent staining, × 40).

Laser confocal microscopy showed that green fluorescence migrated along the distribution of blood vessels 24 hours (A, B) and 72 hours (C, D) following cold light irradiation, and CD31 was mainly expressed in vascular endothelial cells of the ischemic penumbra. Fluorescence intensity was weaker in the acute cerebral infarction group (A, C) compared with the hypoxic preconditioning group (B, D). Green: Fluorescein isothiocyanate (FITC)-labeled CD31 expression.

Moreover, vascular endothelial growth factor fluorescence intensity in the ischemic penumbra was significantly stronger in the acute cerebral infarction and hypoxic preconditioning groups 24 hours after cold light irradiation compared with 72 hours after cold light irradiation (Table 2).

CD31 fluorescence intensity

Statistical analysis showed that CD31 fluorescence intensity in the ischemic penumbra was significantly stronger in the acute cerebral infarction and hypoxic preconditioning groups compared with the control and sham-surgery groups 24 and 72 hours after cold light irradiation ($P < 0.01$). CD31 fluorescence intensity in the ischemic penumbra was significantly increased in the hypoxic preconditioning group compared with the acute cerebral infarction group 24 and 72 hours after cold light irradiation ($P < 0.01$). Moreover, CD31 fluorescence intensity in the ischemic penumbra was significantly weaker in the acute cerebral infarction and hypoxic preconditioning groups 24 hours after cold light irradiation compared with 72 hours after cold light irradiation (Table 3).

Table 2 Influence of hypoxic preconditioning on vascular endothelial growth factor expression (fluorescence intensity) in ischemic penumbra of mice with acute cerebral infarction

Group	Time after cold light irradiation (hour)	
	24	72
Normal control	5.08±1.03	5.17±0.99
Sham-surgery	5.24±1.01	5.32±1.08
Acute cerebral infarction	43.98±4.94[a]	29.66±4.71[ac]
Hypoxic preconditioning	52.43±5.76[ab]	38.21±4.98[abc]

Results are expressed as mean ± SD of six mice from each group. [a]$P < 0.01$, vs. normal control and sham-surgery groups; [b]$P < 0.01$, *vs*. acute cerebral infarction group; [c]$P < 0.01$, *vs*. cold light irradiation for 24 hours (one-way analysis of variance, least significant difference test for paired comparison). Higher fluorescence intensity represents more vascular endothelial growth factor expression.

Table 3 Influence of hypoxic preconditioning on CD31 expression (fluorescence intensity) in the ischemic penumbra of mice with acute cerebral infarction

Group	Time after cold light irradiation (hour)	
	24	72
Normal control	7.73±1.06	8.08±1.01
Sham-surgery	8.11±1.23	8.34±1.23
Acute cerebral infarction	20.12±4.34[a]	24.97±4.39[ac]
Hypoxic preconditioning	28.54±5.94[ab]	33.41±4.16[abc]

Results are expressed as mean ± SD of six mice from each group. [a]$P < 0.01$, *vs*. normal control and sham-surgery groups; [b]$P < 0.01$, *vs*. acute cerebral infarction group; [c]$P < 0.01$, *vs*. cold light irradiation for 24 hours (one-way analysis of variance, least significant difference test for paired comparison). Higher fluorescence intensity represents more CD31 expression.

Correlation between vascular endothelial growth factor and CD31 expression in the ischemic penumbra post cerebral infarction

Pearson correlation analysis showed that vascular endothelial growth factor expression was positively correlated with CD31 expression in the ischemic penumbra of the acute cerebral infarction and hypoxic preconditioning groups ($r = 0.571$, $P < 0.01$; Figure 4).

DISCUSSION

Acute ischemic stroke can be severe and is associated with a high death and disability rate. Salvaging ischemic injured tissue after acute cerebral infarction is the key for treatment. Thus, research into protection methods or

drugs for ischemic brain injury has become a large area of research focus[36-41].

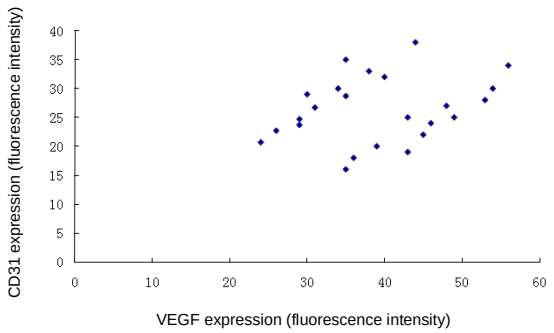

Figure 4 Correlation between vascular endothelial growth factor (VEGF) expression and CD31 expression in the ischemic penumbra of mice with acute cerebral infarction.

Pearson correlation analysis showed positive correlation between VEGF and CD31 expression (r = 0.571, P < 0.01).

Hypoxic preconditioning is an endogenous protective mechanism. It can attenuate hypoxic injury through a series of complex steps to protect the body[7]. Models of cardiac ischemia/reperfusion, cerebral ischemia/reperfusion, *ex vivo* neurons and hippocampal brain section cultures showed that hypoxic preconditioning is protective to secondary injuries[1-13]. However, the mechanisms underlying hypoxic preconditioning are very complex, involving animal behavior and metabolism, neuromorphology, spinal cord activity and nerve chemical composition, as well as molecular neurobiology[1-20].

The present study established a photochemical model of acute cerebral infarction in mice because the cranium of mice is thin, so light irradiation can pass though the bone, preventing craniotomy and increasing the survival rate. Additionally, foci of focal cerebral infarction are more consistent and the infarction process is similar to that following cerebral arterial thrombosis in humans[42]. Cerebral infarction was indeed noted in the acute cerebral infarction and hypoxic preconditioning groups, demonstrating the success of model establishment.

In this study, we prolonged observation duration post ischemia because 72 hours post acute cerebral infarction is the peak stage of cerebral edema, infarct volume and neurological function impairment[43]. Results from this study showed that hypoxic preconditioning reduced infarct volume and improved neurological function at this time point.

However, the neuroprotective mechanism of hypoxic preconditioning remains poorly understood. Some studies used general hypoxic preconditioning in mice and a middle cerebral artery occlusion model to investigate the influence of hypoxic preconditioning on ischemic brain injury. Results showed that hypoxic preconditioning can improve behaviors in model mice and reduce infarct volume and degree of edema. Hypoxic preconditioning can antagonize the reduction of new protein kinase C membrane translocation in the infarct area and reduce cAMP response element binding protein phosphorylation in the ischemic cortex. Preconditioning can also reduce cAMP response element binding protein 2 hydrolysis fragments in the ischemic penumbra, and increase phosphorylation of mitogen- and stress-activated kinase 1 and cAMP response element binding protein, thereby protecting the brain against middle cerebral artery occlusion-induced injury[44-45]. The present study also demonstrated the protective effects of hypoxic preconditioning through its influence on angiogenesis.

Angiogenesis followed by blood vessel formation is an area of focus in ischemic cerebrovascular disease research[21-25]. Vascular endothelial growth factor is a multifunctional cytokine. It can promote vasopermeability of arteriole and arterial blood vessels, proliferation and migration of vascular endothelial cells and induce angiogenesis[25-30]. Vascular endothelial growth factor and receptor expression in brain tissues is increased after ischemia, especially in vascular endothelial cells and neurons of the ischemic penumbra, thereby protecting ischemic brain tissue[25-30]. CD31 is a platelet endothelial cell adhesion factor and is expressed in blood vessel endothelium. It has been regarded as a clinical index to reflect angiogenesis[31-35]. Thus, the present study observed changes of vascular endothelial growth factor and CD31 to investigate the protective mechanism of hypoxic preconditioning.

Results showed that vascular endothelial growth factor was mainly expressed in neurons in the early ischemic period (24 hours), and in endothelial cells in later ischemia (72 hours). It is likely that after ischemia or other stress conditions, the body first protects tissues and cells with important functions, but poor tolerance. Collateral circulation is then constructed through protecting endothelial cells and promoting endothelial cell proliferation to increase blood supply and stably protect the ischemic tissues for a long period of time[25-30]. In the present study, little vascular endothelial growth factor and CD31 was expressed in the normal control and sham-surgery groups, indicating that angiogenesis is not active under normal conditions. However, vascular

endothelial growth factor and CD31 expression was significantly increased after acute cerebral infarction, indicating that angiogenesis is increased post ischemia to promote recovery of ischemic injury. Further results showed that vascular endothelial growth factor and CD31 expression was significantly increased in hypoxic preconditioning compared with the acute cerebral infarction group, and vascular endothelial growth factor expression was positively correlated with CD31 expression, indicating that hypoxic preconditioning can enhance angiogenesis in the ischemic penumbra after acute cerebral infarction and promote functional recovery. This finding suggests that hypoxic preconditioning may have protective effects on brain injury in patients with acute cerebral infarction.

Vascular endothelial growth factor functions by binding to receptors. After binding to the receptors, vascular endothelial growth factor is phosphorylated and regulates Ca^{2+} influx to function *via* the protein tyrosine kinase pathway: Vascular endothelial growth factor specifically acts on vascular endothelial cells to promote division. vascular endothelial growth factor binds receptors on vascular endothelial cells, flt-1 and flk-1 to construct collateral circulation in ischemic tissues, thereby increasing reperfusion and oxygen supply and attenuating ischemic injury. In addition, vascular endothelial growth factor directly protects nerve cells and vascular endothelial cells to promote neural regeneration[46-47]. Palmer *et al* [48] found newly generated neurons around blood vessels of the dentate gyrus and proposed neurogenesis due to vascular endothelial growth factor. Another study showed that vascular endothelial growth factor could stimulate neurogenesis in brain cell culture or in the dentate gyrus and subependymal region of mice[49]. vascular endothelial growth factor has also been shown to protect cortical neurons against hypoxic injury, stimulate axon growth and improve survival of ganglion cell in rat neck and dorsal root ganglion[50]. An *in vitro* HN33 cell model of brain ischemia showed that vascular endothelial growth factor could increase the number of surviving cells deprived of oxygen and glucose for 24 hours, but no angiogenesis was observed. These findings demonstrate the direct protective effects of vascular endothelial growth factor[51], and may indicate a novel molecule that could be used for the treatment of ischemic injury.

In summary, hypoxic preconditioning promoted angiogenesis by increasing vascular endothelial growth factor and CD31 expression in the ischemic penumbra after acute cerebral infarction, thereby protecting brain tissues against ischemic injury. However, further studies are needed to observe the long-term effects of hypoxic preconditioning as we only observed vascular endothelial growth factor and CD31 expression 24 and 72 hours after acute cerebral infarction.

MATERIALS AND METHODS

Design

A randomized and controlled animal study.

Time and setting

Experiments were conducted in the Institute of Cerebrovascular Disease, Xuanwu Hospital, Capital Medical University, China from June to September 2012.

Materials

Ninety-six BALB/c mice of clean grade, aged 6–8 weeks, weighing 18–22 g, of either gender, were provided by the Animal Department of Capital Medical University, China (license No. SCXK (Army) 2007-004). All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[52].

Methods

Establishment of the hypoxic preconditioning model

Mice in the hypoxic preconditioning group were placed in a 125-mL wide-mouthed bottle. The bottle was immediately sealed with the rubber stopper until the animals exhibited a first gasp as a tolerance limit of hypoxia. This was regarded as the first hypoxic exposure. The procedures were conducted in triplicate to reproduce a model of hypoxic preconditioning[1-10]. Mice from other groups were not exposed to hypoxic preconditioning.

Establishment of the acute cerebral infarction model

After establishment of the hypoxic preconditioning model for 30 minutes, the acute cerebral infarction model was induced using the photochemistry method[42]. Briefly, mice were anesthetized by intraperitoneal injection of 0.35 g/kg chloral hydrate at 25°C, followed by slow injection of 100 mg/kg rose Bengal (5%; Sigma, St. Louis, MO, USA) *via* the tail vein. The mouse head was fixed onto stereotaxic apparatus (type 51600; Stoelting Co., Illinois, USA), and a median incision was made at the head to expose the left cranium. The optical fiber probe of cold light [150 W, 24 V metal halide lamp (LG-150; Xuzhou Hengda Optical Electronic Instrument, Jiangsu, China); ultra-violet ray and infrared ray were filtered, with single green beam at 530 nm] was perpendicularly attached to

the exposed cranium at the site, 2 mm left of the sagittal suture and 2 mm posterior to the coronal suture. The irradiation field was 3 mm in diameter. The cold light was started 5 minutes after rose Bengal injection at an intensity of 2 lx. The incision was sutured after irradiation for 10 minutes, and disinfected with iodine tincture. The sham-surgery group was irradiated with cold light, but not injected with rose Bengal. Evans blue (1%) was injected 1 hour prior to collecting the brain tissue to allow for observation of infarct volume.

Determination of infarct volume

Six mice from each group were sacrificed 72 hours after establishment of the photochemical model. The infarct volume (length × width × height, mm^3)[17] was measured with vernier calipers (precision, 0.02 mm; Santo 8012, Shanghai Santo, Shanghai, China).

Assessment of neurological function

Six mice from each group were sacrificed 72 hours after establishment of the photochemical model. Neurological function was assessed according to the Garcia test including spontaneous activity; symmetry in four-limb movement; forepaw outstretching; climbing; body proprioception; and response to whisker touch. Each item was rated 0–3, and the sum of six tests gave a total score[7]. Higher scores indicate better neurological function.

Immunofluorescent staining for vascular endothelial growth factor and CD31 expression in the ischemic penumbra

Six mice from each group were sacrificed at 24 and 72 hours after establishment of the photochemical model. Brain tissue in the ischemic penumbra was harvested, frozen in liquid nitrogen, and sectioned (10 μm) using a freezing microtome (CM1900; Leica, Heerbrugg, Switzerland). Briefly, sections were washed with 0.1 mol/L PBS three times, 5 minutes each, and incubated with blocking goat serum (1:10 dilution; Wuhan Boster, Wuhan, Hubei Province, China). Subsequently, sections were incubated with mouse anti-human vascular endothelial growth factor monoclonal antibody and mouse anti-mouse CD31 monoclonal antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C after discarding the blocking serum. Sections were washed with 0.1 mol/L PBS twice, 5 minutes each, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG (1:100; Santa Cruz Biotechnology) at 37°C for 30 minutes. Sections were then washed with 0.1 mol/L PBS twice, 5 minutes each, and mounted with PBS glycerol (pH 8.5–9.5). The edges of the cover slip were smeared with nail polish. A Bio-Rad Radiance 2100 laser scanning confocal microscope (Hercules, CA, USA) was used to scan positive staining. Scanning parameters included excitation light wavelength 554 nm, observation light wavelength 575 nm, objective 40 × magnification with spot scanning (Zoom, 1.0). Lasersharp 2000 software (4.5.3; Bio-Rad) was used to obtain images. Fluorescence intensity of 30 randomly selected sections from each group was determined using LaserSharp 2000 software (Bio-Rad, Hercules, CA, USA) and the mean value was calculated.

Statistical analysis

Results were expressed as mean ± SD. Comparisons were conducted using one-way analysis of variance and paired comparison was performed with least significant difference test using SPSS 10.0 (SPSS, Chicago, IL, USA). Correlation between vascular endothelial growth factor and CD31 was analyzed using Pearson correlation analysis. A value of $P < 0.05$ was considered statistically significant.

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