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Research report

Morroniside promotes angiogenesis and further improves microvascular circulation after focal cerebral ischemia/reperfusion

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ABSTRACT

Preservation of cerebral microvascular functional integrity is crucial for protecting and repairing the brain after stroke. Our previous study demonstrated that morroniside promoted angiogenesis 7 days after stroke. The current study aimed to further evaluate the long-term effects of morroniside on angiogenesis and to examine whether angiogenesis induced by morroniside could improve blood flow velocity. Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO), and morroniside was then administered once per day at a dose of 270 mg/kg. New vessel formation and the expression of ephrinB2/VEGFR2 signaling pathway components were examined 14 days after MCAO to examine angiogenesis and the associated mechanisms. The dynamics of regional cerebral blood flow (rCBF) and the number of vessels of the leptomeningeal anastomoses were analyzed to characterize microvascular circulation 3 days after MCAO. We demonstrated that morroniside promoted angiogenesis by regulating the ephrinB2/VEGFR2 signaling pathway 14 days post-ischemia. By 3 days post-ischemia, morroniside improved rCBF and increased the number of vessels of the leptomeningeal anastomoses. Moreover, morroniside decreased the infarct volume and improved neurological function 14 days after MCAO. Our findings suggest that morroniside promoted long-term angiogenesis, thereby improving microvascular circulation and neurological function. It suggested that the angiogenic mechanism of morroniside might be mediated by the ephrinB2/VEGFR2 signaling pathway.

1. Introduction

Ischemic stroke is a devastating neurological disorder and one of the leading causes of morbidity and mortality (Mozaffarian et al., 2016). After ischemic stroke, blood supply to the injured brain is decreased, leading to dysfunction of the brain tissue in the lesioned area. The pathophysiological processes implicated in ischemic brain damage are complex and extensive, including bioenergetic failure, acidotoxicity, excitoxicity, oxidative stress and inflammatory reaction (Amantea et al., 2014; Brassai et al., 2015; Zhou et al., 2016). Thrombolytic therapy remains the only beneficial therapy for ischemic stroke, but the use of thrombolytic therapy is restricted to a short therapeutic window of time following the infarct (Gursoy-Ozdemir et al., 2012). Restoring cerebral blood flow to the ischemic region sooner may save the brain tissue from death and improve patient recovery (Green, 2008). It has been suggested that augmenting microvascular circulation may be neuroprotective during stroke.

Angiogenesis is a critical process in microvascular formation as well as revascularization after cerebral ischemia. Efficient and functional vessel formation in the ischemic boundary zone can provide patients with sufficient blood flow and reduce the infarct volume (Chen et al., 2009). The growth of newly formed microvessels provides the necessary neurotrophy to support newly generated neurons (Chen et al., 2005). These vessels also serve as routes for neuroblast movement into the recovering peri-infarct regions. Thus, Improvement of microvascular circulation through enhanced

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angiogenesis might be a novel strategy for angiogenesis-based therapies after stroke.

Morroniside is one of the most abundant iridoid glycosides extracted from *Cornus officinalis*. We have previously reported the anti-oxidant and anti-apoptotic effects of morroniside (Wang et al., 2008, 2009, 2010a). Further studies demonstrated that morroniside promoted angiogenesis 7 days after stroke (Sun et al., 2014). In the present study, we determined the long-term effects of morroniside on angiogenesis and tested whether the natural compound morroniside could enhance the blood flow velocity, eventually improved neurological function. Our study represents an attractive therapeutic strategy for stroke treatment by promoting angiogenesis to improve microvascular circulation via exogenous drugs.

2. Materials and methods

2.1. Morroniside extraction and identification

The sarcocarp of *C. officinalis* was purchased from Tong Ren Tang Company, Beijing, China. Morroniside was extracted from the sarcocarp of *C. officinalis* and purified as previously described (Wang et al., 2010a). The final purity was determined to be 98.5%.

2.2. Animals

Male Sprague-Dawley (SD) rats weighing 260–280 g were purchased from Beijing Vital River Experimental Animal Co. (Beijing, China) and were housed under a 12/12 h dark/light cycle and specific-pathogen-free (SPF) conditions. The ambient temperature and relative humidity were maintained at $22 \pm 2\,^{\circ}C$ and $55 \pm 5\%$, respectively, and the animals were given standard chow and water ad libitum for the duration of the study. The animal protocols used in these studies were approved by the Animal Care and Use Committee of Xuanwu Hospital of Capital Medical University, China.

2.3. MCAO model

After the rats were fasted without water deprivation for 12 h, focal ischemia was induced in enflurane-anesthetized rats for 30 min via intraluminal vascular occlusion of the middle cerebral artery as described previously (Atchaneeyasakul et al., 2016; Cao et al., 2001; Kumar et al., 2016; Longa et al., 1989). A piece of nylon monofilament was inserted into the left internal carotid artery via arteriotomy and lodged in the narrow proximal anterior cerebral artery to block the middle cerebral artery (MCA) at its origin. After 30 min of ischemia, reperfusion was established by filament withdrawal. When the cerebral and rectal temperatures returned to normal levels, the animals were allowed to regain consciousness and were placed under warm conditions for an additional 3 h. Sham operations were performed using the same anesthesia and surgical procedures, but without insertion of the intraluminal filament.

The rats were randomly divided into three groups: (1) sham-operated group, (2) vehicle-treated ischemic model group, and (3) $270\,mg\,kg^{-1}\,day^{-1}$ morroniside-treated ischemic group. Morroniside was dissolved in normal saline and administered intragastrically once daily starting at 3 h after MCAO. The vehicle control groups of ischemic and sham-operated rats received an equal volume of normal saline. Body weights were collected every two days after MCAO.

2.4. Laser speckle flowmetry

The anesthetized rats were placed in a stereotaxic apparatus. After the midline scalp was shaved, disinfected and sliced, the galea and periosteum overlying the parietal bone were swept. The regions of interest (ROIs), each with a 2.5 mm × 5 mm area that was centered at 2.5 mm posterior and 2.5 mm lateral to the bregma over the left and right cortex, were thinned using a high speed dental drill (SDE-H37L, Marathon, Korea) until the inner cortical layer of bone was encountered. Images were acquired at 23 fps (exposure time T = 5 ms) by a laser speckle imaging system PeriCam PSI (Perimed, Stockholm, Sweden) with a laser diode (780 nm; Dolphin BioTech Ltd., Shanghai, China) over the skull and processed by the random process estimator (RPE) method. One blood flow image was generated by averaging the numbers obtained from 20 consecutive raw speckle images. The cerebral blood flow (CBF) in a region of interest was obtained by using the pallet software installed in the Omegazone imaging system. CBF was measured at three time points: before MCAO, before reperfusion after MCAO and 3 days after MCAO. During the CBF measurement, animals were maintained under 1.5% isoflurane anesthesia and the body temperature was maintained at 37 °C.

2.5. Latex perfusion

At 3 days after MCAO, latex perfusion was performed to visualize the leptomeningeal anastomoses as previously reported (Wang et al., 2013). Under deep anesthesia, the auricle of the right atrium was incised to allow venous outflow. The left ventricle of the heart was cannulated and injected at 150 mm Hg with 100 mL of saline and 20 mL of latex compound (Bai Ta, Beijing, China) mixed with 50 μL/mL carbon black (Bokusai, Fueki, Inc., Tokyo, Japan). The brain was then removed from the skull, and photographs of the surface of the brain were taken with a stereomicroscope (Olympus, Tokyo, Japan) to assess the number of vessels of the leptomeningeal anastomoses in ipsilateral hemispheres.

2.6. Immunofluorescence analysis

Single-immunofluorescence staining was used to visualize lectin-labeled vessels. Double-immunofluorescence was used to visualize cells labeled with lectin and VE-cadherin, 5-bromo-2′-deoxyuridine (BrdU), p-ephrinB2, or p-VEGFR2. BrdU (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally twice daily during days 7–13 after MCAO. Next, rats were perfused with 4% paraformaldehyde in PBS. Ice-cold sections (20 μm) of the brains were prepared using standard protocols. For BrdU staining, the sections were pretreated with 50% formamide/280 mM NaCl/30 mM sodium citrate at 65 °C for 2 h, incubated in 2 N HCl at 37 °C for 30 min, and rinsed in boric acid (0.1 M, pH 8.5) at room temperature for 10 min. After incubation with a blocking solution, all sections were incubated with primary antibodies, including mouse anti-BrdU (1:200, Roche, Indianapolis, IN, USA) and lectin (1:400, Vector Laboratories, Burlingame, CA, USA). Other immunofluorescence staining was conducted using ice-cold sections (20 μm) permeabilized with 0.1 M phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 30 min, blocked for 2 h at room temperature with 5% donkey serum (Jackson ImmunoResearch Laboratories, Philadelphia, PA, USA), and incubated with combinations of the following primary antibodies: lectin (1:400, Vector Laboratories, Burlingame, CA, USA), rabbit anti-VE-cadherin (1:200, Abcam, Cambridge, MA, USA), rabbit anti-p-ephrinB2 (Y316) (1:200, Abcam, Cambridge, MA, USA), mouse anti-p-VEGFR2 (Y1175) (1:200, Abcam, Cambridge, MA, USA) for 48 h at 4 °C. Appropriate Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies were used to detect the primary antibody (Life Technologies, Carlsbad, CA, USA). A mounting medium with 4′, 6′-diamidino-2-phenylinidole (DAPI) (Abcam, Cambridge, MA, USA) was used to label cell nuclei. The fluorescence signals were visualized using a Nikon 80i fluorescence microscope.