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Angiogenesis effects of 4-methoxy benzyl alcohol on cerebral ischemia-reperfusion injury via regulation of VEGF-Ang/Tie2 balance

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Abstract

Introduction: Angiogenesis facilitates the formation of microvascular networks and promotes neurological deficit recovery after cerebral ischemia-reperfusion injury (CIRI). This study investigated the angiogenesis effects of 4-methoxy benzyl alcohol (4-MA) on CIRI.

Methods: The angiogenesis effects of 4-MA and the potential underlying mechanisms were assessed based on a middle cerebral artery occlusion/reperfusion (MCAO/R) rat model and a hind limb ischemic (HLI) mouse model. Immunofluorescence was conducted to detect microvessel density and western

blotting and polymerase chain reaction were performed to determine the expression of angiogenesis-promoting factors. In addition, we investigated whether the angiogenesis effects of 4-MA caused damage to the blood-brain barrier (BBB).

Results: After treatment with 4-MA (20 mg/kg) for 7 days, the neurological deficits recovered and microvessel density in the cerebral cortex increased in the MCAO/R rats. Additionally, 4-MA also regulated the expression of angiogenesis factors, with an increase in Vascular endothelial growth factor (VEGF) and Vascular endothelial growth factor receptor2 (VEGFR-2) expression and a decrease in Angiopoietin 1(Ang-1), Ang-2, and Tie-2 expression in both MCAO/R rats and HLI mice. Moreover, 4-MA increased the expression of angiogenesis-promoting factors without exacerbating BBB cascade damage in MCAO/R rats.

Conclusions: Our results indicated that 4-MA may contribute to the formation of microvascular networks, thus promoting neurological deficit recovery after CIRI.

Keywords: 4-methoxybenzyl alcohol, angiogenesis, vascular maturation, BBB permeability

Introduction

Stroke is the second leading cause of death worldwide and is considered one of the greatest challenges to human health by the World Health Organization (Peng et al. 2019). Ischemic stroke (IS) is common and accounts for 60%–80% of all strokes (Campbell et al. 2019). The pathological reasons for cerebral arterial occlusion in IS are complex and can result in ischemia, hypoxia, and necrocytosis of cerebrally damaged areas as well as corresponding neurological deficits (Zhang et al. 2012). Furthermore, after IS, thrombus autolysis and the use of thrombolytic agents, such as recombinant tissue plasminogen activator (r-tPA), can also cause cerebral ischemia-reperfusion injury (CIRI), neurological damage, and morphological changes in cerebrally injured regions (Liu et al. 2018; Yamashita et al. 2009). As such, IS demonstrates high disability and mortality rates. Effective prevention has therefore become an important field in basic and clinical research. Currently, the best IS

treatments include rapid reperfusion and effective circulation in the cerebral cortex by intravenous thrombolysis and surgery in order to ensure blood supply to the ischemic area, thereby limiting reversible damage to tissue and reducing disability and mortality (Knecht et al. 2017; Powers et al. 2019). However, most clinical IS patients receive a poor prognosis despite treatment because the therapeutic time window for effective r-tPA is narrow and the anatomical characteristics of cerebral vessels increase surgical difficulty (Reeves et al. 2005). Several studies have reported that neurons in central ischemic areas can suffer irreversible necrocytosis after 8–10 min of cerebral ischemia (Heo et al. 2005), whereas peripheral ischemic areas experience milder insults due to residual perfusion from collateral blood vessels, resulting in damaged nerve function but intact neurons, thus forming a salvageable ischemic penumbra (IP) (Arai et al. 2009; Xiao et al. 2020). In recent years, many IS studies have investigated IP rescue and confirmed the positive effect of IP function repair on IS prognosis (Lange et al. 2016). In the central nervous system (CNS), neurons and vessels exhibit close functional connectivity and crosstalk guided by similar signals, with both residing together and neurons interacting with corresponding capillaries (Miller 2002; Quaegebeur et al. 2011; Zhai and Feng 2019). Thus, the key factor in maintaining nerve function is blood flow within the brain. Increasing and re-establishing the blood supply to the IP is an effective way in which to save this area (Chimowitz 2013; Moskowitz et al. 2010; Seet et al. 2012). Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels, with new capillaries able to be formed within a few days of ischemia (Arai et al. 2009; Paronis et al. 2020). This can effectively re-establish the blood vessel network to supply the ischemic area within a relatively short time. Thus, promoting angiogenesis can accelerate the repair of nerve function in ischemic areas.

There is a certain time correlation between the expression of early CIRI pro-angiogenesis factors and permeability of the blood-brain barrier (BBB). At 2–18 h after CIRI, the BBB is destroyed, tight junction proteins claudin-5 and occludin are down-regulated (Komarova et al. 2017; Lakhan et al. 2013), and the first permeability peak appears. When hypoxic factors induce angiogenesis in the ischemic area, the

expression levels of pro-angiogenesis factors Vascular endothelial growth factor A(VEGF-A) and Angiopoietin 2(Ang-2) increase, while the expression of Ang-1 decreases. At 18–36 h, intracranial pressure increases, fibrin and platelets block microvessels, and BBB permeability decreases. At 48–72 h, the pro-angiogenesis factors VEGF-A, Angiopoietin 1(Ang-1), and Ang-2 gradually increase, thus promoting endothelial cell proliferation and budding(Jin et al. 2010). At the same time, the second BBB permeability peak appears(Nourhaghighi et al. 2003), suggesting that angiogenesis regulators may be involved in the formation of the second BBB permeability peak.

As a traditional Chinese medicinal herb, *Gastrodia elata* Blume (GEB) can treat many symptoms in the nervous system, such as language paralysis, trances, headaches, and dizziness, which commonly occur with IS (Duan et al. 2015). We previously showed that ethyl acetate extracts (EtOAc) of GEB can reduce ischemic injury induced by transient focal cerebral ischemia in rats. This may be due, at least partially, to attenuation of the apoptotic pathway (Duan et al. 2015) and decrease in BBB permeability (He et al. 2016).

4-Methoxy benzyl alcohol (4-MA, Fig. 1) is a major active compound in GEB (Duan et al. 2013). Our previous study indicated that 4-MA can protect the brain against CIRC in focal cerebral ischemic rats and promote the migration, proliferation, and tube formation of primary brain microvascular endothelial cells in such rats *in vitro* (Jia Y 2017). However, the role of 4-MA in promoting the functional development of new blood vessels remains to be elucidated. In the current study, We replicated the middle cerebral artery occlusion/reperfusion (MCAO/R) model in rats to explore the angiogenesis effect of 4-MA and the potential mechanism after CIRC. The hind limb ischemia (HLI) model in mice was also used to determine whether it can promote angiogenesis in peripheral ischemic sites. The model can better simulate the pathological state of lower limb ischemia in clinical patients, and reflect the multiple stages of severe ischemia and insufficient blood supply in lower limbs, including the process of occurrence, evolution and repair (Magalotti et al. 2013). The microvessel density (MVD) was measured by immunofluorescence, and the

expression of angiogenic factors was detected by Western blotting and quantitative polymerase chain reaction (qPCR). In addition, we also investigated whether the angiogenesis of 4-ma impairs BBB.

Methods

Animals

Adult male Sprague Dawley (SD) rats (250–300 g) were used in all experiments. The rats were reared adaptively under standard conditions (i.e., 25 ± 1 °C room temperature, 65%–70% relative humidity, and 12-h light and dark cycle), with fresh food and drinking water provided *ad libitum*. All animal experimental procedures were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Furthermore, this study was approved by and carried out in accordance with the guidelines of the Experimental Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine (Kunming, China) (R-062016003). All reasonable efforts were made to minimize animal suffering.

Preparation of MCAO/R rat model

The MCAO/R model was prepared via the suture method described in our previous research (Bederson et al. 1986; He et al. 2017; He et al. 2016). Rats were fasted overnight for 12 h before the experiment. After anesthesia using box induction with 5% isoflurane, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated. The proximal ends of the CCA and ECA were ligated. A small cut was made at the stump of the CCA, with the spherical end of a thread bolt then inserted 18 ± 2 mm into the ICA. After 2 h, the thread bolt was pulled out for reperfusion. Body temperature was maintained at 37.0 ± 0.5 °C during the entire procedure. Rats in the sham group underwent the same surgery, but without suture insertion.

Evaluation of neurological deficits

Neurological scores were determined as per earlier research (Bederson et al. 1986; He et al. 2017; He et al. 2016). Briefly, the severity of neurological deficits was evaluated using a 5-point deficit score system 2 h after brain ischemia (0 = normal motor function; 1 = flexion of torso and of contralateral forelimb on lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = leaning to the contralateral side at rest; and 4 = no spontaneous motor activity). Neurological score recording was performed once a day at a fixed time for 7 days.

Preparation of 4-MA

In brief, 200 mg of 4-MA was first weighed out with an analytical balance, after which 1 mL of Tween-80 was added to aid dissolution and dilution in double-distilled water to a volume of 100 mL. The solution was then stored at 4 °C in the dark.

Groups and 4-MA administration in MCAO/R rats

After 2 h of reperfusion, rats were divided into three groups based on neurological scores: (1) 4-MA group: rats were treated with 20 mg/kg 4-MA (J&K Scientific, Beijing, China); (2) sham group: rats were treated with the same volume of water; and (3) model group: rats were treated with 1 mL/100 g distilled water. Intragastric administration of the test substance was performed once a day for 7 days. The rats were placed into cages in groups for recovery with free access to food and water. All groups consisted of at least six rats and three independent experiments were conducted for each treatment.

Immunochemical analysis of cerebral cortex in MCAO/R rats

After MCAO/R, rats were administered treatment for 7 days (drug delivery as described previously). Rats were first anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), followed by 100 mL of normal saline for cardiac perfusion and 200 mL of phosphate-buffered saline (PBS) perfusion containing 4% paraformaldehyde (pH 7.4). Brain tissues were harvested, fixed in 4% paraformaldehyde solution at 4 °C for 24 h, and dehydrated in a 20% to 30% sucrose gradient.

Frozen 20- μ m-thick brain sections were cut using a freezing microtome (CRYOSTAR NX50, Thermo Fisher Scientific, USA). The brain sections were then blocked by 50% goat serum (Solarbio, Beijing, China) and incubated at 4 °C for 12 h with specific primary antibodies: i.e., rabbit anti-NeuN antibody (1:200, Abcam, UK), rabbit anti-CD34 antibody (1:300, Abcam, UK), and rabbit anti-VEGF-A antibody (1:100, Abcam, UK). After washing three times in PBS, the NeuN and CD34 sections were incubated with the secondary antibody goat anti-rabbit IgG H&L (Alexa Fluor 488, 1:500, Abcam, UK) at 37 °C for 2 h, and the VEGF-A sections were incubated with the secondary antibody goat anti-rabbit IgG H&L (Cy3, 1:500, Abcam, UK) at 37 °C for 2 h. The sections were then washed with 0.01 M PBS (Solarbio, Beijing, China) and labeled using DAPI (Fluoroshield Mounting Medium with DAPI, Abcam, UK). Images were obtained using a fluorescence microscope (Ci-L, Nikon, Japan). Positive NeuN and VEGF-A expression was visualized under a microscope (20 $\times$ objective), and five images of each region were taken for counting fluorescent area using ImageJ software (<http://rsb.info.nih.gov/ij/>). In addition, the three regions with the highest CD34-positive density were selected under a microscope (4 $\times$ objective), and five images of each region were taken for counting (20 $\times$ objective).

Western blot analysis in cerebral cortex of MCAO/R rats

Western blot analysis was performed as described previously (He et al. 2017; He et al. 2016). The right parietal lobe cortex was homogenized in radio immunoprecipitation assay buffer with protease inhibitors (Solarbio, Beijing, China). The homogenates were centrifuged at 7 000 $\times g$ for 3–5 min at 4 °C, and the supernatant was collected for western blot analysis. Protein concentration in the supernatants was determined using BCA kits (Beyotime, Shanghai, China). Samples containing 100 μ g of protein were loaded onto 10% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride membranes (Amersham, Piscataway, Nanjing, China). The membranes were incubated at 4 °C for 12 h in specific primary antibodies: i.e., VEGF-A (1:200, Abcam, UK), VEGFR-2 (1:250, Cell Signaling Technology, USA), Ang-1 (1:500,

Abcam, UK), Ang-2 (1:1 000, Abcam, UK), and Tie-2 (1:250, R&D Systems, USA). After thrice washing with Tris-buffered saline Tween, the membranes were then incubated in a solution containing rabbit anti-goat IgG (H+L) (1:1 000, Proteintech, USA) at 37 °C for 2 h. The immunoreactive bands were visualized using an ECL western blot detection kit (BI, USA) on a light-sensitive film and imaged with a western blot imaging system (UVP). Images were analyzed with ImageJ software by their gray values.

Preparation of HLI mouse model

The left femoral artery complete occlusion hind limb ischemic model was prepared as described previously (Jazwa et al. 2013; Wang et al. 2014). The mice were anesthetized with isoflurane (Yiping Pharmaceutical, Hebei, China) using an animal respiratory anesthesia machine (Matrx, USA), with a 37 °C electric heating plate placed under the abdomen of the mice to ensure a constant temperature. The skin was opened with a 0.5-cm incision in the groin, followed by careful separation of the femoral nerve, vein, and artery. The mice were subjected to unilateral hind limb ischemia by complete occlusion of the left femoral artery. Blood flow in both hind limbs was measured by a laser speckle instrument (PeriCam PS, Sweden). After operation, the flow ratio was reduced to less than 50% of the standard. All mice were then divided randomly into three groups: (1) 4-MA group: mice were treated with 28 mg/kg 4-MA; (2) sham group: mice were treated with the same volume of water; and (3) model group: mice were treated with 1 mL/100 g distilled water. Intragastric administration of the testing substances was performed once a day for 7 days. The mice were placed into cages in groups for recovery with free access to food and water. After the operation, the blood flow of both hind limbs was monitored for 7 days by laser speckle to determine dynamic changes in hind limb blood flow perfusion.

Laser speckle scanning of lower limb blood flow in mice

Mice were anesthetized with isoflurane and placed on a 37 °C constant temperature heating plate. Hair around the groin area was removed with depilatory cream, followed by cleaning with warm normal saline and disinfection with iodophor.

The foot soles of the lower limbs were placed in the infrared cross projection area of the laser speckle spectrometer, and the position was adjusted and aligned. Blood flow in the hind limbs of mice was monitored, and the basic blood flow value of the lower limbs was recorded.

Immunochemical analysis (Ki67/tomato-lectin/DAPI) of gastrocnemius muscle in HLI mice

Frozen 10-um-thick sections of gastrocnemius muscle were cut using a freezing microtome (CRYOSTAR NX50, Thermo Fisher Scientific, USA). The sections were blocked by 50% goat serum (Solarbio, Beijing, China) and incubated in specific primary antibody rabbit anti-Ki67 (1:100, Abcam, USA) at 4 °C for 12 h. After washing three times with 0.01 M PBS (Solarbio, Beijing, China), the sections were incubated with goat anti-rabbit IgG H&L (Alexa Fluor 488, 1:500, Abcam, USA) secondary antibody and tomato-lectin (Texas Red-labeled *Lycopersicon esculentum* (Tomato)-Lectin, 1:250, Vector Laboratories, USA) at 37 °C for 2 h. The sections were then washed with 0.01 M PBS (Solarbio, Beijing, China) and labeled with DAPI (Fluoroshield Mounting Medium with DAPI, Abcam, USA). Images were obtained with a fluorescence microscope (Ci-L, Nikon, Japan). Positive microvessels (Ki67/tomato-lectin/DAPI) were observed by the treble-labeling method.

Quantitative real-time PCR

Total RNA was extracted from the gastrocnemius muscle of HLI model mice using TRIZOL reagent (Invitrogen, USA) as per the manufacturer’s protocols. Equal amounts of RNA were reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara, Japan). Primers for all tested subunits of angiogenesis-promoting factors and GAPDH in HLI model mice are listed in Table 1. Real-time PCR was performed using an Applied Biosystems QuantStudio 5 (ABI, USA) with 20-μl reaction mixtures containing cDNA, primers, and PowerUp™ SYBR Green Master Mix (ABI, USA). The PCR amplifications were carried out in two stages (40 cycles): hold stage, uracil-DNA glycosylase (UDG) activation at 50 °C for 2 min and

Dual-Lock™ DNA at 95 °C for 2 min; PCR stage: denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Results of the log-linear phase of the growth curve were analyzed and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method with GAPDH as an internal standard (Yang et al. 2016).

BBB permeability determination in early damage stage in MCAO/R rats

To clarify the time points of the two permeability peaks in the BBB during early CIRI, BBB permeability was determined. Rats were divided randomly into 10 time point groups: i.e., 15 min, 30 min, 1 h, 2 h, 4 h, 12 h, 24 h, 48 h, 72 h, and 96 h after reperfusion. The MCAO/R model was duplicated in rats, which were then dissected at different time points, with 2% Evans blue (EB, Sigma, USA) solution intravenously injected (0.4 mL/100 g) 2 h before dissection. After rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), the thoracic cavity was opened and perfused with normal saline. Brain tissue was harvested, and the hemispheres were separated and weighed, then dried at 105 °C for 24 h for measurement of dry weight. The hemispheres were then incubated in formamide solution (Sigma, USA, 1 mL/each hemisphere brain tissue) at 50 °C for 24 h. Optical density values of the formamide solution at 610 nm were measured with a multi-plate reader (Infinite M200 PRO, Tecan, Switzerland). Results were calculated according to a standard curve.

EB and FITC-dextran double-labeling fluorescence in early damage stage in MCAO/R rats

Rats were randomly divided into three groups, i.e., 4-MA, sham operation, and model groups (drug delivery as described previously). The MCAO/R model was duplicated, with 2% EB (Sigma, USA) solution injected intraperitoneally (0.4 mL/100 g, 15 min after ischemia) and test substances administered intragastrically. At 48 h after reperfusion, rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), followed by an intraperitoneal injection of 5% FITC-dextran (Sigma, USA) solution (0.18 mL/100 g). After 5 min, brain tissues were

harvested, fixed in 4% paraformaldehyde, and dehydrated in a 20% to 30% sucrose gradient.

Frozen 50- μ m-thick brain sections were cut using a freezing microtome (CRYOSTAR NX50, Thermo Fisher Scientific, USA). After drying, the sections were washed three times in 0.01 M PBS (Solarbio, Beijing, China). Images were obtained by a fluorescence microscope (Ci-L, Nikon, Japan). BBB leakage (EB) and microvessels (FITC-dextran) were observed using the double-labeling method. Areas were counted as follows: five regions with FITC-dextran positive (green) expression were selected under a microscope (10 $\times$ objective), and EB exudation (red) of each section was observed in the central and peripheral cerebral ischemic areas. Images were analyzed with ImageJ software and microvessel density was evaluated based on FITC-dextran fluorescent areas.

Results

Neural repair effects of 4-MA in MCAO/R rats

Compared with the sham group, obvious neurological deficits were observed in the MCAO/R rats. In the MCAO/R group, neurological scores increased significantly ($P < 0.001$, Fig. 2A) and NeuN expression decreased significantly ($P < 0.001$, Fig. 2B and C). With 4-MA (20 mg/kg) pretreatment, however, neurological scores decreased significantly ($P < 0.001$, Fig. 2A) and NeuN expression increased significantly ($P < 0.001$, Fig. 2B and C).

Angiogenesis effects of 4-MA in MCAO/R rats

Compared with the sham group, the cerebral blood flow ratio decreased significantly ($P < 0.001$, Fig. 3A and B) and CD31 ($P < 0.001$, Fig. 3C) and VEGF-A expression ($P < 0.001$, Fig. 3D) increased significantly in the model group. With 4-MA (20 mg/kg) pretreatment, however, the cerebral blood flow ratio increased significantly ($P < 0.001$, Fig. 3A and B) and CD31 ($P < 0.001$, Fig. 3C) and VEGF-A expression ($P < 0.01$, Fig. 3D) decreased significantly.

Effects of 4-MA on protein expression of angiogenesis-promoting factors in MCAO/R rats

Compared with the sham group, the expression levels of VEGF-A ($P < 0.01$, Fig. 4A) and Ang-1 ($P < 0.05$, Fig. 4C) increased significantly and the expression levels of VEGFR-2 (Fig. 4B), Ang-2 (Fig. 4D), and Tie-2 (Fig. 4E) showed an increasing trend in the model group. Compared with the model group, after 4-MA (20 mg/kg) pretreatment, Ang-2 expression (Fig. 4C) increased significantly and VEGFR-2 (Fig. 4B), Ang-1 (Fig. 4C), Ang-2 (Fig. 4D), and Tie-2 expression (Fig. 4E) showed an increasing trend.

Angiogenesis effects of 4-MA in HLI mice

Compared with the sham group, the hind limb blood flow ratio in the model group decreased ($P < 0.001$, Fig. 5A and B), whereas, after pretreatment with 4-MA (28 mg/kg), the hind limb blood flow ratio increased ($P < 0.01$, Fig. 5A and B). Furthermore, the capillary density of the ischemic gastrocnemius muscle increased upon IS. After 7 days, the gastrocnemius muscle on the ischemic side of the sham group was arranged in a rhomboid order, with uniform distribution of tomato-lectin between the muscle fibers and almost no expression of Ki67 (Fig. 5C). In the model group, however, the gastrocnemius muscle fibrocytes were obviously atrophic, connective tissue was hyperplastic, and tomato-lectin and Ki67 showed increased expression (Fig. 5C). After administration of 4-MA for 7 days, the expression of tomato-lectin and Ki67 in the gastrocnemius muscle increased in the 4-MA group compared with that in the model group (Fig. 5C).

Effects of 4-MA on protein expression of angiogenesis-promoting factors in HLI mice

Compared with the sham group, the expression levels of VEGFR-2 ($P < 0.05$, Fig. 6B), Ang-1 ($P < 0.01$, Fig. 6C), Ang-2 ($P < 0.05$, Fig. 6D), and Tie-2 ($P < 0.01$, Fig. 6E) increased significantly in the HLI group. Compared with the HLI group, after pretreatment with 4-MA (20 mg/kg), the expression levels of VEGF-A ($P < 0.001$,

Fig. 6A), VEGFR-2 ($P < 0.001$, Fig. 6B), Ang-1 ($P < 0.05$, Fig. 6C), Ang-2 ($P < 0.01$, Fig. 6D), and Tie-2 ($P < 0.01$, Fig. 6E) increased significantly.

Effects of 4-MA on BBB permeability in early stage of CIRI

In the early stage of CIRI, as pathological damage progressed, the permeability of the BBB increased significantly, but showed a biphasic permeability phenomenon, with two permeability peaks at 4 h and 48 h of reperfusion ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; Fig. 7A and B). Compared with the sham group, the expression of FITC-dextran and EB declined (Fig. 7C and E) and microvessel density decreased significantly ($P < 0.001$, Fig. 7D and F) in the MCAO/R rats after reperfusion for 4 h and 48 h. Furthermore, compared with the model group, after pretreatment with 4-MA (20 mg/kg), the expression of FITC-dextran and EB increased (Fig. 7C and E) and microvessel density increased significantly ($P < 0.001$, Fig. 7D and F).

Discussion

Angiogenesis is an endogenous repair mechanism that can rapidly re-establish the vascular network in peripheral areas around ischemic sites to repair damaged tissue (Herbert and Stainier 2011; Kovacs et al. 1996; Seto et al. 2016). Both survival time and prognosis in IS patients are closely related to neovascularization density, with better recovery found in patients with high neovascularization density than in those with low neovascularization density (Arenillas et al. 2007; Greenberg 2014; Zhang et al. 2002). Thus, angiogenesis can have a positive impact on development and prognosis in IS patients. However, the self-protection and repair mechanism of the brain is restricted by many physiological and pathological factors. For example, advanced age, increased blood pressure, hyperlipidemia, and increased blood sugar reduce vascular regulation and endothelial function, resulting in a reduction in the compensatory building capacity of collateral blood circulation in the brain (Iadecola and Davisson 2008; Omura-Matsuoka et al. 2011; van Weel et al. 2006), thus limiting the self-repair effect of endogenous angiogenesis. With the extension of time, the endogenous self-repair effect gradually weakens, and it is difficult to avoid the formation of cerebral infarction. Therefore, the use of exogenous factors to mobilize

blood vessel rebirth and reconstruct and improve blood supply has practical significance for reducing infarct volume, promoting structural remodeling of damaged brain tissue, and accelerating damaged nerve function repair.

To the best of our knowledge, this study is the first to report on the role of 4-MA in promoting angiogenesis in the pathological process of ischemia. Results showed that 4-MA increased cerebral blood flow and microvessel density in the cerebral cortex of MCAO/R rats (Fig. 2) and increased blood flow and microvessel density in lower limb ischemic areas in HLA mice (Fig. 4). The evidence of increased microvessel density was derived from the positive expression of CD34, Ki67, and tomato-lectin. CD34 is expressed in the proliferation, movement, and migration stages of endothelial cells, but is not expressed after the formation of tight junctions between cells and the maturation of blood vessels (Knapp et al. 1995). Therefore, it can be used to detect early neovascularization and neovascularization density in tissues. Ki67 is an antigen with non-histone proteins and is present in all active stages of mitosis and can be used to evaluate cell proliferation (Chan et al. 2020; Razavi and Yahyaabadi 2018). Tomato-lectin is a carbohydrate-binding protein and can be used to label vascular elements in fixed tissue sections (Mazzetti et al. 2004; Robertson et al. 2015). The present results showed that 4-MA increased the positive expression of CD34 in the cerebral cortex of MCAO/R rats and increased the positive expression of Ki67 and tomato-lectin in the gastrocnemius muscle of HLA mice. Furthermore, within the central nervous system, blood vessels and nerves are distributed together, and each neuron has corresponding capillaries (Quaegebeur et al. 2011; Ruan et al. 2015; Yang et al. 2011). Therefore, the key factor for maintaining neural function is cerebral blood flow (Strbian et al. 2008). Angiogenesis can establish a new vascular network in a relatively short time, reconstruct blood supply to ischemic tissue, and provide nutritional support for nerve repair. Our study showed that 4-MA promoted nerve repair and increased the neurological deficit scores and expression levels of neurotrophic factors in the brain tissue of MCAO/R rats (Fig. 1).

After rupture of the BBB following IS, reactive oxygen species (ROS) induce morphological changes in astrocytes to form reactive astrocytes, which can lead to the

complete reconstruction of the extracellular matrix (ECM) to form an ECM bundle (Iadecola 2013). Endothelial cells then migrate through ECM bundles to establish new capillary buds (Shao et al. 2017). This process is strictly controlled by the balance between angiogenesis (pro-angiogenesis) and vascular homeostasis (anti-angiogenesis) factors. Many angiogenic factors have been identified, including VEGF, angiopoietin, platelet-derived growth factor (PDGF), transforming growth factor (TGF), basic fibroblast growth factor (bFGF), matrix metalloproteinase (MMP), nitric oxide (NO), and prostaglandins (Beck and Plate 2009). Key signaling molecules such as VEGF and its receptor (VEGFR) and Ang and its receptor (Tie-2) play important regulatory roles in the angiogenesis process. VEGF/VEGFR acts during the early stage of angiogenesis in the IP and promotes the formation of a primitive vascular network, while Ang/Tie-2 mainly acts during the middle and late stages of angiogenesis to promote vascular remodeling and maturation (Eklund and Saharinen 2013; Gerber et al. 2002; Hur et al. 2006; Olsson et al. 2006; Passos-Silva et al. 2013). Regulating the dynamic balance of VEGF/VEGFR-Ang/Tie2 may be an effective strategy to promote angiogenesis and maturation. Our results showed that 4-MA regulated the dynamic balance of VEGF/VEGFR-Ang/Tie2 to promote angiogenesis. Specifically, 4-MA administration up-regulated the expression levels of VEGF and VEGFR-2, but down-regulated the expression levels of Ang-1, Ang-2, and Tie-2 (Fig. 4, Fig. 6).

In the early stage of IS, VEGF-A and Ang-2 can affect the structure and permeability of the BBB (Jin et al. 2010; Lange et al. 2016; Nourhaghighi et al. 2003). Our results showed that 4-MA promoted angiogenesis but did not aggravate BBB cascade damage in the early stage of IS. Of note, 4-MA administration increased EB and FITC-dextran expression in the brain tissue of MCAO/R rats.

The process of angiogenesis includes an increase in vascular permeability, degradation of the original vascular basement membrane, and proliferation, migration, budding, and orderly arrangement of endothelial cells (Stetler-Stevenson 1999; Zhang et al. 2002), as well as the reconstruction and maturation of neovascularization. Thus, one limitation of this research is the lack of a time-dependent study on 4-MA

430 promoting angiogenesis. Based on our positive results, however, we will conduct a
431 longer-term study in the future.

432 In conclusion, 4-MA exposure promoted angiogenesis, at least partially, by
433 regulating the dynamic balance of VEGF/VEGFR-Ang/Tie2, and promoted neural
434 repair after IS.

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Table 1

Angiogenesis-promoting primer sequences used in HLI mice

Gene	Primer	Primer sequence
GAPDH	Forward	5'- GTAAGAAACCCTGGACCACCC -3'
	Reverse	5'- GAGGGAGATGCTCAGTGTTGG -3'
VEGF-A	Forward	5'- GTCTGCTCTGCGCTCTGTA -3'
	Reverse	5'- AGGGAGCAAAGGTCACGAAA -3'
VEGFR-2	Forward	5'- CTGCGCTCACCTCCTGTTTA -3'
	Reverse	5'- AATGTGGCTACTTCCGGGTG -3'
Ang-1	Forward	5'- CCAGCAGCAAGTGGTTATGTC -3'
	Reverse	5'- CCAAGTGGCGATTCTGTTGAC -3'
Ang-2	Forward	5'- TGTTGTAGTGACACCCAGCC -3'
	Reverse	5'- CCTGTGGGCAGAGACCTTTT -3'
Tie-2	Forward	5'- CCCATCTGGTTACTACGGACC -3'
	Reverse	5'- TTGCCATCCTTGAGAGCAGAG -3'

Figure legends

Figure 1. Structure of 4-methoxy benzyl alcohol (4-MA)

Figure 2. Neural repair effects of 4-MA in MCAO/R rats. (A) Five-point method was used to investigate neurological scores following reperfusion for 7 days. (B) Western blot analysis of NeuN protein expression after MCAO/R rats were administered 4-MA for 7 days. (C) Immunochemical analysis of NeuN-positive expression after MCAO/R rats were administered 4-MA for 7 days. Scale bar, 50 μ m. Significant differences between groups are indicated as: $\Delta\Delta\Delta P < 0.001$, compared with sham group; $***P < 0.001$, compared with model group. Values are means $\pm$ SEM (n = 6).

Figure 3. Angiogenesis effects of 4-MA in MCAO/R rats. (A and B) Laser speckle images and quantitative analysis of cerebral blood flow ratio in rat cerebral ischemic hemispheres after reperfusion for 7 days. (C) Immunochemical analysis of CD34-positive expression after MCAO/R rats were administered 4-MA for 7 days. Scale bar, 50 μ m. (D) Immunochemical analysis of VEGF-A-positive expression after MCAO/R rats were administered 4-MA for 7 days. Scale bar, 50 μ m. Significant differences between groups are indicated as: $\Delta\Delta\Delta P < 0.001$, compared with sham group; $**P < 0.01$, $***P < 0.001$, compared with model group. Values are means $\pm$ SEM (n = 6).

Figure 4. Effects of 4-MA on protein expression of angiogenesis-promoting factors in MCAO/R rats. (A-E) Significant differences between groups are indicated as: $\Delta\Delta P < 0.05$, $\Delta P < 0.05$, compared with sham group; $*P < 0.05$, compared with model group. Values are means $\pm$ SEM (n = 6).

Figure 5. Angiogenesis effects of 4-MA in HLI mice. (A and B) Laser speckle images and quantitative analysis of hind limb blood flow ratio in mice after 7 days of ischemia. Significant differences between groups are indicated as: $\Delta\Delta\Delta P < 0.001$, compared with sham group; $*P < 0.05$, compared with model group. Values are means $\pm$ SEM (n = 6). (C) Positive expression of Ki67, tomato-lectin, and DAPI was detected using treble-labeling immunofluorescence of

neovascularization in gastrocnemius muscle of HLI mice for 7 days. Scale bar, 100 μ m. Ki67 (green) expression is an important marker of cell proliferation; tomato-lectin (red) binds to vascular endothelial cells in rodents and marks vessels effectively; DAPI (blue) labels nuclei. We examined neovascularization by labeling immunopositive reactions of Ki67 and tomato-lectin.

Figure 6. Effects of 4-MA on relative mRNA expression of angiogenesis-promoting factors in HLI mice. (A-E) qPCR analysis of angiogenesis-promoting factors in gastrocnemius muscle of HLI mice for 7 days. Significant differences between groups are indicated as: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, compared with sham group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with model group. Values are means $\pm$ SEM (n = 6).

Figure 7. Effects of 4-MA on BBB permeability in early stage of CIRC. (A-B) Changes in Evans blue (EB) leakage in MCAO/R rat cerebral cortex at different time points. (C-D) Microvessel density and permeability of BBB in MCAO/R rats after reperfusion for 48 h, observed using EB and FITC-dextran double-labeling fluorescence. Significant differences between groups are indicated as: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$, compared with sham group; $**P < 0.01$, $***P < 0.001$, compared with model group. Values are means $\pm$ SEM (n = 4). EB (red fluorescence) evaluates degree of vascular and BBB damage; FITC-dextran (green fluorescence) shows blood vessels via blood flow. EB and FITC-dextran show yellow fluorescence when superimposed and indicate undamaged microvessels.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 6

Figure 7