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Original Article

Herbal Formula *Danggui-Shaoyao-San* Promotes Neurogenesis and Angiogenesis in Rat Following Middle Cerebral Artery Occlusion

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ABSTRACT: Current studies demonstrated that traditional Chinese herbal formula *Danggui-Shaoyao-San* (DSS) is not only used for the treatment of menstrual disorder, but has also found its use in neurological diseases. However, the neuroprotective role of DSS on ischemia-induced brain injury is still unclear. The aim of the present study is to explore the effect of DSS in ischemic brain injury. Total 30 adult female Sprague–Dawley rats underwent 90 min transient middle cerebral artery occlusion (MCAO). DSS (600 mg/kg) was administered through the intragastric route at the time of reperfusion and then performed every day thereafter until sacrifice. Results showed that DSS treatment significantly improved neurobehavioral outcomes (N=10 per group, *P*<0.05). Immunohistochemical staining showed that microvessel density in the perifocal region of DSS-treated rats was significantly increased compared to the saline-treated group (N=4 per group, *P*<0.01). Similarly, the numbers of $BrdU^+/DCX^+$ cells in the subventricular zone were increased in DSS-treated rats compared to the saline-treated group (*P*<0.05). Furthermore, we demonstrated that DSS treatment activated vascular endothelial growth factor (N=4 per group，*P*<0.05) and promoted eNOS phosphorylation (N=4 per group，*P*<0.05). Thus, we concluded that DSS promoted focal angiogenesis and neurogenesis, and attenuated ischemia-induced brain injury in rats after MCAO, suggesting that DSS is a potential drug for ischemic stroke therapy.

Key words: Ischemic stroke, Dangui-Shaoyao San, Angiogenesis, Neurogenesis

Ischemic stroke is the 4th leading cause of death in the United States and the leading cause of disability worldwide. Currently, the intravenous use of thrombolytic agent, tissue plasminogen activator (tPA), is the only FDA-approved treatment for ischemic stroke. However, the time window for tPA treatment appears to be only effective within about the first 4.5 hours after the initial onset of symptoms [1], a mere 1-2% stroke patients can benefit from tPA. Therefore, therapeutics for the post-acute phase, which is a wider treatment window, will be helpful for improving functional recovery after ischemic stroke.

Spontaneous neurogenesis and angiogenesis in the post-acute phase are highly coordinated responses and may contribute to improvement in neurologic function after stroke [2]. Ischemic stroke triggers neurogenesis in the subventricular zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the dentate gyrus in the hippocampus. The production of neuroblasts increases and they migrate toward the damaged brain tissue to form

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functional neurons by integrating into the neuronal circuitry. Angiogenesis, the generation of new blood vessels, is most prominent in the ischemic boundary zone and is correlated with reduced injury in animal models and human stroke patients [3, 4]. Accumulating experimental studies showed that promoting post-ischemic angiogenesis and neurogenesis can improve neurological function [5, 6], suggesting it is a promising therapeutic target for ischemic stroke.

Danggui-Shaoyao-San (DSS), a famous traditional Chinese medicinal prescription containing a mixture of Chinese herbs, was first recorded in the "Synopsis of Prescriptions of the Golden Chamber" (early in 3rd century A.D.). DSS is used widely in oriental countries such as China, Korea and Japan [7-9] to relieve menorrhagia and other abdominal pains of women. Estrogen modulation is believed to be one of its mechanisms. It was reported that DSS modulated the estrogen level in parous ovariectomized rats and stimulated estrogen production *in vivo* [10, 11]. Recent studies found that the function of DSS is not limited to modulation of estrogen level, but is also widely used for the treatment of cognitive impairment and depression [10, 12-16]. Zhou et al reported that DSS reduced infarct size 24 h post-stroke in transient middle cerebral artery occlusion (MCAO) mice [17]. The medicinal serum of DSS could reduce hypoxia-induced myocardial cell injury too [18]. DSS was also reported to increase blood flow in the hippocampus after global cerebral ischemia [19]. All these studies suggest that DSS is a potential agent for the treatment of ischemic/hypoxia-induced injury. To the best of our knowledge however, the effect of DSS in promoting angiogenesis and neurogenesis after ischemic stroke is unknown.

Vascular endothelial growth factor (VEGF) confers neuroprotection and promotes neurogenesis and cerebral angiogenesis after ischemic stroke [20-22]. VEGF signaling promotes endothelial nitric oxide synthase (eNOS) phosphorylation and is related to the reduction of brain injury [23, 24]. Since eNOS is expressed in endothelial cells and neurons in the brain [25], we speculated that DSS could mitigate ischemia-induced brain injury through a VEGF/eNOS-dependent manner.

Here, we studied the effect of DSS following ischemic stroke using a rat model of transient middle cerebral artery occlusion (MCAO) and investigated the possible underlying mechanism of DSS.

MATERIALS AND METHODS

Experimental design

All animal experiments were approved by Animal Care and Use Committee of Xuanwu Hospital, Capital Medical University, and conducted according to National Institutes of Health guidelines. A total of 100 adult female Sprague–Dawley (SD) (280 to 320 g) rats were purchased from Vital River, a Charles River company. Animals were maintained on a 12-hour light/dark cycle with unlimited access to food and water. Rats were randomly divided into three groups: sham, MCAO and MCAO+DSS. The MCAO+DSS group was administered DSS (600 mg/kg/day) via the intragastric route at the time of reperfusion and then every day thereafter until they were scheduled to be sacrificed [17]. The MCAO group was administered with an equal volume of sterile saline solution. All three groups received 5-bromo-2-deoxyuridine (BrdU) to label dividing cells. BrdU (Sigma) was intraperitoneally injected at a dose of 50 mg/kg/day at 11 days after MCAO for three consecutive days.

Focal cerebral ischemia

Female Sprague–Dawley rats (280 to 320 g) were anesthetized and MCAO was induced by intraluminal occlusion for 90 min with a nylon monofilament suture as described previously [26]. In brief, the right common carotid artery and the right external carotid artery (ECA) were exposed. The ECA was then dissected distally, ligated, and coagulated. The right MCA was occluded using a heparinized intraluminal filament (0.28 mm, rounded tip). After 90 min, the suture was withdrawn. During surgery, rectal temperature was maintained at 37±0.5°C with a thermostat-controlled heating pad. Sham-operated rats underwent an identical surgery except that the MCA was not occluded.

Preparation of DSS

DSS is composed of the following 6 raw herbs: (1) Danggui, also known as *Angelica sinensis* (Oliv.) Diels (Umbelliferae), (2) Baishao, also known as *Paeonia lactiflora* (Ranunculaceae), (3) Fuling, also known as *Poria cocos* (Polyporaceae), (4) Baizhu, also known as *Atractylode smacrocephala* (Compositae), (5) Zexie, also known as *Alisma orientalis* (Alismataceae), and (6) Chuanxiong, also known as *Ligusticum chuanxiong* (Umbelliferae). These materials were purchased from Tongrentang Pharmaceutical Company (Beijing, China), and were then authenticated by Dr. Weipeng Yang in the China Academy of Chinese Medical Sciences. The DSS dilution was prepared as described previously [13]. In brief, the 6 raw herbs were mixed in their dry weight ratios of 3:16:4:4:8:3. The mixture was soaked in distilled water (1:8 w/v) for 30 minutes at room temperature, boiled for 1.5 h, and the extract filtered thereafter. The boiling and extraction procedures were repeated three times. The extracted filtrate was concentrated using a rotary

evaporator, and the final concentration of the extract is 1 g/ml (equivalent to the dry weight of the raw materials). The DSS extract was then stored at 4 ℃.

Neurobehavioral test

Neurobehavioral-tests were performed at 14 days after MCAO. Modified neurologic severity scores of the animals were graded on a scale of 0 to 12, which is a composite of motor, reflex, and sensorimotor integration tests [27]. The higher the score, the more severe the injury was. 10 rats were used for each group. The elevated body swing test (EBST) was used to test asymmetric motor behavior [28]. The rats were held at the base of the tail and raised 15 cm above the testing surface. The initial direction of swing is defined as the turning of the upper body by >10 degrees to either side, and was recorded in 30 trials per rat. The number of turns to each direction (left or right) was recorded for each rat. The total number of swings made to the left was divided by 30 (*n* number of trials) to get a percentage of left-biased swings.

Immunohistochemical analysis

The rats were anesthetized with chloral hydrate (Beijing Chemical Campany, Beijing, China) and were subsequently sacrificed by transcardiac perfusion with physiological saline followed by 4% paraformaldehyde in PBS (pH 7.4). Double immunostaining was performed on brain sections as previously described [29]. The primary antibodies used were mouse monoclonal anti-BrdU (Sigma-Aldrich; 1:500), goat polyclonal anti-DCX (Santa Cruz Biotechnology; 1:200. RECA-1 (Abcam; 1:200) and FITC conjugated *Lycopersicon esculentum* (Tomoto) Lectin, which recognize the rat endothelial cell antigen were used for vessel staining. The secondary antibodies were Alexa Fluor 488-, 594- conjugated donkey anti-mouse or anti-goat, (1:200; Invitrogen, Grand Island, NY, USA). To detect BrdU-labeled cells in brain sections, sections were incubated in 2 N HCl at 37 ℃ for 30 min and rinsed in 0.1 M boric acid (pH 8.5) and then in three washes of PBS. Fluorescence signals were detected with Nikon *Ti Eclipse* Epi-fl Illuminator (Nikon, Japan).

Cell counting

$BrdU^+$ and DCX^+ double-labeled cells in the SVZ, along the lateral walls of the lateral ventricles (beginning at 1.18 mm anterior to the bregma), were counted in three 5 μm paraffin coronal sections per animal (N = 4 per group), spaced 150 μm apart, by an observer blinded to the experimental groups. Results were expressed as the average number of BrdU- and DCX-positive cells in the SVZ.

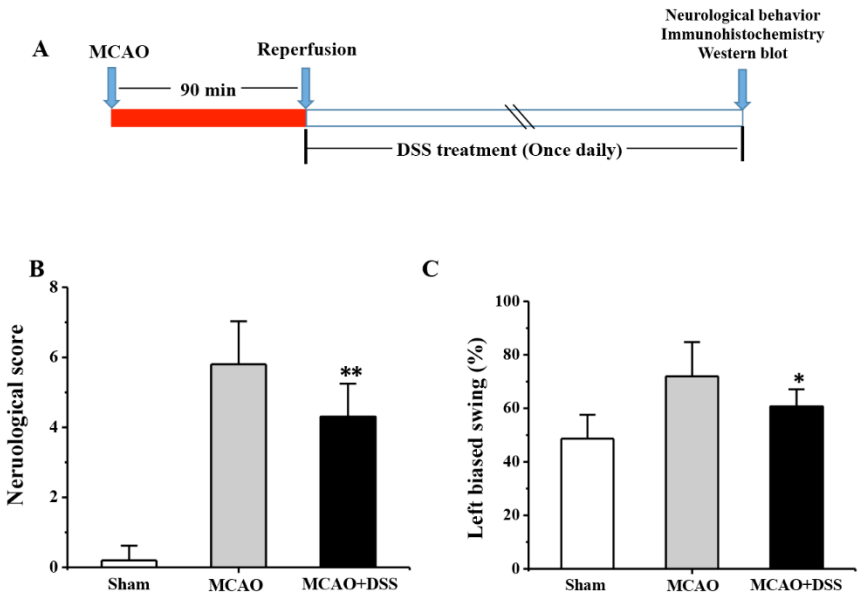

Figure 1. DSS improves neurobehavioral recovery at 14 days following MCAO. (A) Outline of the experiment. Functional recovery was evaluated using (**B**) Neurologic score, and (**C**) Elevated Body Swing Test. **P* < 0.05, ***P* < 0.01 vs MCAO group. Error bars indicate SD. N = 10 per group.

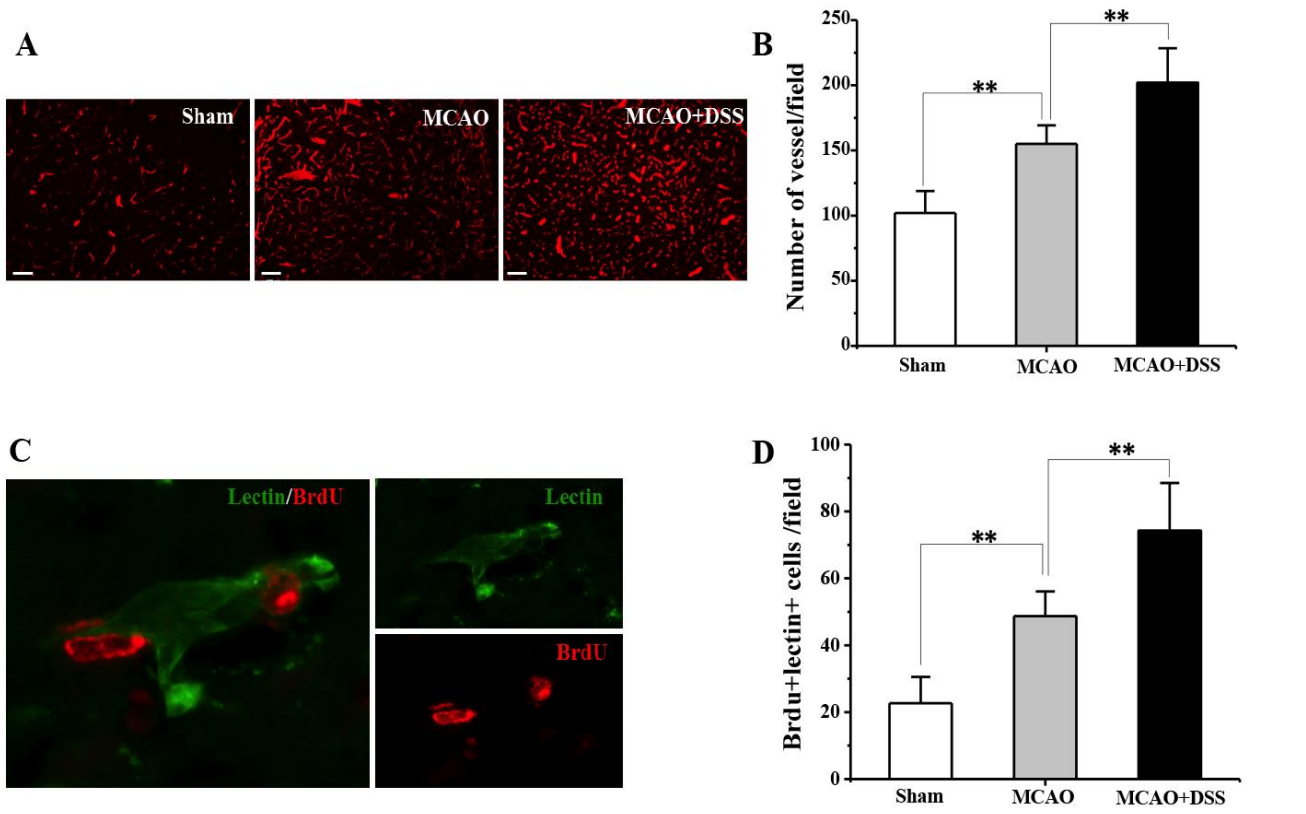

Figure 2. DSS promotes focal angiogenesis at 14 days following MCAO in rats. (**A**) Images show immunostaining for RECA-1$^+$ microvessels in the ischemic penumbra for each group of rats. (**B**) Bar graph shows quantification of microvessels shown in (A). Data are mean ± SD, N = 4 per group. $^{**}P < 0.01$. (**C**) Images represent double-immunostaining for Lectin (green) and BrdU (red) cells. (**D**) Bar graph shows a quantification of Lectin$^+$/BrdU$^+$ cells. Error bars indicate SD, N = 4 per group. $^{**}P < 0.01$.

ELISA Analysis

VEGF levels were quantified using an ELISA kit (Rat VEGF ELISA Kit; R&D systems) according to the manufacturer's protocol. Readings from each sample were normalized for protein concentration.

Western blot analysis

Tissue samples were collected from the striatum and cortex of the ipsilateral hemisphere at 14 days after reperfusion. Western blot analysis was used to assess protein expression of phosphorylated eNOS. Protein (40 μg) was electrophoresed on 10% SDS-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane (Millipore Corporation, USA). The membrane was probed with anti-phosphorylated eNOS antibody (Cell signaling; 1:1000 dilution). The specific reaction was visualized through the use of a chemiluminescent substrate (GE Healthcare, UK). GAPDH was used to verify equal loading. The optical density of protein was measured using Image-Pro Plus software 5.0 (Rockville, MD, USA) according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean and standard deviation (mean ± SD) and statistical tests were performed with SPSS for Windows, version 17.0 (SPSS Inc.). One-way ANOVA following post hoc test (Student-Newman-Keuls) was used for between-groups comparison. In all cases, $P<0.05$ was the criterion for significance.

RESULTS

DSS treatment improves neurobehavioral outcomes

Neurological deficits, including body posture and sensorimotor integration in the DSS-treated group improved significantly at 14 days after reperfusion

compared to the MCAO group (Figure 1A) (P<0.01). Similarly, motor deficits also improved based on EBST (Figure 1B) (P<0.05).

DSS promotes focal angiogenesis in rats after MCAO

To investigate the effect of DSS on focal angiogenesis after MCAO in rats, we first performed RECA-1 microvessel immunostaining in the ischemic perifocal region to evaluate the number of microvessels at 14 days following MCAO. The RECA-1$^+$ cells in the ischemic perifocal regions were significantly increased in MCAO group (159.4 ± 16.1) compared to the sham group (103.5 ± 23.7) (Figure 2A and 2B, P<0.01). DSS treatment (203.9 ± 34.1) increased the RECA-1$^+$ cells compared to the MCAO group (Figure 2A and 2B, P<0.01). We further evaluated the effect of DSS on endothelial cell proliferation. DSS treatment enhanced the number of Lectin$^+$/BrdU$^+$ cells (74.3 ± 19.1) compared to the MACO group (49.2 ± 9.5) (Figure 2C and 2D, P<0.01).

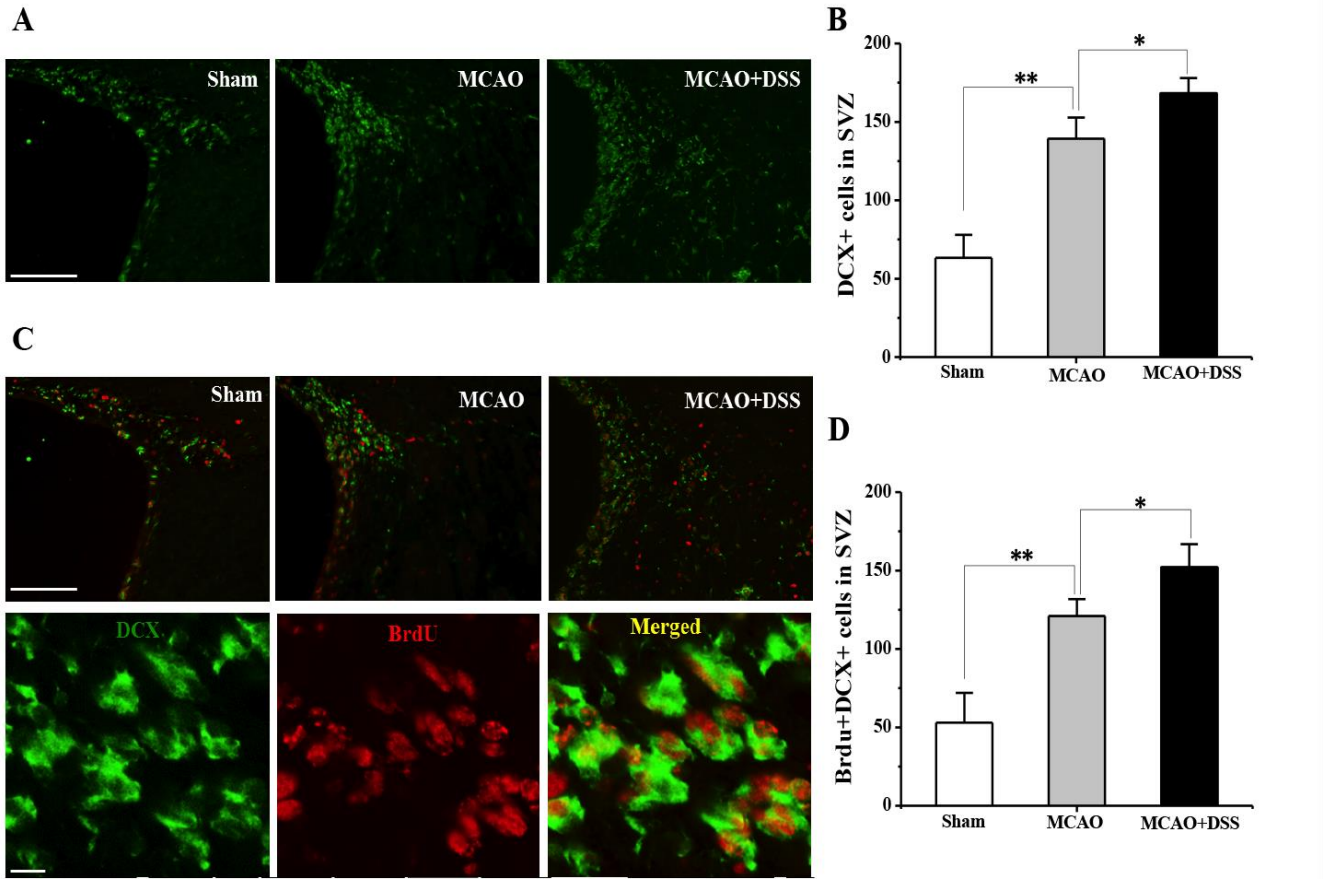

Figure 3. DSS enhances neurogenesis in the SVZ at 14 days following MCAO. (**A**) Images show immunostaining for DCX$^+$ cells in SVZ in each group at 14 days after MCAO (Scale bar, 100 μm). (**B**) Bar graph shows a quantification of the number of DCX$^+$ cells in the SVZ for each group. Error bars indicat SD, N = 4 per group. *P < 0.05, **P< 0.01. (**C**) *Upper line*: Images show double-immunostaining for DCX$^+$/BrdU$^+$ cells in the SVZ at 14 days after MCAO for each group (Scale bar, 100 μm). *Bottom line:* Images show immunopositive cells at higher magnification (Scale bar, 10 μm). (**D**) Bar graph showed a quantification of DCX$^+$/BrdU$^+$ cells in the SVZ. Error bars indicate SD, N = 4 per group. *P < 0.05, **P < 0.01.

DSS enhances neurogenesis in the subventricular zone (SVZ)

To further investigate the effect of DSS on focal neurogenesis after MCAO, we analyzed the presence of neurogenesis in the SVZ. Doublecortin (DCX) is a microtubule-associated protein expressed in neural stem cells and immature neurons, and thus can be used to visualize migrating neuroblasts. We performed DCX immunostaining at 14 days after MCAO. The number of

DCX^+ cells in MCAO group （139.3 ± 13.5）was significantly increased compared to sham group （63.2 ± 14.7）(Figure 3A, $P<0.01$). The number of DCX^+ cells was significantly increased in the DSS-treated group (168.4 ± 9.7) compared to MCAO groups (Figure 3A, $P<0.05$). To confirm the effect of DSS on cell proliferation, $BrdU^+/DCX^+$ cells in the SVZ were assessed. Double-label immunohistochemistry showed that the number of $BrdU^+/DCX^+$ cells in the SVZ was significantly increased in the DSS-treated group（152.4 ± 14.9）compared to MCAO groups (121.3 ± 10.7) (Figure 3B, $P<0.05$), suggesting that DSS promoted neurogenesis in the SVZ after MCAO.

DSS promotes VEGF expression and activated eNOS following MCAO

To explore the mechanisms underlying neuroprotection by DSS administration after ischemic stroke, we performed ELISA analysis to quantify VEGF levels. We found that VEGF expression levels were significantly increased in the DSS-treated group (57.6 ± 5.9 pg/mg) compared with MCAO groups (44.5 ± 4.4 pg/mg) at 14 d after ischemia in the striatum ($P<0.01$). In the cortex, VEGF expression levels were significantly increased in the DSS-treated group (63.4 ± 5.7 pg/mg) compared with MCAO groups (52.3 ± 9.6 pg/mg) ($P<0.05$) (Figure 4A) Western blot analysis showed that there was an increase in eNOS phosphorylation at 14 days following ischemia-reperfusion, suggesting that augmented MCAO induced eNOS phosphorylation (Figure 4C and 4D, $P<0.01$).

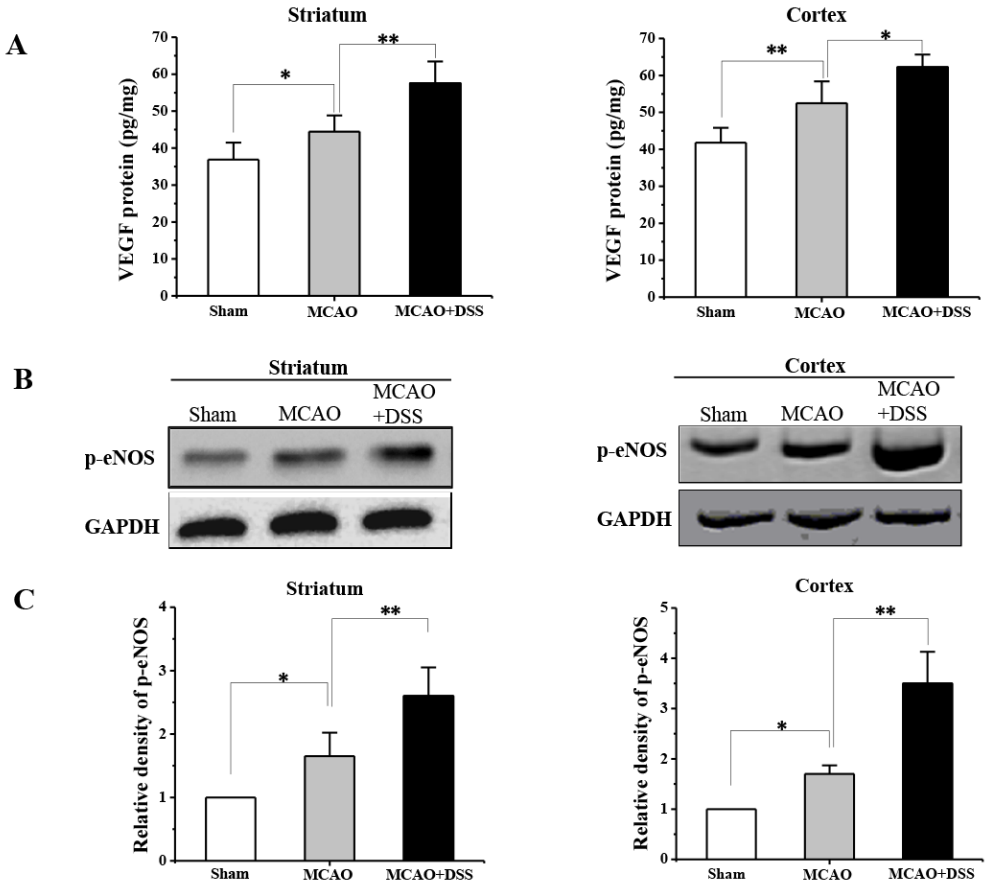

Figure 4. DSS promotes VEGF expression and activated eNOS following MCAO. (**A**) Bar graph shows a quantification of VEGF expression for each group. Error bars indicate SD, N = 4 per group. *$P < 0.05$, **$P < 0.01$. (**B**) Images show the expression of phosphorylated eNOS after 14 days after MCAO for each group. (**C**) Bar graph shows a quantification of p-eNOS/GAPDH ratio. Error bars indicate SD, N = 4 per group. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

In this study, we showed the neuroprotective effect of DSS treatment in that it reduces ischemic brain injury. DSS promoted focal angiogenesis and neurogenesis at 14 days after MCAO. We also found an increase of VEGF and eNOS activation in the cortex and striatum, which suggested that the neuroprotective effect of DSS was in part ascribed to enhancing focal eNOS expression.

DSS has been widely studied in emmeniopathy. In recent years, its use is extended to brain diseases. For example, Hatip-Al-Khatib et al. reported that DSS increased acetylcholine and blood flow in global cerebral ischemia rats [19]. A clinical study also showed that DSS increased regional cerebral blood flow in the posterior circulation in patients with mild cognitive impairment and Alzheimer's disease [12]. Song et al. found that long-term administration of DSS could increase nerve growth factor in olfactory-bulb-lesioned mice [30]. Zhou et al. also reported that DSS reduced infarct size, brain edema and BBB leakage at 24 h after MCAO in rats [17]. In the present study, we are the first to demonstrate that DSS treatment improves neurobehavioral outcomes after MCAO at 14 days after MCAO. Since the time window of neuro-restorative therapies is likely to be far longer than that for acute neuroprotection [31], we first demonstrated DSS as a potential candidate for future stroke therapy in the subacute phase via daily administration of DSS after reperfusion to promote angiogenesis and neurogenesis.

After ischemic stroke, angiogenesis and neurogenesis are implicated in neurorepair [21]. Conditions that promote focal angiogenesis and neurogenesis improve outcome of stroke via promoting functional recovery [3, 5]. To date, several reports showed that Chinese herbal compounds and individual herbs could promote neurogenesis and angiogenesis in cerebral ischemia models and could improve neural functional reconstruction and restoration [32-34]. In this study, we detected significant increases in the number of $BrdU^+$ cells and microvessels, suggesting that DSS could promote angiogenesis and neurogenesis after ischemic stroke.

A multitude of cellular and molecular mechanisms have been associated with neuroprotection of DSS that include modulation of neurotransmitter, regulation of cerebral blood flow and reduction of NO and malondialdehyde [13, 17, 19]. In this study, we first reported that DSS improved neuronal outcome through the promotion of angiogenesis and neurogenesis. Various intracellular signaling and molecular cascades are involved in ischemic neovascularization. The VEGF/eNOS pathway is important for angiogenesis and neurogenesis [24]. Numerous studies show that brain injury upregulates VEGF, which is critical for angiogenesis and neurogenesis [20, 24]. Intraventricular administration of VEGF-A for 3 days starting at 24 hours after MCAO in rats increased postischemic BrdU labeling in neuronal cells in the DG and SVZ [35]. Intravenous VEGF-A also increased microvessel density in the cortical ischemia penumbra [36]. The level of eNOS regulates both angiogenesis and neurogenesis in brain tissue. Agents that increased NO level induced endothelial cell proliferation and migration *in vivo* and *in vitro* [37]. $eNOS^{-/-}$ mice were reported to have decreased endothelial cell proliferation and exhibited a decrease of DCX^+ cell expression in the SVZ after stroke [25]. Furthermore, the ELISA analysis showed that $eNOS^{-/-}$ mice did not decreased VEGF expression compared with wild-type mice. These results indicate that eNOS is a downstream mediator for VEGF and angiogenesis in the ischemic brain [25]. In the present study, our results showed that DSS increased VEGF expression and eNOS activation. Integration of our results with previous findings and literature suggests that the neuroprotective effect of DSS was in part ascribed to enhancing focal VEGF/eNOS pathway. Simultaneously, we also found an increase in $BrdU^+/DCX^+$ cells and vascular density after DSS administration. Our findings thus provide new insights into the possible regulatory mechanisms of DSS for neuro-restorative therapy.

In conclusion, we demonstrated that DSS is a valuable therapy for ischemic stroke because it promotes focal angiogenesis and neurogenesis. Furthermore, we identified that the VEGF–eNOS signaling pathway is involved in this process. DSS is a widely used agent in ancient traditional Chinese medicine formulations. This study provides a novel insight into a potential curative treatment for ischemic stroke and explored one possible mechanism through which DSS acts.

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Disclosures/Conflict of Interest

None.

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