

# Effects of acupuncture on angiogenesis-associated factor expression in ischemic brain tissue following cerebral infarction in rats

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## Abstract

**Background:** We aimed to investigate changes into the levels of angiogenesis-associated factors following cerebral infarction and acupuncture intervention and reveal the underlying molecular mechanisms involved in promoting angiogenesis.

**Methods:** Model rats with middle cerebral artery occlusion (MCAO) were randomized into electroacupuncture (EA), model control (MC), and blank control (control) groups. Changes in the degree of neurological impairment following cerebral infarction and angiogenesis in the ischemic center and peripheral area were observed using immunofluorescence double-labeling. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting were used to detect changes in the Ang-1, Ang-2, PDGF-B, and bFGF levels. Moreover, the effects of EA intervention were evaluated.

**Results:** The neurological severity score of each phase in the EA group was lower than that into the simultaneous phase in the MC group. The proliferation of vascular endothelial cells in the EA group was higher than that in the MC group at 12 hours to 7 days. The Ang-1 and Ang-2 mRNA and protein levels in the EA group were significantly higher than those in the MC group. PDGF-B levels in the EA group were significantly higher than those in the MC group at 3 to 6 hours and 3 to 12 days, and protein levels were high at 6 hours and 3 to 12 days. bFGF mRNA levels at 24 hours to 12 days and bFGF protein at 3 to 12 days were significantly elevated in the EA group than those in the MC group.

**Conclusions:** EA at Shui Gou (DU 26) significantly improved the neurological symptoms of MCAO rats, promoted vascular endothelial cell proliferative activity around the infarct area, significantly advanced the time of proliferation of vascular endothelial cells, and upregulated the expression of angiogenesis-related factors, thereby promoting angiogenesis. Thus, EA may significantly improve the prognosis of cerebral infarction.

**Keywords:** Angiogenesis, Cerebral infarction, DU 26, Electroacupuncture

**Graphical abstract:** <http://links.lww.com/AHM/A43>.

## Introduction

The pathological process of cerebral infarction is complicated and is triggered by cerebral ischemia<sup>[1]</sup>. The purpose of cerebral infarction treatment is to restore the compromised function of nerve cells. However, the regeneration

and proliferation of nerve cells require nutritional support from the blood vessels. Cerebrovascular blockage determines the fate of the brain cells in the dominant area, and their smooth functioning depends on whether the region can regain compensatory blood flow. The regeneration of cerebral microvessels and improvement of microcirculation following ischemia can promote the proliferation, differentiation, and survival of neural stem cells<sup>[2-3]</sup>. The formation of new blood vessels can be observed in the ischemic penumbra area following infarction, and their density is positively correlated with patient prognosis<sup>[4-7]</sup>. In 1993, Von Kummer and Forsting<sup>[8]</sup> indicated that the infarct size in patients with middle cerebral artery (MCA) obstruction mainly depends on collateral blood supply. An efficient collateral blood supply is crucial for limiting the extent of cerebral ischemic infarction and spontaneous or drug-induced recanalization. Recently, some experts<sup>[9-10]</sup> emphasized that timely restoration of blood circulation during cerebral infarction is vital to minimizing the degree and scope of nerve cell degeneration caused by ischemia. Although reperfusion may cause injury, its benefits outweigh its disadvantages. Improving cerebral circulation is critical in the treatment of cerebral infarction. Currently, improving cerebral circulation and the compensatory function of collateral vessels in the peripheral areas of cerebral infarction have gained importance in the treatment of this disease.

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The establishment of collateral circulation following cerebral infarction includes early vasodilation (collateral circulation initiation) and subsequent angiogenesis (collateral circulation reconstruction). Cerebral infarction has been treated with acupuncture in the Tianjin University of Traditional Chinese Medicine First Affiliated Hospital for more than 30 years and Xingnao Kaiqiao method has been established<sup>[11]</sup>. Because acupuncture is effective, it plays an important role in promoting collateral circulation. Our previous study<sup>[12]</sup> showed that electroacupuncture (EA) at Shui Gou(DU26) could promote the proliferation of vascular endothelial cells around the infarct area in middle cerebral artery occluded (MCAO)-model rats. Furthermore, we established for the first time that EA could significantly advance the occurrence of vascular endothelial cell proliferation, which confirmed that EA at DU 26 can promote angiogenesis of the penumbra following cerebral infarction, thus promoting the reconstruction of collateral circulation. In addition, we found that the function and morphology of cerebrovascular vessels were critically impaired following cerebral infarction. Acupuncture at DU 26 could dilate cerebrovascular vessels, improve autonomic movement and energy metabolism, promote the timely start of cerebral collateral circulation, and increase compensatory blood flow; thus, the general goal of this study was to determine how acupuncture plays a positive role in angiogenesis.

Based on the above theoretical basis, this study aimed to reveal the molecular mechanism of acupuncture treatment of cerebral infarction from the perspective of promoting angiogenesis by observing the mRNA and protein expression patterns of angiogenesis-related factors Ang-1, Ang-2, PDGF-B, and bFGF in ischemic brain tissue of rats. Our findings will provide a scientific experimental basis for the clinical application of acupuncture treatment for cerebral infarction.

## Materials and methods

### Animals

A total of 114 specific-pathogen free, healthy male Wistar rats, each weighing 180 to 200 g, were obtained from the Experimental Animal Center of the Chinese Academy of Military Medical Sciences [Permit number: SCXK (Beijing) 2019-0010]. All rats were housed in separate cages (five per cage) at the Animal Experiment Center of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences at room temperature (25 ± 1)°C. A week's worth of food and clean water were provided to the rats. Before the operation, rats were fasted for 12 hours, with *ad libitum* access to water. Minimal pain and the use of experimental animals were observed during the experiment. Rats were randomized into the model control (MC) and EA groups ( $n = 54$  rats in each group) and the blank control group (control,  $n = 6$ ) based on a random number table method; the first two groups were further divided into nine phase groups according to the time intervals: 1, 3, 6, 9, 12, and 24 hours and 3, 7, and 12 days following the operation. Each group consisted of six rats in each phase.

### Preparation of the MCAO rat model

In the EA and MC groups, the Longa intracavitary suture method<sup>[12–13]</sup> was used to block the MCA on the right side of the rat head to establish the MCAO model. Nylon wire with a 0.21 mm diameter was used. In addition, a 10% chloral hydrate solution (3 mL/kg, i.p.) was used for anesthesia, and the paraffin-impregnated nylon wire was introduced through the proximal end and bifurcation of the common carotid artery. The length of the sutures was (18 ± 0.5) mm. The suture and common carotid artery were then ligated, and the skin was sutured.

### EA treatment

In the EA group, DU 26 acupoint location (as per the “Atlas of Common Animal Acupoints” edited by Hua Xingbang) was acupunctured immediately following the operation. A 0.5” acupuncture needle was used to obliquely puncture the rat’s nasal septum 2 mm upward and to insert a needle approximately 2 mm below this site as a reference electrode. The DU 26 and reference electrode were connected to the positive and negative electrodes of the EA device respectively. Electrical stimulation of the 15 Hz/2 mA density wave was provided, followed by acupuncture for 20 minutes. The 1, 3, 6, 9, 12, and 24-hour groups were subjected to acupuncture only once, and the rats were sacrificed immediately following the completion of treatment for sampling. The other time-stage groups received acupuncture once a day, and the rats were sacrificed for sampling immediately following the completion of treatment at 3, 7, and 12 days. The MC and control groups were similarly fixed without any treatment.

### Neurological evaluation

Neurological severity scores<sup>[14]</sup> (NSSs) were determined for each group of rats before and after surgery. Later, the NSS for 3, 7, and 12 days was assessed only once daily. A postoperative NSSs of ≥1 is indicative of a successful operation, and thus, can be included in the experiment. Rats that died before the observation time point or experienced subarachnoid hemorrhage or internal carotid artery bifurcation hemorrhage during the acquisition of brain samples were excluded from further studies.

### Sample processing

Rats were deeply anesthetized using 10% chloral hydrate in the abdominal cavity at the specified time, and the chest was opened rapidly. Next, the left ventricle was quickly perfused with 0.9% normal saline (4°C) and 4% polyformic acid solution (4°C), and the brain was decapitated. Considering the bifurcation of the middle and anterior cerebral arteries as a midpoint, 2 mm of brain tissue was harvested just before and after this point. Brain samples were fixed at 4°C in 4% paraformaldehyde overnight, dehydrated using a sucrose gradient, and embedded. Frozen sections of 10-μm thickness were prepared and stored in a slide box at room temperature for immunofluorescence double-label staining.

Tissue from the right MCA blood supply area (approximately 3 mm before and after the bregma)

was cut from the coronal region for reverse transcription-polymerase chain reaction (RT-PCR) and western blotting.

#### Immunofluorescence double-label staining

Primary antibodies [CD31 (1:100) (1 mL, Item No.: MCA1334GA; AbD Serotec, UK) and Ki67 (1:1,000) (1 mL Item No.: ab15580; Abcam, UK)] were added after the samples were blocked with goat serum for 1 hour. After overnight incubation at 4°C, fluorescein-labeled secondary antibodies [goat anti-rabbit rhodamine red-X IgG (1:100) (1 mL, Item No.: CW0149; ComWin Biotech, China) and goat anti-mouse Cy2 IgG (1:100) (1 mL, Item No.: CW0161; ComWin Biotech)] were added drop-wise. Incubation was performed for 2 hours at room temperature, and the samples were rinsed using 0.01 M phosphate-buffered saline. The slides were mounted with anti-fluorescence quenching tablets, and the images were captured using a microscope (Leica, Germany). With the Image Pro-Plus 6.0 image analysis system used, three non-coincident  $\times 400$  fields were randomly analyzed for each tissue section. The average of the three values was used to define the number of positive cells, while  $\geq 10\%$  was defined as positive.

#### Reverse transcription-polymerase chain reaction

Brain tissues (50 mg) were used to extract RNA [E.Z.N.A. HP Total RNA kit (R6812-01), Omega, USA]. With double distilled water (ddH<sub>2</sub>O) (RNase/Dnase free) used,<sup>2</sup> micro-ultraviolet spectrophotometer K5500 (Kaiao Technology, China) was calibrated, and then, the absorbance of 1  $\mu$ L brain RNA samples was measured at 260 and 280 nm. The total RNA of each sample was diluted to a final concentration of 1  $\mu$ g/ $\mu$ L, reverse-transcribed into cDNA, followed by RT-PCR. The reaction was carried out in a MicroAmp<sup>®</sup> Fast Optical 96-well reaction barcoded plate, with GAPDH as the housekeeping gene and ddH<sub>2</sub>O as the negative control. The thermocycling protocol was as follows: 1) Uracil-N-glycosylase (UNG) enzyme digestion at 50°C for 10 minutes; 2) pre-denaturation at 95°C for 10 minutes; 3) 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The experiments were conducted in triplicate. ABI 7,500 Fast Real-Time PCR System software (Roche Lightcycler480, Switzerland) was used to analyze the real-time fluorescence quantitative PCR experiment results and calculate the mRNA expression levels of each experimental index (Table 1).

#### Western immunoblotting

The protein levels were determined using a BCA assay kit (BOSTER, China). Briefly, 10  $\mu$ L protein was collected from the brain tissues of the right MCA blood supply area of rats in each experimental group, boiled at 100°C for 5 minutes, separated on a 10% SDS-PAGE gel, and transferred onto a polyvinylidene difluoride membrane. With standard molecular weight protein markers as a reference, sample protein bands were cut according to their molecular weights. They were immersed in an incubation box containing 5% non-fat milk at

room temperature for 2 hours. Primary diluent Ang-1 antibody (Abcam, 1:25,000), Ang-2 antibody (Abcam, 1:2,000), bFGF antibody (Abcam, 1:100), PDGF antibody (Abcam, 1:10,000), and GAPDH (Abcam, 1:10,000) were added. The samples were incubated at room temperature for 1 hour and then at 4°C overnight. It was followed by washing three times with Tris Buffered Saline with Tween20 (TBST) (0.05% Tween 20) solution for 10 minutes. Subsequently, the samples were immersed in the corresponding secondary antibody diluent (Abcam, 1:5,000), incubated for 1 hour at room temperature, and washed with TBST. The electrochemiluminescence (ECL) solution was added with dark conditions maintained, and 30 seconds later, protein expression was observed using a BIO-RAD gel imaging system and gray scanning. The absorbance value of the target band and internal reference band were analyzed using ImageJ software, with the ratio of these two values being utilized to measure relative protein expression.

#### Statistical analysis

Statistical tests were performed using SPSS version 16.0. The data obtained in the experiment were normally distributed and expressed as the mean  $\pm$  standard deviation (mean  $\pm$  SD). One-way analysis of variance (ANOVA) was used for multiple comparisons between diverse groups. When the variance was observed to be homogeneous, it was subjected to the least significant difference (LSD) analysis. However, when the variance was uneven, it was subjected to Dunnett's T3 test.  $P < 0.05$  was the significance threshold.

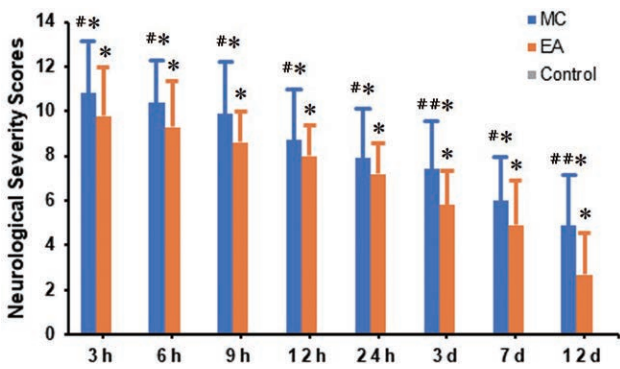
## Results

#### Neurological evaluation

Each group consisted of six rats in each phase. Before the operation, the NSSs of all rats were normal (NSSs = 0). Additionally, the control group's NSSs was zero after the procedure. The EA and MC groups were considered for the score observation process from 3 hours onward because the MCAO rats from the 1-hour group were not fully awake. The NSSs of the EA and MC groups was higher than that of the control group in each phase ( $P < 0.01$ ). However, the NSSs of the EA group was considerably lower than that of the MC group in each phase ( $P < 0.05$ ), with the most significant difference at 3 and 12 days ( $P < 0.01$ ), as shown in Figure 1.

**Table 1**  
Probe for experiment

TaqMan <sup>®</sup> Gene Expression Assays-Ang1 (Rn01504818_m1)	ThermoFisher, USA
TaqMan <sup>®</sup> Gene Expression Assays-Ang2 (Rn01756774_m1)	ThermoFisher, USA
TaqMan <sup>®</sup> Gene Expression Assays-PDGF-B (Rn1502596_m1)	ThermoFisher, USA
TaqMan <sup>®</sup> Gene Expression Assays-bFGF (Rn00570809_m1)	ThermoFisher, USA
TaqMan <sup>®</sup> Gene Expression Assays-GAPDH (Rn01775763_g1)	ThermoFisher, USA



**Figure 1.** Neurological severity scores. \* $P < 0.01$  vs. control group; ## $P < 0.01$ , # $P < 0.05$  vs. MC group. All comparisons were made at the same time point. EA: electroacupuncture; MC: Model control.

### Angiogenesis in ischemic penumbra area following infarction

Immunofluorescence double-label staining analysis of cerebral arteries showed no vascular endothelial cell proliferation surrounding the infarct area in the MC group, for 1 to 12 hours following MCAO. Proliferation began to appear at 24 hours and reached a maximum after 3 days. Later, it decreased on day 7 and disappeared on day 12. In the EA group, no proliferation of vascular endothelial cells was observed for 1 to 9 hours following MCAO. Proliferation began at 12 hours, continued to increase at 24 hours, and reached a maximum at 3 days. Later, it decreased on day 7 and disappeared on day 12. The proliferation trend of vascular endothelial cells in the EA group following MCAO was similar to that in the MC group. However, the proliferation appeared earlier and was higher. No proliferation of vascular endothelial cells was observed on the contralateral side of the infarct area in any of the phases in the two groups. In addition, no proliferation was observed in the controls at each phase, as shown in Figure 2 and Table 2.

### Increased mRNA and protein expression of angiogenesis-related factors following EA

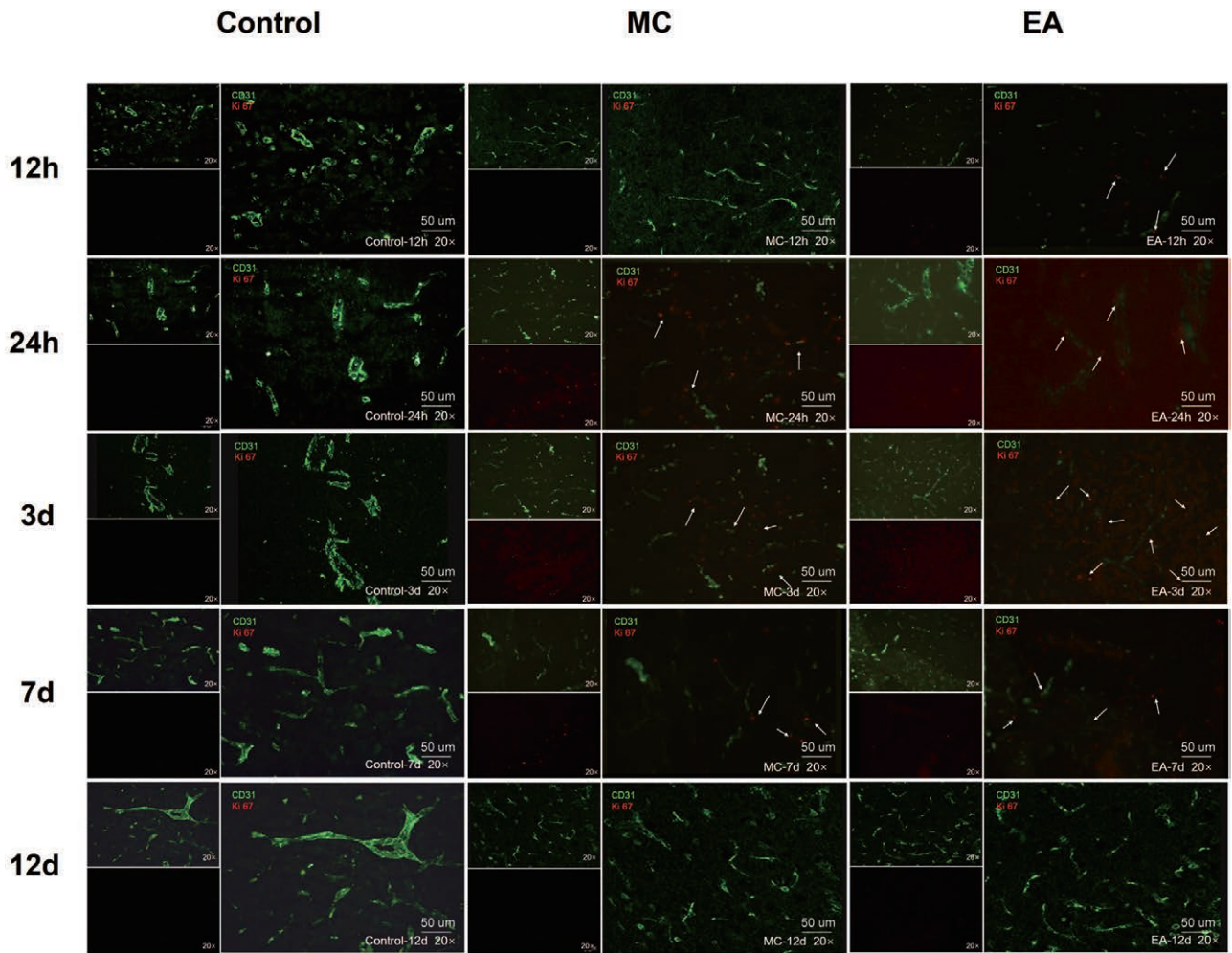
After the operation, the expression of angiogenesis-associated factors in the cerebral vascular region differed among the groups ( $P < 0.05$ ,  $P < 0.01$ ). The Ang-1 mRNA expression levels in the EA and MC groups from 12 hours to 12 days were significantly higher than those in the controls ( $P < 0.05$ ,  $P < 0.01$ ). In addition, the Ang-1 mRNA expression levels in the EA group were significantly higher than those in the MC group from 3 to 12 days ( $P < 0.05$ ), with a marked difference at day 7 ( $P < 0.01$ ). Ang-1 protein levels in the EA and MC groups from 6 hours to 12 days were significantly increased compared to those in the control ( $P < 0.01$ ). Moreover, the Ang-1 protein levels in the EA group were considerably higher than those in the MC group from 3 to 12 days ( $P < 0.05$ ). Ang-2 and PDGF-B mRNA expression levels in each phase were significantly increased in the EA and MC groups compared to those in the controls ( $P < 0.05$ ,  $P < 0.01$ ). The Ang-2 mRNA expression levels in each phase in the EA group were higher than those in the MC group in a similar phase. However, the difference was significant from 3 to 24 hours ( $P < 0.05$ ), with a highly significant

difference at 9 to 12 hours ( $P < 0.01$ ). PDGF-B mRNA expression levels in the EA group were higher than those in the MC group at 3 to 6 hours and 3 to 12 days ( $P < 0.05$ ). Ang-2 and PDGF-B protein levels in each phase in the EA and MC groups were elevated relative to the controls ( $P < 0.01$ ). The Ang-2 protein level in each phase in the EA group showed a higher trend than that in the MC group in a similar phase. The difference was significant from 3 to 12 hours ( $P < 0.05$ ), with a highly significant difference at 3 to 6 hours and 12 hours ( $P < 0.01$ ). In contrast, the PDGF-B protein levels in the EA group were significantly higher than those in the MC group at 6 hours and 3 to 12 days ( $P < 0.05$ ) and highly significant at 7 to 12 days ( $P < 0.01$ ). From 9 hours to 12 days, bFGF mRNA expression levels in the MC group were higher than those in the controls ( $P < 0.05$ ,  $P < 0.01$ ), whereas the expression levels in the EA group were higher at 3 hours and 9 to 12 days ( $P < 0.05$ ,  $P < 0.01$ ). In addition, compared with that in the MC group, the bFGF mRNA expression level in the EA group was significantly elevated from 24 hours to 12 days ( $P < 0.05$ ) and was highly significant at 3 to 12 days ( $P < 0.01$ ). The bFGF protein levels in the MC group were higher than those in the controls at 24 hours to 3 days ( $P < 0.05$ ), whereas the EA group levels were higher at 12 hours to 12 days ( $P < 0.05$ ,  $P < 0.01$ ). Furthermore, the bFGF protein levels in the EA group were higher than those in the MC group at 3 to 12 days ( $P < 0.05$ ) and were highly significant at 12 days ( $P < 0.01$ ), as shown in Figures 3 and 4.

### Discussion

Angiogenesis has vital clinical significance because it promotes the repair of neural structure and function following cerebral infarction by stimulating the endogenous repair mechanism<sup>[15]</sup>. Rapid restoration of blood supply in the ischemic penumbra area is a fundamental goal of acute cerebral infarction treatment<sup>[10,16]</sup>. Anatomical studies have indicated that the facial and trigeminal nerve branches are distributed around DU 26 points. Stimulation of DU 26<sup>[17]</sup> may lead to activation of the terminal branches of the trigeminal nerve and facial nerve, causing excitation of the sphenopalatine ganglion and modulation of cerebral vasomotion. Electrical stimulation at 2 Hz can cause the parasympathetic nerve to release acetylcholine, whereas electrical stimulation at  $\geq 10$  Hz can selectively release peptide neurotransmitters, such as vasoactive peptides<sup>[18]</sup>. Previous studies have indicated that p-Akt expression in positive cells in the hippocampal CA1 region, dentate gyrus, and cortex in the moderate-intensity (2–4 mA) EA group was significantly higher than that in the high-intensity (5–7 mA) EA and control group<sup>[19]</sup>. Based on these results, we chose 15 Hz/2 mA as the criterion for EA in our experiments. Our previous study<sup>[11]</sup> showed that EA at DU 26 could promote the proliferation of vascular endothelial cells around the infarct area in MCAO model rats. It is consistent with the present results, confirming that EA at DU 26 can promote angiogenesis of the penumbra following cerebral infarction.

We found that with the prolongation of ischemic time, the NSSs of the two groups showed a downward trend and there were significant differences in each phase



**Figure 2.** EA promote the proliferation of vascular endothelial cells in ischemic penumbra area of the MCAO rat. CD31 is positively expressed by staining the membrane of vascular endothelial cells in green. Ki67 is positively expressed by staining the nucleus of vascular endothelial cells in red. The number of Ki67-positive cell nuclei when CD31 and Ki67 were expressed together was used to indicate the proliferation number of vascular endothelial cells. EA: Electroacupuncture; MCAO: Middle cerebral artery occlusion.

**Table 2**  
**Semi-quantitative analysis of the proliferation of vascular endothelial cells around the infarct zone of MCAO rats (n = 6)**

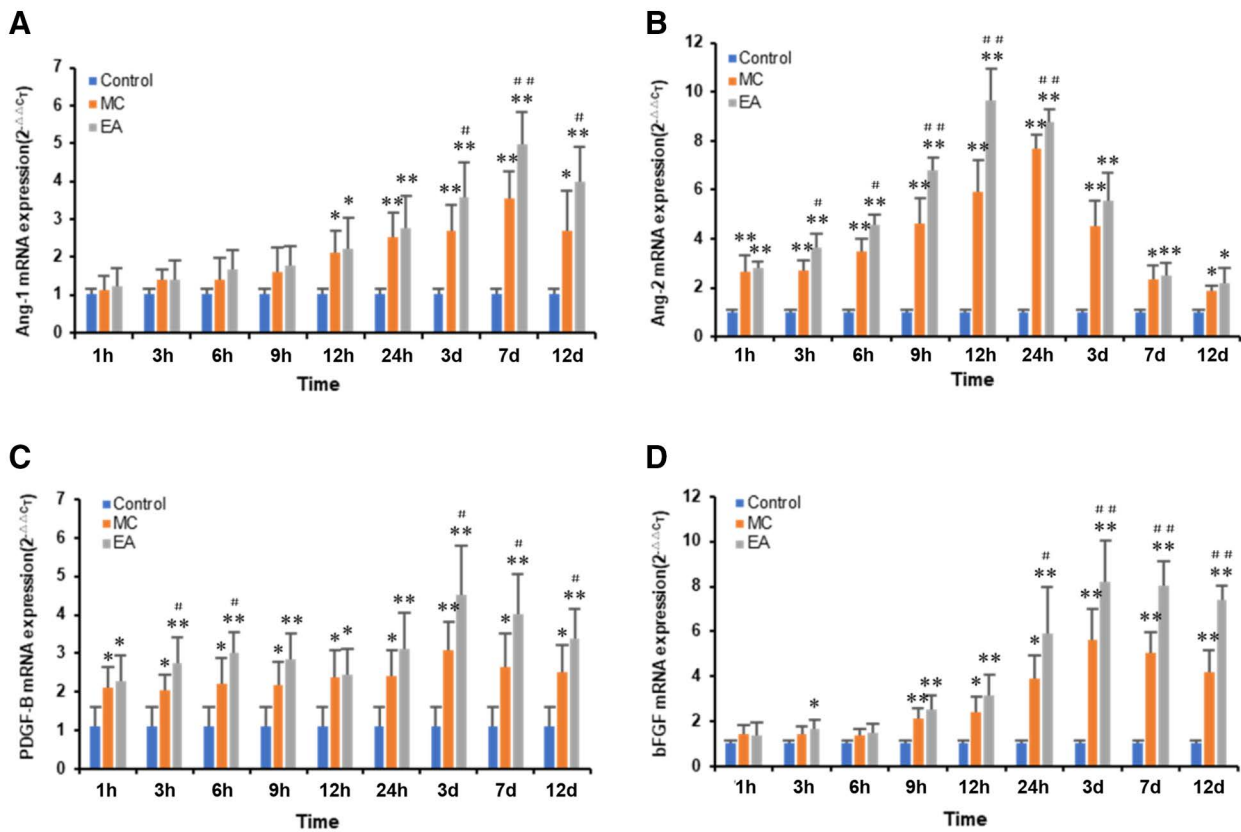
Cerebral ischemia time	The MC group	The EA group	The control group
1 h	-	-	-
3 h	-	-	-
6 h	-	-	-
9 h	-	-	-
12 h	-	+	-
24 h	+	++	-
3 d	++	+++	-
7 d	+	++	-
12 d	-	-	-

EA: Electroacupuncture; MC: Model control; MCAO: Middle cerebral artery occlusion.

"-" indicates no positive staining; "+" indicates one to three endothelial cells; "++" indicates four to six endothelial cells; "+++" indicates seven to nine endothelial cells; "++++" indicates more than nine endothelial cells.

( $P < 0.05$ ), indicating that acupuncture can significantly alleviate the neurological symptoms of MCAO rats and slow down the trend of nerve function aggravation, with the most significant improvement at 3 and 12 days ( $P < 0.01$ ). Simultaneously, Ki67 and CD31

immunofluorescence double-labeled detection was performed on rat brain tissue. We found that around the infarction area, co-expression of Ki67 and CD31 began to appear in the EA group 12 hours following MCAO, and co-expression of Ki67 and CD31 began to appear in the MC group 24 h following MCAO. With the prolongation of ischemia time, co-expression gradually increased, reached a peak at day 3, and subsequently, the proliferation level decreased. However, co-expression was still observed on day 12 in the EA group but not in the MC group. We found that the co-expression of Ki67 and CD31 within 24 h may be due to early endothelial cell proliferation and migration to form microvascular cavities. Co-expression since 3 d may be the expression of endothelial cells that aggregated and proliferated into mature blood vessels. At this time, the stronger CD31 fluorescence signal may be due to the maturation and stability of the endothelial cells. In addition, the proliferation and expression of endothelial cells in capillary branches were observed from day 7 to 12, and it was possible to establish branch connections of the vascular network through endothelial cell proliferation at this time, thus forming a new capillary network. In addition, the newly formed capillaries in the marginal zone of the ischemic brain tissue of rats in the EA group at day 12

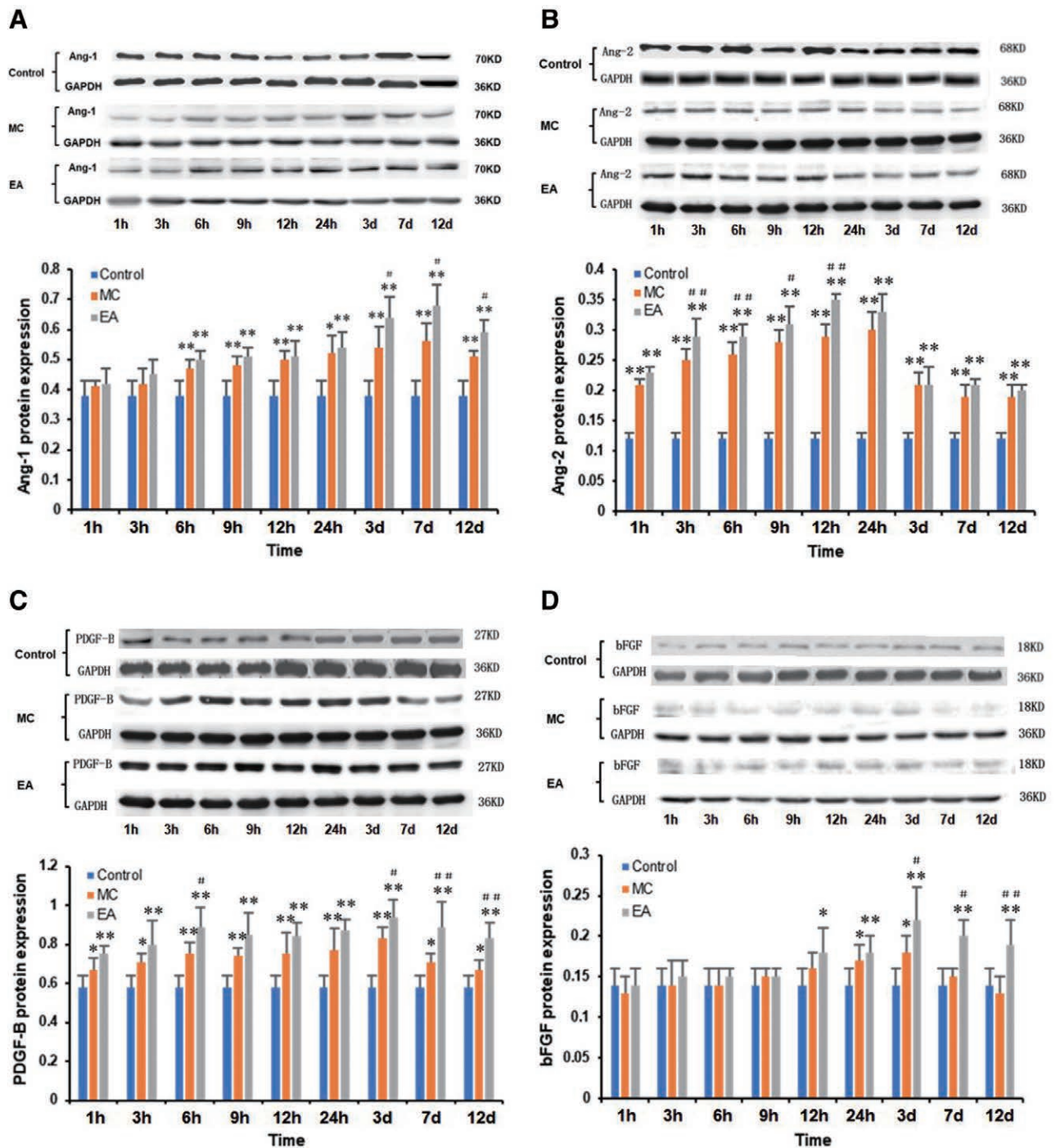


**Figure 3.** Ang-1, Ang-2, PDGF-B, and bFGF mRNA in the cerebral vascular of rats by RT-PCR detection. (A) Ang-1 mRNA levels. (B) Ang-2 mRNA levels. (C) PDGF-B mRNA levels. (D) bFGF mRNA levels. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control group; # $P < 0.05$ , ## $P < 0.01$  vs. MC group. All comparisons were made at the same time point. MC: Model control; RT-PCR: reverse transcription-polymerase chain reaction.

had complicated branches and large diameters. This may be because EA can promote the upregulation of angiogenesis-related factors and accelerate endothelial cell proliferation and migration to form a new blood vessel network.

Angiogenesis is a complex process resulting from the coordinated effects of pro-angiogenic and anti-angiogenic factors (vascular balance theory). Their stability determines the start time and progression of the subsequent angiogenesis process. Under conditions such as hypoxia, inflammation, or external agents, angiogenesis-related factors directly or indirectly activate endothelial cells in autocrine or paracrine forms to initiate the process of angiogenesis. Previous experiments<sup>[20]</sup> have confirmed that EA at DU 26 can activate the endogenous Sonic Hedgehog signaling pathway and promote the early and high expression of related factors. The Shh pathway has a very close relationship with angiogenesis-related factors and can induce the upregulation of bFGF and balance Ang-1 and Ang-2 expression<sup>[21]</sup>. By stimulating the expression of multiple downstream signal transduction pathways, Ang-1 regulates angiogenesis, forms stable blood vessels, and inhibits the expression of cell surface adhesion molecules, inflammatory cell adhesion, and apoptosis<sup>[22–24]</sup>. A dynamic balance consisting of low Ang-2 and high Ang-1 levels within the central nervous system is important for blood–brain barrier integrity. The binding of Ang-1 to Tie receptors contributes to the induction of phosphorylation, which can prevent apoptotic death of cells of the vascular

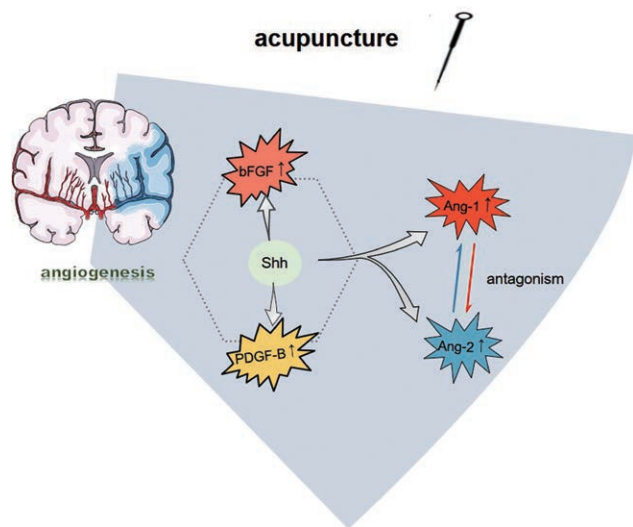
endothelium. However, the binding of Ang-2 to the Tie-2 receptor does not cause receptor phosphorylation but blocks Ang-1–mediated Tie-2 activation, thereby promoting blood vessel lengthening<sup>[25–26]</sup>. We found that the expression of Ang-1 mRNA in the MC group began to upregulate at 6 hours whereas that in the EA group at 3 hours. The expression of Ang-2 mRNA in the MC group was upregulated at 3 hours whereas that in the EA group at 1 hour. The expression of Ang-1 and Ang-2 in each phase in the EA group was consistently higher than that in the MC group. This result shows that EA can promote the early high-level expression of Ang-2 to initiate the angiogenesis mechanism in advance, thereby prolonging the angiogenesis process, whereas Ang-1 appears to be highly expressed at 3 day to induce the maturation and stability of new blood vessels. It can be speculated that EA induces the expression of angiogenesis-related factors by modulating the Shh pathway and coordinating their interactions, thereby promoting the growth, maturation, and stability of microvessels in the neurovascular network (Figure 5). bFGF is an FGF family protein<sup>[27]</sup> that shares vital biological functions, including proliferation, differentiation, migration, and apoptosis. It also plays a crucial role in the early and middle stages of angiogenesis and is a neurotrophic factor that can directly nourish neurons, accelerate neural stem cell differentiation and proliferation, and promote neural remodeling<sup>[28–29]</sup>. We found that the expression of bFGF mRNA in the MC group began to increase at 6h whereas that in the EA group at 1 hour. The mRNA and protein expression levels



**Figure 4.** Ang-1, Ang-2, PDGF-B, and bFGF protein in the cerebral vasculature of rats by Western Blotting. (A) Ang-1 protein levels. (B) Ang-2 protein levels. (C) PDGF-B protein expression. (D) bFGF protein expression. \**P* < 0.05, \*\**P* < 0.01 vs. control group; #*P* < 0.05, ##*P* < 0.01 vs. MC group. All comparisons were made at the same time point. MC: Model control.

in each phase in the EA group were higher than those in the MC group, and the expression level remained higher from 9 hours to 12 days. This result indicates that EA can stimulate the stress expression of endogenous bFGF, thereby promoting the proliferation and migration of endothelial cells. Shh signaling pathway inhibitors can also suppress PDGF-induced vascular smooth muscle cell proliferation<sup>[30]</sup>. PDGF-B is synthesized and secreted by endothelial cells. It promotes blood vessel maturation by recruiting pericytes and stabilizing developing blood vessels<sup>[31]</sup> and is important during the late stage of angiogenesis. Ischemia can cause transient upregulation of

PDGF-B and bFGF expression<sup>[32-34]</sup> in the cortex of the infarct area. EA can promote the early high-level expression of PDGF-B and bFGF, thus protecting nerves<sup>[35]</sup> and promoting angiogenesis during the healing and remodeling of ischemic cells and tissues. Therefore, these four angiogenesis-related factors, Ang-1, Ang-2, PDGF-B, and bFGF, may combinedly promote the formation of the new blood vessel network, save dying neurons, reduce brain tissue damage, complete the vascular remodeling process much earlier than in the MC group, and continue to promote angiogenesis in the infarcted area. We speculate that after cerebral infarction, angiogenesis



**Figure 5.** Electroacupuncture induces the expression of angiogenesis-associated factors by mediating the Shh pathway.

around the infarct area is not only to increase cerebral blood perfusion and remove metabolites, but a response to the reorganization of nerve function in this region, that is, the high metabolic rate in the process of neurological reorganization requires more blood to provide nutrients and quickly clear metabolites, and the increase in demand mediates the increase of angiogenesis, which also belongs to the body's protective mechanism against local damage. This inference needs further study, and we will design experiments to explore this mechanism in depth in future studies. Moreover, EA may promote angiogenesis by mediating the Shh signaling pathway, but its specific mechanism still needs to be studied in depth.

## Conclusions

Based on the above research results, we conclude that EA at DU 26 can significantly improve neurological deficit symptoms in MCAO rats and promote the upregulation of mRNA and proteins of angiogenesis-related factors Ang-1, Ang-2, PDGF-B, and bFGF. Our study also indicates that EA could advance the expression phase, thereby promoting the regeneration, maturation, and stabilization of microvessels. Thus, EA may be an important technique for the treatment of cerebral infarction.

## Conflict of interest statement

The authors declare no conflict of interest.

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## Author contributions

Jing Li participated in the planning and execution of the experiments and the conception and design of the study.

Jing Li and Yajie Sun wrote the first draft of this manuscript. Yajie Sun conducted experiments. Rainer Georgi and Bernhard Kolberg revised the manuscript and participated in statistical analysis. Lihong Yang conducted the immunofluorescence double-label staining experiments. All the authors have read and approved the final manuscript.

## Ethical approval of studies and informed consent

The rat experimental were overseen and approved by the Beijing Municipal Science & Technology Commission and the Administrative Commission of Zhongguancun Science Park [Approval number: SCXK (Beijing) 2019-0010].

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## Data availability

All data generated or analyzed during this study are included in this published article.

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