

Protective effect of troxerutin and cerebroprotein hydrolysate injection on cerebral ischemia through inhibition of oxidative stress and promotion of angiogenesis in rats

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Abstract. Brain ischemia, including cerebral ischemia and cerebrovascular ischemia, leads to poor oxygen supply or cerebral hypoxia, and causes brain tissue death or cerebral infarction/ischemic stroke. The troxerutin and cerebroprotein hydrolysate injection (TCHI), is widely applied in China to improve blood supply in ischemic brain tissues and to enhance neuroprotective effects in clinical practice. However, the benefits and detailed underlying mechanism elaborating the effectiveness of TCHI in cerebrovascular diseases require further investigation. Therefore, in the present study, experimental *in vivo* and *in vitro* models were employed to investigate the potential mechanisms of TCHI on cerebral ischemic injury. The results demonstrated that TCHI increased the lactate dehydrogenase levels in the brain homogenate and conversely decreased lactic acid levels. TCHI was further observed to significantly increase superoxide dismutase activity and decrease malondialdehyde levels in ischemic brain tissues. In addition, TCHI significantly induced vascular maturation processes, including proliferation, adhesion, migration and tube formation in cultured human umbilical vein endothelial cells. Additionally, TCHI significantly stimulated microvessel formation in the rat aortic ring and chick chorioallantoic membrane assays. Taken together, these results provided strong evidence that TCHI stimulated angiogenesis at multiple steps, and indicated that TCHI attenuated cerebral ischemic damage through the amelioration of oxidative stress and promotion of angiogenesis.

Introduction

Brain ischemia, also known as cerebral ischemia or ischemic stroke, is a condition involving insufficient blood flow to the brain to meet metabolic demand and is a leading cause of mortality worldwide, causing serious long-term disability (1,2). The treatment strategies of brain ischemia, particularly acute ischemic stroke, currently include thrombolytic, antiplatelet aggregation and neuroprotective therapy, among which thrombolytic therapy remains the sole effective treatment. However, there is a lack of effective therapies to improve functional recovery in the cerebral post-ischemic phase, while the treatment for ischemic stroke is restricted to a narrow therapeutic time window and holds the risk of hemorrhage (3). Thus, the mechanism of cerebral ischemia and the treatment of post-stroke patients remain under investigation (2).

Oxidative stress, characterized by excessive production of reactive oxygen species (ROS), is a recognized mechanism of cerebral ischemic injury (4,5). As a result, antioxidants are considered to be a conventional therapeutic strategy in the treatment of cerebral ischemic injury. Superoxide dismutase (SOD) is a first-in-line endogenous defense against ROS and specific scavengers of the superoxide anion, which eliminates oxygen-free radicals to prevent excessive superoxide anion concentration damage and serves an important role in maintaining the normal physiological activity of the body. Furthermore, as the product of lipid peroxidation induced by oxygen-free radicals, the malondialdehyde (MDA) content in tissues effectively reflects the degree of cell damage and free radical attacks in the body (6). A number of studies have confirmed that SOD, lactate dehydrogenase (LDH) and MDA levels in brain tissue were abnormal following cerebral ischemia (7,8). In addition, recent studies demonstrated that the promotion of angiogenesis and the formation of new blood vessels from pre-existing vessels may be a novel strategy to reduce the infarct volume and improve neurobehavioral recovery following ischemic stroke (2,9). Enhancement of endothelial cell functions, including adhesion, migration, proliferation and differentiation, is critical for inducing neovascularization to provide the required nutrients, oxygen

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and blood-flow for ischemic tissues during the process of angiogenesis.

Troloxerutin, is a flavonol used as a vascular protective and antioxidative agent, and cerebroprotein hydrolysate includes brain protein hydrolysate, sialic acid. The troloxerutin and cerebroprotein hydrolysate injection (TCHI) has been approved by the State Food and Drug Administration of China for the amelioration of cerebral ischemic conditions based on neuroprotective effects exerted in clinical practice (10). Troloxerutin has the effect of lowering blood viscosity, inhibiting platelet aggregation, promoting the formation of collateral circulation, improving microcirculation and eliminating free radicals, and can further effectively inhibit the formation of thrombi and promote the repair of damaged nerve tissue (11). Bayer *et al* (12) demonstrated that sialic acid increases blood vessel formation. Other studies have suggested that sialic acid interacts with extracellular matrix (ECM) components and growth factors, regulating cell adhesion, migration and proliferation (13). Endothelial cells express several integrin heterodimers, including $\alpha\beta3$, $\alpha5\beta1$ and $\alpha\beta5$. Among these, integrin $\beta3$ is a critical cell adhesion molecule in angiogenesis (14). The expression of integrin $\beta3$ on the endothelial cell surface activates and promotes endothelial cell proliferation, thereby promoting angiogenesis (15). A previous clinical study suggested that TCHI improves neurological recovery in patients with acute cerebral infarction (16). It was further demonstrated that TCHI supports a shortening of coma duration, and improves the quality life and long-term outcomes (17). Therefore, it is speculated that TCHI protects against cerebral ischemic injury via attenuation of oxidative stress or promotion of angiogenesis. However, the detailed mechanism underlying the effectiveness of TCHI in cerebrovascular diseases requires further investigation.

In the present study, experimental *in vitro* and *in vivo* models were employed to investigate the underlying mechanisms of TCHI in the protection of cerebral tissues from ischemic injury.

Materials and methods

Drug. TCHI (drug batch no., 160602; Shandong Buchang Pharmaceutical Co., Ltd., Heze, China) is a compound preparation made with sterilized water, troloxerutin ($C_{33}H_{42}O_{19}$) and porcine brain extracts. The components of TCHI include troloxerutin (40 mg/ml), active peptides, a variety of amino acids and a variety of gangliosides (100 μ g/ml), with a total nitrogen content of 0.5 mg/ml. Edaravone (drug batch no., 170704; Nanjing Xiansheng Dongyuan Pharmaceutical Co., Ltd., Nanjing, China), a neuroprotective drug that has the properties of a free radical scavenger and could potentially reduce oxidative stress, was used to help with recovery following a stroke and to treat amyotrophic lateral sclerosis.

Animals. Male SD rats ($n=66$, 250 ± 20 g, 6-8 weeks old) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and housed in a room with a 12-h light-dark cycle maintained at $22 \pm 2^\circ\text{C}$ and with a relative humidity of $60 \pm 2\%$. Food and water were supplied to all rats *ad libitum*. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and approved by the Ethics Committee of Xi'an Jiaotong University, School of Medicine (Xi'an, China).

Middle cerebral artery occlusion (MCAO). A modified model of MCAO was used to achieve permanent focal ischemia, as described previously (18,19). Briefly, animals were anesthetized by intraperitoneal injection of 7% chloral hydrate (350 mg/kg), and the right side of the common carotid artery was exposed and isolated. The middle cerebral artery (MCA) was occluded by inserting a monofilament nylon suture (diameter 0.265 mm) into the internal carotid artery. When resistance was encountered, the insertion was stopped, and the ischemia time was counted. The length of the nylon line inserted was 18-20 mm. The wound was sutured, and the rat was closely monitored for post-operative recovery.

Groups and drug administration. Six groups of rats (11 rats/group) were included in the present study, as follows: Sham-operated group, in which rats underwent the same surgical procedure as the model group, with the exception of insertion of the nylon suture; MCAO model group, in which rats were subjected to ischemia; three TCHI groups, in which animals received 0.5, 1.0 and 2.0 ml/kg TCHI respectively (this drug is metabolized in mice and the concentration at each dose is described, rather than the total concentration of the three doses as this description is more similar to the human dosage regimen), following MCAO; and positive control group, in which rats received edaravone (5 mg/kg) treatment following MCAO. Edaravone is a synthetic antioxidant agent that neutralizes free radicals, and can be used to relieve reperfusion injury associated with cerebral ischemia and oxidation (20). TCHI and edaravone were administered three times after surgery, at 6 and 24 h; Sham-operated group and MCAO model group were administered of Saline immediately after surgery, at 6 and 24 h.

Neurological function assessment. The animal behavior of each rat was carefully evaluated at 6 and 24 h after surgery. The animals were scored for neurological damage as follows: 0, normal spontaneous movement; 1, failure to fully extend contralateral forepaw; 2, circling to affected side; 3, partial paralysis on affected side; and 4, no spontaneous motor activity.

Measurement of cerebral infarction range. At 2 h following the final drug administration, the brain was carefully removed and cut into six coronal sections with a thickness of 2 mm. Next, the sections were quickly stained with triphenyl tetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution at temperature of 37°C for 30 min, followed by incubation with 10% buffered paraformaldehyde for fixation. Following staining, the non-ischemic tissue was stained red, while the infarcted area appeared white. The white tissue was carefully stripped and weighed. The cerebral infarct range was calculated as the percentage of the infarction tissue weight to the weight of ischemic hemisphere.

Determination of lactic acid (LD), LDH, SOD and MDA levels in brain tissue. The rat brain tissue of the surgical side was weighed, homogenized with normal saline to prepare 10% brain homogenate and stored in a -20°C refrigerator. The homogenates were centrifuged at $300 \times g$ for 10 min at 4°C , and the supernatant was used to measure the LH, MDA, SOD and MDA activities were evaluated in a 96-well plate using their respective activity

assay kits (cat. nos. A0201, A0031, A001-3 and A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions, while the protein content was measured by biuret method, as described previously (21).

MTT assay. Human umbilical vein endothelial cells (HUVECs) were provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and fetal calf serum was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. (Hangzhou, China). HUVECs were seeded in 96-well plates (3×10^4 cells/ml) and cultured in a highly humidified atmosphere of 5% CO₂ at 37°C for 24 h. Subsequently, the medium was replaced with 200 μ l medicated medium containing various concentrations of TCHI (0, 2, 10, 50 and 250 μ g/ml). Following incubation for 24 h, the medicated medium was replaced with 180 μ l serum-free medium and 20 μ l MTT, and incubated at 37°C for 4 h. The supernatant was discarded, and the precipitate was dissolved by adding 150 μ l DMSO. The contents of the wells were dissolved using a shaker for 15 min and the absorbance was measured using a microplate reader at a wavelength of 490 nm.

Tube formation assay. A 48-well plate was coated with 100 μ l Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37°C for 30 min to permit solidification. HUVECs (2×10^4 cells/well in 500 μ l RPMI 1640 medium) and different concentration of TCHI (0, 2, 10, 50 and 250 μ g/ml) were added to the plate. Subsequent to incubation for 24 h, the tube formation was observed by microscopy, and images of the cells were captured.

Adhesion assay. HUVECs were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μ g/ml Streptomycin and 100 IU/ml penicillin containing 0, 2, 10, 50 and 250 μ g/ml TCHI in a highly humidified atmosphere of 5% CO₂ at 37°C for 24 h. Following incubation for 24 h, HUVECs (2×10^6 cells/well) were seeded on a Matrigel-coated 24-well plate and incubated for 1 h. Subsequent to discarding the medium, the plate was washed with PBS three times and observed under a microscope, and images were captured to examine cell adhesion.

Cell migration assays. HUVECs (2.5×10^5 /well) were seeded in a 6-well cell culture plate and then incubated at 37°C in a 5% CO₂ atmosphere for 24 h. When confluence reached 90%, the HUVEC monolayer on the plate was scratched with a 200 μ l pipette tip and washed three times by PBS. Fresh medium containing various concentrations of TCHI (0, 2, 10, 50 and 250 μ g/ml) was then added. Following incubation at 37°C for 0, 12 and 24 h, images of the plates were obtained to examine the cell migration.

Chick chorioallantoic membrane (CAM) assay. Fertilized chicken eggs (Xi'an Xinfengyuan livestock breeding specialized cooperatives, Xi'an, China) were cleaned with 1% benzalkonium bromide and then incubated at 37°C in an incubator with 60-70% relative humidity for 7 days. On day 8 of incubation, a 1-cm-diameter window was carefully created

on the broader side of the egg and then sterile gelatin sponges saturated with normal saline (serving as the control), 10 μ g/ml TCHI or recombinant bovine basic fibroblast growth factor (bFGF; 4,200 IU/ml; Zhuhaiyisheng Biological Pharmaceutical Co., Ltd., Guangdong, China) were placed inside the egg, and permeable sticky tape was immediately placed over the window. bFGF is a cytokine with a fundamental role in angiogenesis and served as a positive control drug in the CAM assay. After 3 days of incubation, CAMs were fixed with 4% paraformaldehyde for 10 min at room temperature. Images of blood vessels around the gelatin sponges were captured, and the number of these vessels was counted under a microscope.

Rat aortic ring assay. For the rat aortic ring assay, male SD rats (n=2, 250 \pm 20 g, 6-8 weeks old) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and housed in a room with a 12-h light-dark cycle maintained at 22 \pm 2°C, with a relative humidity of 60 \pm 2%. Food and water were supplied to all rats *ad libitum*. Then, 48-well plates were covered with 100 μ l Matrigel at 4°C and incubated at 37°C in 5% CO₂ for 30 min. The rats were anesthetized with 20% urethane, and then their aortas were isolated and cleaned of the residual blood in the lumen and redundant adipose tissue. Aortas were cut into 1-mm long rings, placed on the Matrigel-covered wells of the plates and covered with another 100 μ l Matrigel. Artery rings were cultured in the RPMI 1640 complete medium, or with medium containing 2, 10, 50 or 250 μ g/ml TCHI. After 6 and 9 days of incubation, the microvessel growth was measured under an inverted microscope and images of the artery rings were captured.

RT-PCR. The total RNA was isolated using RNA fast 2000 kit (Fastagen, Shanghai, China) according to the manufacturer's protocols. The RNA was subsequently reverse transcribed into cDNA using Prime Script RT Master Mix Perfect Real-Time kit (DRR036A; Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. The primers sequences used (Sangon Biotech Co., Ltd., Shanghai, China) were: β 3, forward 5'-GCC AGCACCATCTCTTTACC-3' and reverse 5'-GCACTCTCT CCCTTTGAGGA-3', with a length of 112 bp; β -actin, forward 5'-TGACGTGGACATCCGCAAAG-3' and reverse 5'-CTG GAAGGTGGACAGCGAGG-3', with a length of 205 bp. The cycling protocol for PCR involved incubating the samples at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final cycle of incubation at 72°C for 2 min. The amplification products were analyzed by electrophoresis (Beijing Junyi, Beijing, China) in agarose gels and detected under UV illumination (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after staining with nucleic acid dye (DuRed; FanBo Biochemicals, Beijing, China). Images were analyzed using a quantitative analysis system (Quantity One Analysis Software, version 4.6.2; Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are expressed as the mean \pm standard deviation. Statistical analysis was performed with SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to compare differences between groups, and a value of P<0.05 was considered to denote a statistically significant difference.

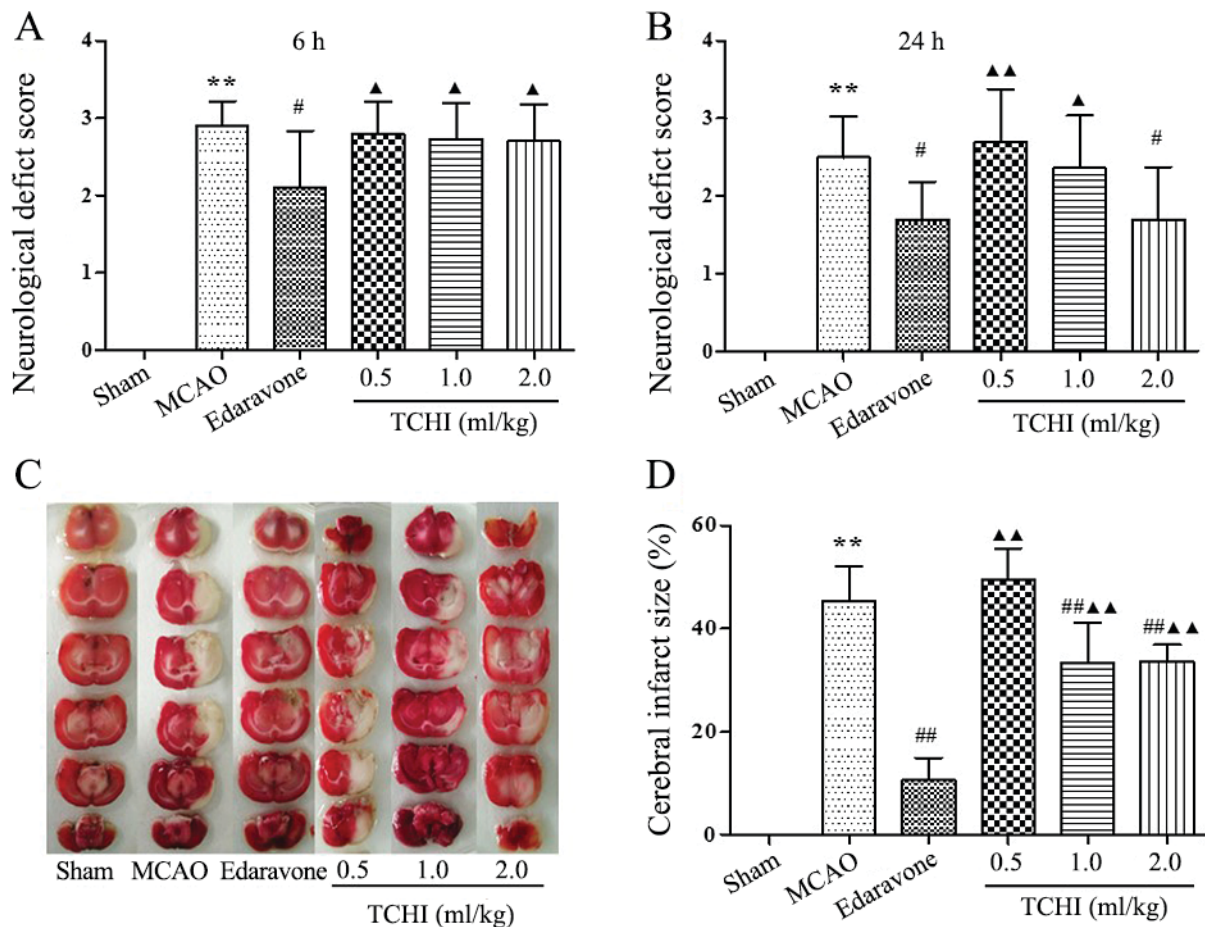


Figure 1. Effect of TCHI on neurological scores and infarct volume in MCAO rats. Neurological scores at (A) 6 h and (B) 24 h following MCAO are displayed. (C) Coronal sections from ischemic rat brains stained with triphenyltetrazolium chloride. (D) Cerebral infarct area was decreased following TCHI treatment in rats with an MCAO. Data are expressed as the mean \pm standard error of the mean ($n=10-11/\text{group}$). ** $P<0.01$ vs. sham group; # $P<0.05$ and ## $P<0.01$, vs. MCAO group; ▲ $P<0.05$ and ▲▲ $P<0.01$ vs. edaravone group. TCHI, trolox and cerberin hydrolysate injection; MCAO, middle cerebral artery occlusion.

Results

TCHI improves neurological outcomes and attenuates infarct volume following cerebral ischemic injury in rats. Initially, in order to examine the protective effects of TCHI in an MCAO animal model, tail-vein injection of TCHI (0.5, 1 and 2 ml/kg), edaravone (5 mg/kg) or vehicle was performed, and neurological deficit scores were determined at 0, 6 and 24 h after the MCAO surgery. The results revealed that there was no neurological deficit in the sham-operated group, while other groups presented various degrees of neurological deficit symptoms at 6 and 24 h following MCAO surgery (Fig. 1A and B). In addition, there was no significant difference in neurological deficit scores between the TCHI dose groups (0.5, 1 and 2 ml/kg) and the MCAO model group at 6 h following surgery (2.80 ± 0.42 , 2.80 ± 0.42 and 2.70 ± 0.48 vs. 2.90 ± 0.32 , respectively; Fig. 1A); Compared with the MCAO model and TCHI groups, the edaravone group significantly reduced neurological deficit scores at 6 h following surgery (2.90 ± 0.32 , 2.80 ± 0.42 , 2.80 ± 0.42 and 2.70 ± 0.48 vs. 2.10 ± 0.88 , respectively; $P<0.05$; Fig. 1A). However, compared with the MCAO model group, the neurological deficit score of the edaravone groups and 2 ml/kg TCHI group was significantly reduced at 24 h following

surgery (2.50 ± 0.53 vs. 1.70 ± 0.48 , 1.70 ± 0.67 , respectively; $P<0.05$; Fig. 1B). Compared with MCAO model group, 0.5 and 1 ml/kg TCHI groups, edaravone group significantly reduced neurological deficit scores at 24 h following surgery (2.50 ± 0.53 , 2.70 ± 0.67 , 2.36 ± 0.67 vs. 1.70 ± 0.48 , respectively; $P<0.05$; Fig. 1A). No significant difference was observed in neurological deficit scores between the 2 ml/kg TCHI group and the edaravone group at 24 h following surgery (1.70 ± 0.67 vs. 1.70 ± 0.48 ; Fig. 1A). Subsequently, the cerebral infarct volume was determined using TTC staining. Compared with the sham-operated group, the infarct volume was significantly increased in the model group ($P<0.01$; Fig. 1C and D), the cerebral infarct volume was $45.35 \pm 6.75\%$ in rats of the MCAO model group, 32.78 ± 7.86 and $33.66 \pm 3.19\%$ in rats treated with 1 and 2 ml/kg TCHI and $10.59 \pm 4.40\%$ in rats treated with 5 mg/kg edaravone, respectively. Thus, the results of the present study revealed that, in MCAO rats treated with TCHI at doses of 1 and 2 ml/kg and 5 mg/kg edaravone, the percentage of the infarct volume was significantly reduced compared with that in the untreated model group ($P<0.01$; Fig. 1C and D). In addition, compared with the edaravone group, the percentage of the infarct volume was significantly higher compared with that in the TCHI groups ($P<0.01$; Fig. 1C and D).

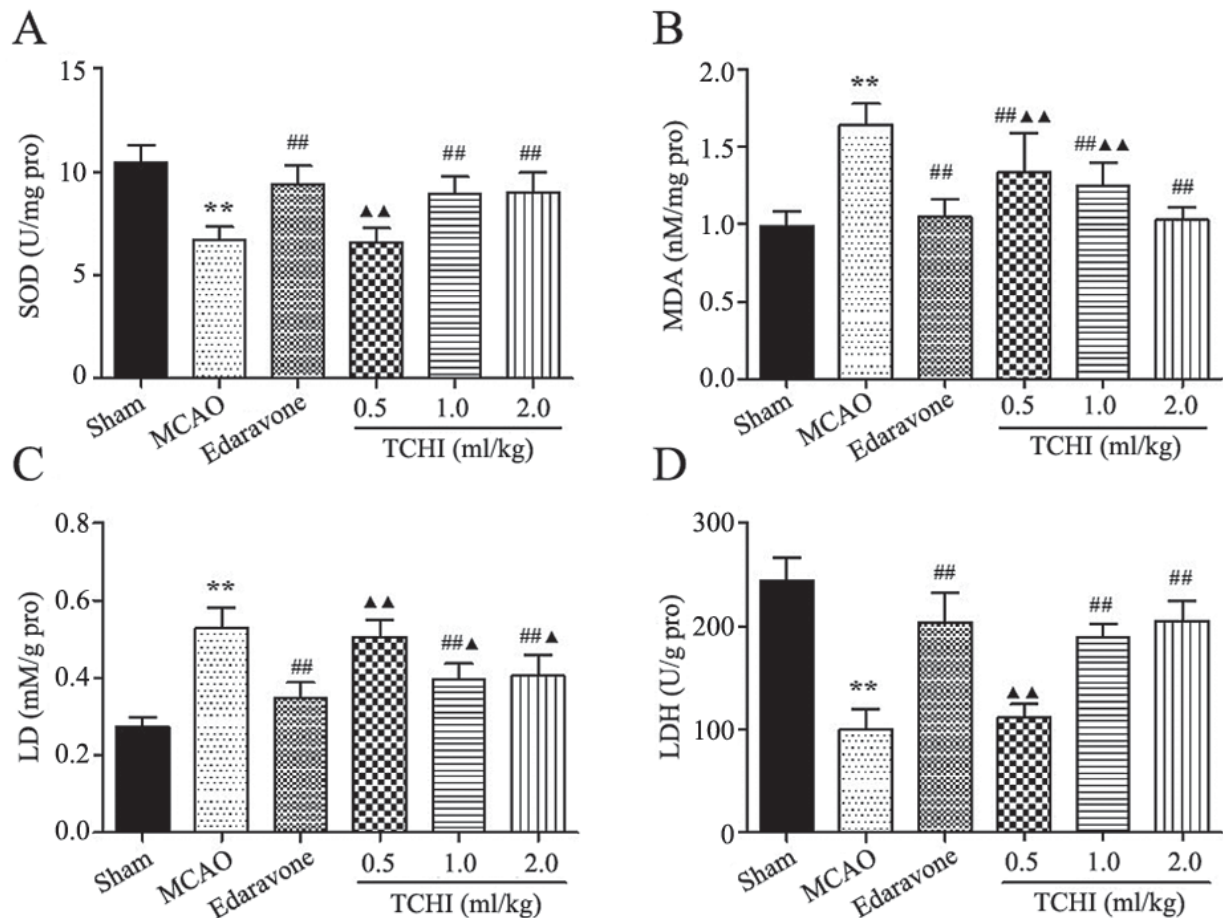


Figure 2. Effect of TCHI on SOD, MDA, LD and LDH levels following MCAO. Representative levels of (A) SOD, (B) MDA, (C) LD and (D) LDH at 24 h following MCAO. Data are expressed as the mean \pm standard error of the mean (n=10-11/group). **P<0.01 vs. sham group; ##P<0.01 vs. MCAO group; ▲P<0.05 and ▲▲P<0.01 vs. edaravone group. TCHI, troloxerutin and cerebroprotein hydrolysate injection; SOD, superoxide dismutase; MDA, malondialdehyde; LD, lactic acid; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion.

TCHI protects against oxidative damage. To investigate the potential antioxidative mechanisms, SOD activity was determined. SOD is one of the most important endogenous antioxidant enzymes in defending against oxidative stress. The results revealed that SOD activity was significantly decreased at 24 h following MCAO surgery, as compared with the sham group (P<0.01). At concentrations of 1 and 2 ml/kg, TCHI and 5 ml/kg edaravone significantly increased SOD activity compared with that in the MCAO model group (8.97 ± 0.82 , 9.03 ± 0.95 and 9.40 ± 0.89 , vs. 6.74 ± 0.61 U/mg, respectively; P<0.01; Fig. 2A). No significant difference was observed in SOD activity between the 1 and 2 ml/kg TCHI groups and the edaravone group. The MDA levels in brain tissues were determined at 24 h following MCAO surgery. The data demonstrated that the MCAO group exhibited significantly increased MDA levels compared with the sham group (1.64 ± 0.14 vs. 0.99 ± 0.10 nM/mg; P<0.01). Treatment with TCHI and edaravone significantly decreased the MDA levels compared with the MCAO model group (1.33 ± 0.26 , 1.26 ± 0.14 , 1.03 ± 0.08 and 1.05 ± 0.12 , respectively vs. 1.64 ± 0.14 nM/mg; P<0.01; Fig. 2B). In addition, the MDA level of the edaravone group was significantly lower than that of the 0.5 and 1 mg/kg TCHI groups. Although no significant difference in MDA level was observed between the 2 ml/kg TCHI group and the edaravone group.

These results suggested that TCHI was an effective antioxidant protecting against cerebral ischemic injury.

TCHI decreases LDH and LD levels in the brain tissue of rats with MCAO. The levels of LDH and LD were also detected in rat brain tissues. The results revealed that LD levels significantly decreased in the TCHI treatment groups (1 and 2 ml/kg) and the edaravone group compared with those in the model group (0.39 ± 0.04 , 0.41 ± 0.05 and 0.34 ± 0.04 , vs. 0.53 ± 0.05 mM/g, respectively; P<0.01; Fig. 2C). The LD level of the TCHI groups was significantly higher compared with the edaravone group (0.51 ± 0.05 , 0.39 ± 0.04 and 0.41 ± 0.05 vs. 0.34 ± 0.04 mM/g, respectively; Fig. 2C). In addition, at the doses of 1 and 2 ml/kg TCHI and 5mg/kg edaravone, LDH levels were significantly increased compared with those in the model group (189.59 ± 12.88 , 205.71 ± 19.37 and 204.34 ± 28.09 , vs. 100.69 ± 19.63 U/g, respectively; P<0.01; Fig. 2D). The LDH level of the 0.5 mg/kg TCHI group was significantly lower compared with the edaravone group (102.20 ± 12.50 vs. 204.34 ± 28.09 , P<0.01; Fig. 2D) although no significant difference was observed in LDH level between the 1 and 2 ml/kg TCHI groups and the edaravone group. The above results suggested that TCHI had a significant effect on the correction of acidosis in the brain.

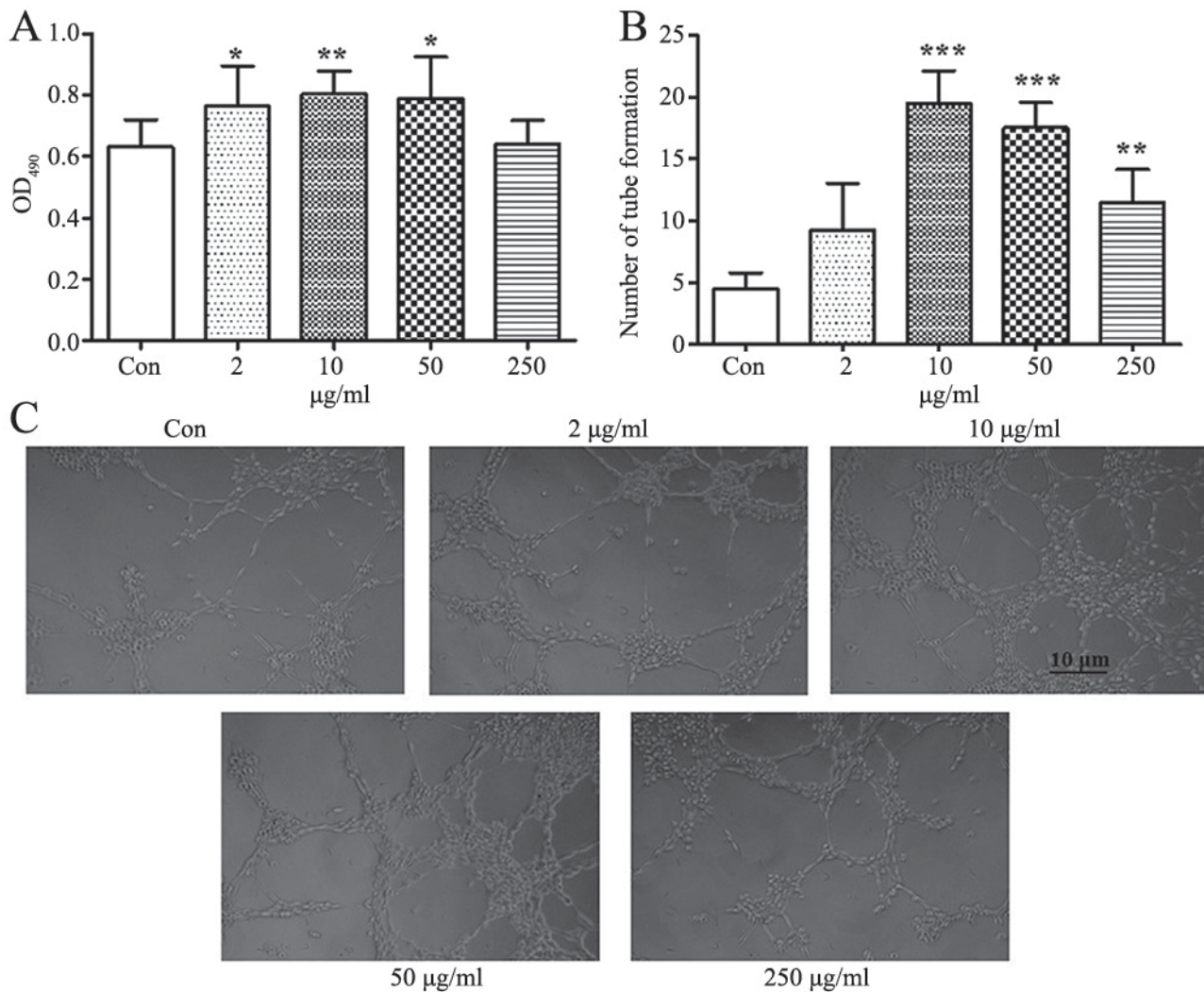


Figure 3. Effects of TCHI on human umbilical vein endothelial cell proliferation and tube formation. (A) Cell proliferation following exposure to TCHI at 2, 10, 50 or 250 $\mu\text{g/ml}$ for 24 h was assessed by an MTT assay. (B) Quantification and (C) cell images (magnification, $\times 100$) of capillary-type tube formation in cells cultured on a layer of Matrigel and incubated with medium containing 2, 10, 50 or 250 $\mu\text{g/ml}$ TCHI at 37°C for 24 h. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, vs. control group. TCHI, troxerutin and cerebroprotein hydrolysate injection; MTT, methylthiazolyl-diphenyl-tetrazolium bromide; OD, optical density.

TCHI increases HUVEC proliferation and tube formation.

The effects of TCHI on endothelial cell proliferation were assessed by an MTT assay. It has previously been reported that troxerutin enhances thymocyte viability and reduces apoptosis at a concentrations of 0.625–10 $\mu\text{g/ml}$, and the peak of viability was observed when a dose of 10 $\mu\text{g/ml}$ was used (22). Concentrations between 2 and 250 $\mu\text{g/ml}$ TCHI were used for the treatment of HUVECs in the current study. Treatment with TCHI (2, 10 and 50 $\mu\text{g/ml}$) significantly increased HUVEC proliferation compared with the control group (0.76 ± 0.13 , 0.80 ± 0.08 and 0.79 ± 0.14 , vs. 0.63 ± 0.09 , respectively; $P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively), while 250 $\mu\text{g/ml}$ TCHI exerted no marked effects on the cell proliferation (Fig. 3A). In addition, the effects of TCHI on HUVEC tube formation were investigated with a Matrigel-based *in vitro* assay. The results revealed that TCHI (10, 50 and 250 $\mu\text{g/ml}$) significantly stimulated tubule formation compared with that observed in the control (19.5 ± 2.65 , 17.5 ± 2.08 and 11.5 ± 2.65 , vs. 4.50 ± 1.29 , respectively; $P < 0.01$; Fig. 3B and C). These data indicated a promoting effect of TCHI on HUVECs during angiogenesis.

TCHI increases HUVEC adhesion and migration.

The adhesion and migration abilities of endothelial cells are essential to vascular development and angiogenesis. To further investigate the proangiogenic activities of TCHI, an endothelial cell adhesion assay was performed. As presented in Fig. 4A and B, with the exception of the concentration of 250 $\mu\text{g/ml}$ TCHI, treatment with 2, 10 and 50 $\mu\text{g/ml}$ TCHI significantly enhanced HUVEC adhesion compared with that in the control (174.78 ± 2.47 , 184.50 ± 1.52 and 177.81 ± 4.85 , vs. 167.07 ± 3.73 , respectively; $P < 0.05$ or $P < 0.01$). Furthermore, the mean migration distances during wound closure and cell migration were observed at 0, 12 and 24 h using scratch assays. The results suggested that low levels of HUVEC migration were observed in the vehicle-treated control, while TCHI (2, 10, 50 and 250 $\mu\text{g/ml}$) strongly enhanced HUVEC migration compared with the control (51.00 ± 6.08 , 59.63 ± 1.10 , 48.87 ± 1.90 and $44.10 \pm 0.53\%$, vs. $21.80 \pm 0.10\%$; $P < 0.01$; Fig. 4C and D).

TCHI promotes angiogenesis in CAM assay. A CAM model was used to confirm the role of TCHI in angiogenesis. As

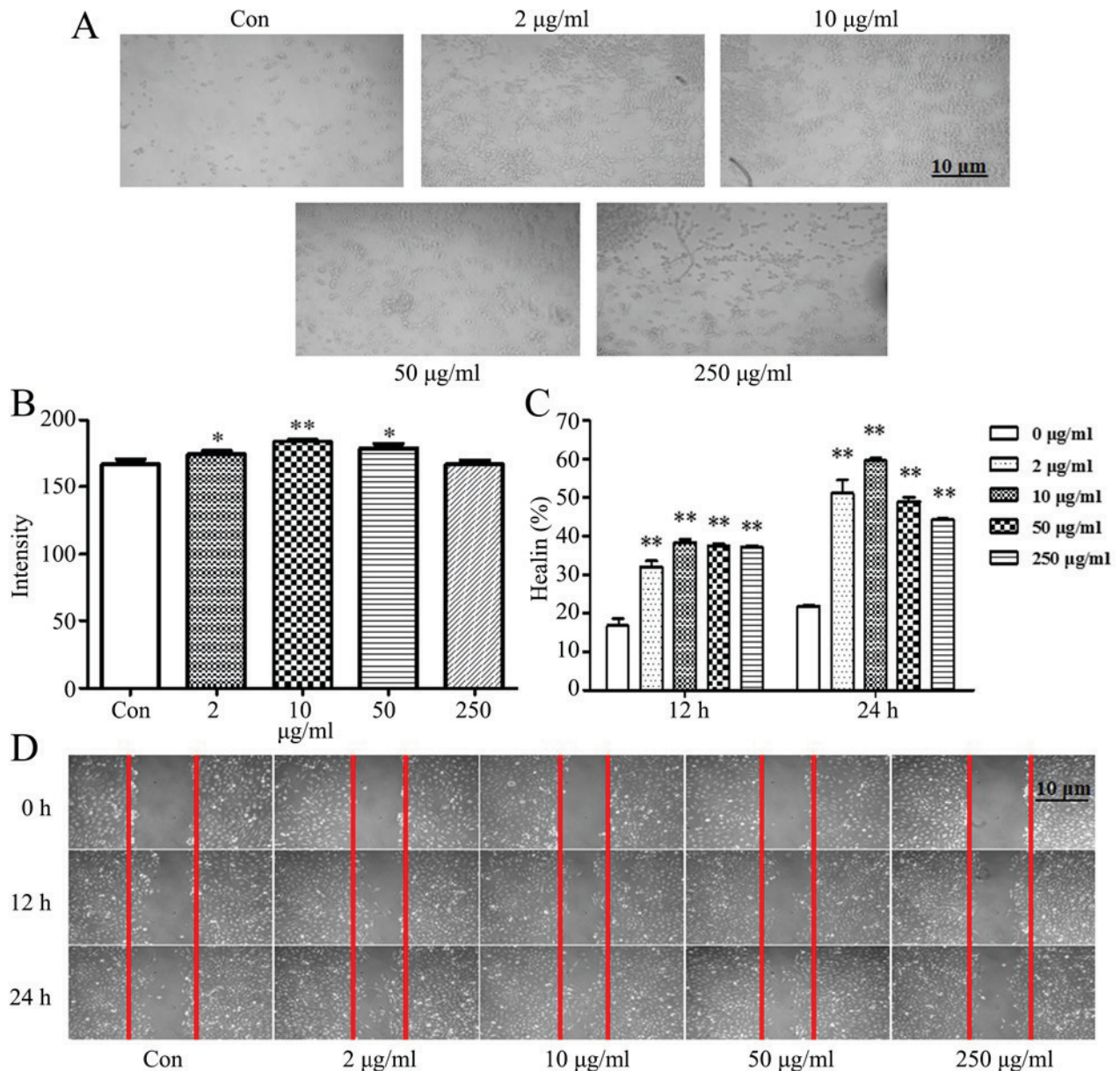


Figure 4. Effects of TCHI on HUVEC adhesion and migration. (A) HUVECs were exposed to TCHI at a concentration of 2, 10, 50 or 250 µg/ml for 24 h, seeded on a Matrigel-coated 24-well plate, incubated for 1 h and observed under a microscope. (B) Adhesion and (C) migration of HUVECs were significantly enhanced by TCHI treatment. (D) Cell migration was examined by a wound healing assay, during which the confluent HUVEC monolayer was wounded with a 200-µl pipette tip and treated with TCHI (2, 10, 50 or 250 µg/ml). At 0, 12 and 24 h, wound healing was visualized with a digital camera. *P<0.05 and **P<0.01 vs. Control. TCHI, troxerutin and cerebroprotein hydrolysate injection; HUVEC, human umbilical vein endothelial cells.

presented in Fig. 5A and B, the blood vessel density significantly increased by 55.32 and 46.81% following exposure to 5 ng/ml bFGF or 10 ng/ml TCHI for 3 days, respectively, as compared with that in the untreated control group (14.60 ± 3.50 and 13.80 ± 3.19 , vs. 9.40 ± 2.37 ; P<0.01; Fig. 5A and B) suggesting a positive impact of TCHI on CAM in angiogenesis.

TCHI promotes microvessel outgrowth from the rat aortic ring. A rat aortic ring model was employed to examine the TCHI-induced angiogenesis *in vitro*. The results demonstrated that TCHI (2, 10, 50 and 250 µg/ml) significantly stimulated microvessel sprouting from the adventitia of cultured aortic rings by 103.84, 261.54, 230.77 and 161.54% compared with that of the control after 6 days of treatment (13.25 ± 4.50 , 23.50 ± 4.73 ,

21.50 ± 1.73 and 17.00 ± 2.94 , vs. 6.50 ± 1.73 , respectively; P<0.05 or P<0.01; Fig. 6A and B). After 9 days of treatment, the number of microvessel increased by 37.23, 184.67, 166.42 and 140.88% in rats treated with 2, 10, 50 and 250 µg/ml TCHI, respectively, relative to that observed in the control group (82.50 ± 9.57 , 91.25 ± 19.31 , 97.50 ± 17.08 and 47.00 ± 9.06 , vs. 34.25 ± 4.92 , respectively; P<0.05 or P<0.01; Fig. 6).

TCHI enhances integrin β3 expression on HUVECs in vitro. Subsequently, integrin β3 mRNA expression was detected in order to further investigate the effects of TCHI on endothelial cells. As discussed earlier, the results demonstrated that 10 µg/ml TCHI presented a strongest pharmacological action on endothelial cell functions, including adhesion, migration

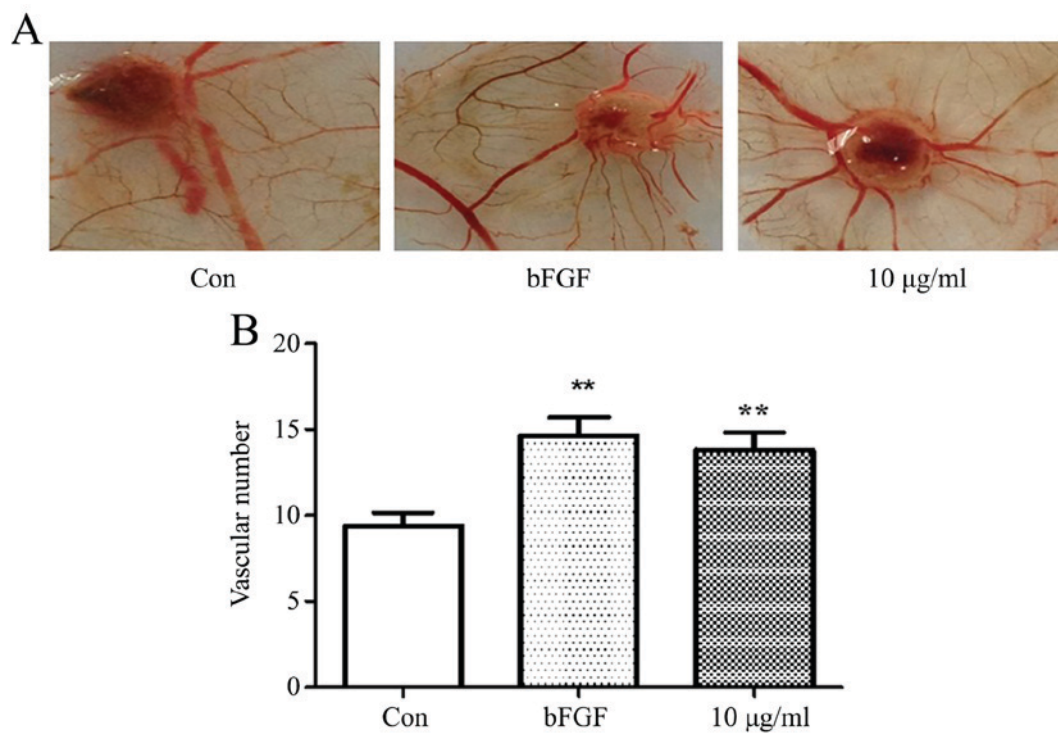


Figure 5. (A) TCHI promoted angiogenesis in the CAM model. (B) Blood vessel density was significantly increased following exposure to 5 ng/ml bFGF or 10 ng/ml TCHI. ** $P < 0.01$ vs. control group. TCHI, troxerutin and cerebroprotein hydrolysate injection; CAM, chick chorioallantoic membrane; bFGF, recombinant bovine basic fibroblast growth factor.

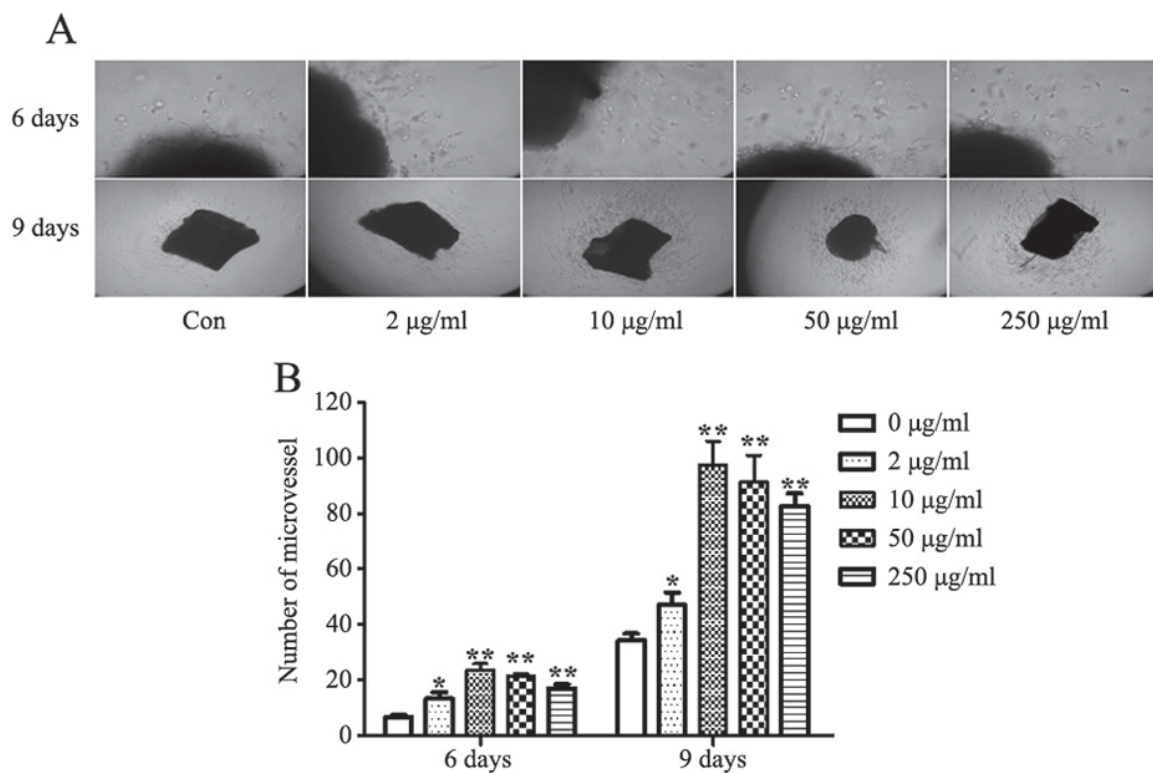


Figure 6. (A) TCHI promoted aortic ring sprouting after 6 and 9 days of treatment. (B) Increased number of microvessels was detected in the rat aortic ring model. * $P < 0.05$ and ** $P < 0.01$ vs. control group. TCHI, troxerutin and cerebroprotein hydrolysate injection.

and capillary formation. Transcriptional expression of integrin $\beta 3$ mRNA on HUVECs following treatment with 10 µg/ml TCHI for 48 h was investigated by RT-qPCR. It was observed

that integrin $\beta 3$ mRNA levels were significantly increased in the 10 µg/ml TCHI group as compared with the control group (1.34 ± 0.08 vs. 1.16 ± 0.09 , respectively; $P < 0.01$; Fig. 7A and B).

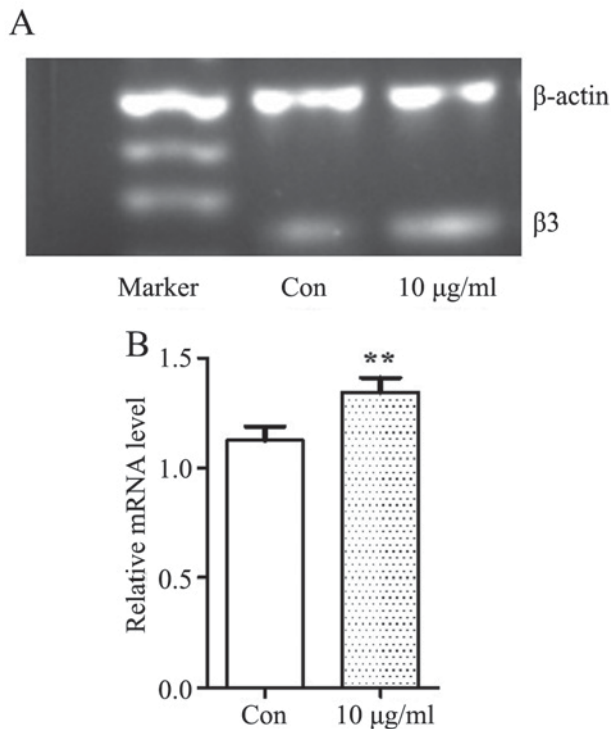


Figure 7. TCHI promoted the expression of integrin $\beta 3$ mRNA on human umbilical vein endothelial cells. (A) Reverse transcription-quantitative polymerase chain reaction assay results and (B) quantified mRNA expression of integrin $\beta 3$ are shown in cells were treated with TCHI for 24 h. TCHI induced a significant increase in integrin $\beta 3$ mRNA expression. Data are expressed as the mean \pm standard error of the mean. ** $P < 0.01$ vs. control group. TCHI, troxerutin and cerebroprotein hydrolysate injection.

This result suggested that TCHI can promote the expression of integrin $\beta 3$ in endothelial cells and enhance the adhesion function of endothelial cells, thus promoting angiogenesis.

Discussion

Troxerutin and cerebroprotein hydrolysate have been observed to have protective effects on acute cerebral infarction in clinical practice (16). However, the mechanisms of action remain unclear. The current study demonstrated that TCHI effectively alleviated neurological symptoms and cerebral ischemic injury in a rat MCAO model. In addition, the results demonstrated that the beneficial effects of TCHI treatment on cerebral ischemia were accompanied by increased LDH and SOD activities, and decreased LD and MDA levels. In addition, TCHI was observed to promote angiogenesis *in vivo* and enhance endothelial cell function *in vitro*.

Following cerebral ischemia and tissue hypoxia, the anaerobic metabolism leads to the production of lactic acid and protons (23). Accumulation of lactic acid results in brain edema, blood-brain barrier dysfunction and free radical production, promoting tissue necrosis by inhibiting uptake of excitatory amino acids (24). LDH is a key enzyme involved in energy metabolism, which converts lactic acid into pyruvate by dehydrogenation (25). The results in the present study demonstrated that severe brain damage resulted in a higher neurological deficit score and lower LDH levels. TCHI treatment increased the LDH levels in brain homogenates and conversely decreased

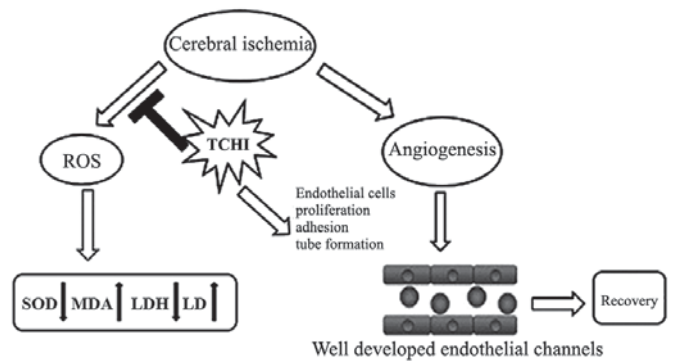


Figure 8. Mechanisms of action illustrating the protective effect of TCHI on neurological function following stroke. TCHI, troxerutin and cerebroprotein hydrolysate injection; ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde; LD, lactic acid; LDH, lactate dehydrogenase.

LD levels, indicating that TCHI reduces the degree of ischemia necrosis and increases the normal brain cell numbers.

Free radicals and ROS are increasingly produced following cerebral ischemia, and oxidative stress is considered as the basic mechanism of brain injury following cerebral ischemia (26,27). ROS levels are modulated by endogenous antioxidant enzymes, including SOD and glutathione peroxidase (GPx). The antioxidant activity of SOD indirectly reflects the ability of scavenging ROS. MDA is an end product of lipid peroxidation and levels reflect the degree of free radical-induced damage (28). Studies have revealed that troxerutin significantly decreases MDA levels and increases GPx activity (29). In the present study, TCHI significantly increased SOD activity and decreased MDA levels following ischemic cerebral injury, suggesting that TCHI attenuates cerebral ischemic injury through the amelioration of oxidative stress. However, the detailed mechanism requires further investigation.

It has previously been confirmed that microvessel density is increased following cerebral ischemia. Post-ischemic angiogenesis improves cerebral blood perfusion, neurological recovery and survival of stroke patients (30). Thus, enhancement of brain revascularization is an important option for the treatment of cerebral ischemia. Angiogenesis comprises a series of events, including endothelial cell proliferation, migration, adhesion and tube formation (31). The effects of TCHI on endothelial cell functions were determined in the present study, and the results demonstrated that TCHI significantly promoted vascular maturation processes in cultured HUVECs, including cell proliferation, adhesion, migration and tube formation. TCHI was further revealed to significantly stimulate microvessel formation in the rat aortic ring *in vitro*. Cell-matrix interactions are crucial for cell migration, and particular ECM proteins have been revealed to regulate endothelial cell migration (32). The scratch assay results obtained in the current study indicated that TCHI treatment promoted endothelial cell migration in a dose-dependent manner. These results provided strong evidence that TCHI stimulated angiogenesis at endothelial cell proliferation, migration, adhesion and tube formation.

The present results further demonstrated that TCHI stimulated the transcription of integrin $\beta 3$ mRNA on endothelial cells. An earlier study confirmed that synergistic interactions

between endothelial cell-specific integrin $\beta 3$ and vascular endothelial growth factor serve a key role in angiogenesis (33). Therefore, the detailed mechanism of TCHI in promoting angiogenesis was further investigated. The tube formation and aortic ring assay results obtained in the current study indicated that TCHI promoted angiogenesis through tube formation. These results were verified by a CAM model, in which TCHI increased blood vessel density and vascular branches. Taken together, these findings implied that TCHI promoted angiogenesis via enhancing endothelial cell migration and inducing the formation of vascular networks.

However, a limitation of the present study was that the association between cerebral ischemia and angiogenesis was not examined *in vivo*. This was due to the complexity of cerebral ischemia and angiogenesis animal models, requiring a much longer time to demonstrate notable changes in comparison with acute animal models (34). Furthermore, we have tried to build animal models, but it is difficult to obtain stable results from the model group; thus, these *in vivo* experiments were delayed for technical reasons and not examined in the present study. Setting up a reliable animal model is currently under investigation.

In conclusion, the findings of the present study suggested that TCHI increased LDH levels and decreased LD levels in rats with cerebral ischemic injury. TCHI also significantly increased SOD activity and decreased MDA levels following ischemia in rat cerebral tissues. In addition, TCHI promoted angiogenesis through increasing the proliferation and enhancing the functions of endothelial cells, including adhesion, migration and capillary formation. Therefore, TCHI reduced experimental ischemic damage through the amelioration of oxidative stress and angiogenesis (Fig. 8). These data are a preliminary elucidation of the role of TCHI in improving cerebral ischemic reperfusion injury, and provided a theoretical basis for the rational use of TCHI in clinical practice.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

WF, SW and WM designed the experiments. WM, XLui, FT, PZ, KC and XLi performed the experiments. QZ, YZ and XZ analyzed the data. WF and WM wrote the manuscript.

WF and XLi revised the manuscript. All authors reviewed the final manuscript.

Ethics approval and consent to participate

All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Xi'an Jiaotong University, School of Medicine (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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