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Original Article

Effect of compound troxerutin and porcine cerebroside injection on the neurological function of the rats with acute cerebral infarction

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Abstract: Objective: We aimed to investigate the effect of compound troxerutin and porcine cerebroside injection (CTPCI) on the neurological function of the rats with acute cerebral infarction and its possible mechanism. Methods: Male specific pathogen-free Sprague-Dawley rats were chosen as the study subjects and were randomly divided into the sham group, the middle cerebral artery occlusion (MCAO) group, the edaravone group, the CTPCI low-dose group, and the CTPCI high-dose group. The MCAO model was induced by suture occlusion technique. The effects of CTPCI on the neurologic deficit and cerebral infarct volume in the MCAO rats were assessed, and the oxidative stress and the levels of angiogenic factors in the rats' brain tissues were detected, in order to explore the cerebral protective mechanism of CTPCI. Results: Twenty-four hours after operation, the CTPCI high-dose group had better neurological function score and lower percentage of cerebral infarct volume than the MCAO group (both $P < 0.05$). Also, the CTPCI high-dose group had higher expression levels of nuclear factor E2-related factor-2 and heme oxygenase-1, lower oxidative stress, and higher expression levels of vascular endothelial growth factor, angiopoietin-1, phosphatidylinositol-3 kinase, and phosphor-protein kinase B than the MCAO group 24 h after operation (all $P < 0.05$). Conclusion: CTPCI can effectively improve the neurological function and reduce the cerebral infarct volume in the rats with acute cerebral infarction. This may be due to the fact that CTPCI can up-regulate the expression levels of nuclear factor E2-related factor-2 and heme oxygenase-1 to reduce the oxidative stress response and up-regulate the expression levels of vascular endothelial growth factor, angiopoietin-1, phosphatidylinositol-3 kinase, and protein kinase B to promote micro-angiogenesis.

Keywords: Compound troxerutin and porcine cerebroside injection, acute cerebral infarction, neural function, oxidative stress, micro-angiogenesis

Introduction

Due to the population aging and people's dietary structure change, the incidence rate of stroke has been rising rapidly each year. Acute cerebral infarction (ACI) is a disease that is caused by vascular stenosis and occlusion due to cerebral atherosclerosis and thrombosis, and the disease accounts for 60% to 80% of the cerebrovascular accidents [1-3]. During the onset of ACI, acute ischemia occurs in the brain cells of patients, resulting in hypoxia and necrosis within a short period of time and eventually neurologic deficit in patients. The disease is life-threatening, has a high mortality rate and a high disability rate, and can bring heavy financial burdens to the family and the society [4]. At present, the main goal of the medical workers in the treatment of ACI is to save the lives of patients and minimize the neural damage to improve patients' neurological prognosis, and how to minimize the neural damage in ACI patients has become a research focus clinically. Over the past 20 years, many cellular, molecular, and genetic studies have been performed to investigate the pathophysiological processes of brain injury in patients with ACI. Oxidative stress (OS), inflammatory response, calcium overload, mitochondrial dysfunction, and cellular apoptosis have been found to play key

roles in the pathological mechanism of brain injury [5, 6]. Also, neovascularization is considered as an essential factor in the recovery of ischemic penumbra [7]. Based on these pathophysiological mechanisms, medicines for the clinical treatment of ACI are being developed continuously.

Compound troxerutin and porcine cerebroside injection (CTPCI) is a compound preparation consisting of troxerutin, monosialotetrahexosylganglioside, active polypeptide, and various amino acids. Troxerutin can improve the microcirculation by inhibiting thrombosis, alleviating vascular injury, and increasing the oxygen content and saturation in blood [8]. Monosialotetrahexosylganglioside can participate in sensing and transmitting the cellular signals and promote the differentiation, regeneration, and repair of nerve tissues [9]. Active polypeptide and amino acids can pass through the blood-brain barrier to regulate and improve neuronal metabolism, promote protein synthesis in brain, and induce neuron differentiation [10]. However, the effect and mechanism of CTPCI in the treatment of ACI remain unclear. Therefore, we established a rat model of middle cerebral artery occlusion (MCAO) to observe the effects of CTPCI on the neurologic deficit and cerebral infarct volume in MCAO rats. We also measured the levels of markers related to OS and angiogenesis in MCAO rats to explore whether CTPCI achieves the cerebral protective effects in ACI via anti-OS and angiogenesis mechanisms.

Materials and methods

Laboratory animals and grouping

Sixty male specific pathogen-free Sprague-Dawley rats (weight: 230-260 g; supplier: Experimental Animal Center of Xi'an Jiaotong University; permit number: SCXK (Shan) 2012-003) were chosen for this study. The rats were randomly divided into the sham group, the MCAO group, the edaravone group (5 mg/kg), the CTPCI low-dose group (0.5 mL/kg), and the CTPCI high-dose group (2.0 mL/kg), 12 rats in each group. All the rats were raised under standard laboratory conditions (12 h-light/12 h-dark, temperature: 22±2°C, relative humidity: 50±5%). Food and water were provided to the rats randomly during their one week of adaptation to the experimental setup. All the animal experiments in this study were approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine.

Medicines and reagents

Medicines: CTPCI (Jilin Buchang Pharma, China; batch number: 160602) and edaravone injection (Nanjing Xiansheng Dongyuan Pharma, China; batch number: 80-170502) were used in this study. CTPCI was a clear yellow to light brown liquid, and 1.0 mL of CTPCI contained 40 mg of troxerutin, 0.5 mg of total nitrogen, and 100 μg of ganglioside.

Main reagents: The main reagents used in this study and their suppliers are listed as follows: triphenyl tetrazolium chloride (TTC, Tianjin Fuchen Chemical Reagent Factory, China; batch number: 20160720), chloral hydrate (Chengdu Kelong Chemical Reagent Factory, China; batch number: 2014031101), Trizol (Invitrogen, USA), reverse transcription kit (GeneCopoeia, USA), polyclonal antibodies of rabbit anti-rat heme oxygenase-1 (HO-1, sc-1796, Santa Cruz Biotechnology, USA), anti-nuclear factor E2-related factor-2 (Nrf2, 12721, Santa Cruz Biotechnology, USA), anti-vascular endothelial growth factor (VEGF, SC-7269, Santa Cruz Biotechnology, USA), anti-angiopoietin-1 (Ang-1, sc-319-824, Santa Cruz Biotechnology, USA), anti-phosphatidylinositol-3 kinase (PI3K, ab38449, Abcam, UK), and anti-phospho-protein kinase B (p-Akt, ab131443, Abcam, UK), test kit for superoxide dismutase (SOD, batch number: 20170213, Nanjing Jiancheng Bioengineering Institute, China), test kit for glutathione peroxidase (GSH-Px, batch number: 20170717, Nanjing Jiancheng Bioengineering Institute, China), test kit for reactive oxygen species (ROS, batch number: 20170207, Nanjing Jiancheng Bioengineering Institute, China), and test kit for malondialdehyde (MDA, batch number: 2017-0207, Nanjing Jiancheng Bioengineering Institute, China).

HO-1 and Nrf2 genes were synthesized by Shanghai Sangon Biotech, China, and the gene sequences are listed in **Table 1**.

MCAO model creation and treatment

MCAO model was established using the improved suture occlusion technique [11, 12]. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g)

Table 1. Primer sequences

Primer	Length (bp)	Sequence
Nrf2	96	Forward: 5'-ACACGGTCCACAGCTCATC-3' Reverse: 5'-TGCCTCCAAAGTATGTCAATCA-3'
HO-1	185	Forward: 5'-ATGCCGGGTCAGGTACTCAG-3' Reverse: 5'-GGCGGTCTTAGCCTCTTCTGT-3'
β-actin	150	Forward: 5'-GTCCCAGTTGGTGACGATGC-3' Reverse: 5'-CCGTCTTCCCCTCCATCG-3'

Note: Nrf2: nuclear factor E2-related factor-2; HO-1: heme oxygenase-1.

Table 2. Longa score in each group (x ± sd)

Group	6 h	24 h
Sham group	0.00±0.00	0.00±0.00
MCAO group	2.90±0.32***	2.54±0.53***
Edaravone group	2.10±0.88$^{\#,a,b}$	1.70±0.48$^{\#\#,aa,ccc}$
CTPCI low-dose group	2.80±0.42	2.50±0.67
CTPCI high-dose group	2.70±0.48	1.70±0.67$^{\#\#,aa,ccc}$

Note: $^{***}P < 0.001$ vs the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the MCAO group; $^{a}P < 0.05$, $^{aa}P < 0.01$ vs the CTPCI low-dose group; $^{b}P < 0.05$ vs the CTPI high-dose group; $^{ccc}P < 0.001$ vs the same group at 6 h after operation. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion

and were fixed in a supine position. After skin preparation and disinfection on the neck, an incision was made along the midline of the neck. The muscle layer was bluntly dissected, and the right common carotid artery and the internal and external carotid arteries were carefully separated. Next, the external carotid artery and the common carotid artery were ligated. The pterygopalatine artery was separated downward from the internal carotid artery. The distal ends of the pterygopalatine artery and the internal carotid artery were temporarily clamped with a rat artery clamp. A small incision was made at the bifurcation of the external carotid artery and internal carotid artery with an ophthalmic scissor, and a smooth nylon fishing line with a spherical head (diameter: 0.265 mm, a mark was made 18 mm from the spherical head) was inserted through the incision. The thread was pushed along the internal carotid artery gently and slowly (insertion depth: 1.8-2.0 cm) until a slight resistance was felt, and the ischemia began from that point. Afterward, the pre-made loose knot was tightened. The incision was ligated and the wound was sutured. The body temperature was maintained at 37°C during the operation. In the sham group, the blood vessels were separated but the nylon thread was not inserted. Two hours after modeling, the rats' neurological function was assessed by modified Longa score [13]. The model was successfully established if the score was no less than 1.

Immediately after the modeling and at 6 h and 24 h after modeling, the rats in the edaravone group, the CTPCI low-dose group, and the CTPCI high-dose group were injected through the tail vein with 5 mg/kg edaravone, 0.5 mg/kg CTPCI, and 2.0 mg/kg CTPCI, respectively. Meanwhile, the rats in the sham group and the MCAO group were injected with the same amount of normal saline (Yichang Sanxia Pharmaceutical, China; specification: 100 mL, 0.9 g).

Neurological function evaluation

The rats' neurological function was evaluated using modified Longa score at 6 h and 24 h after modeling [13]. The scoring was as follows: 0, normal, no neurologic deficit; 1, failure to extend the left forepaw when the rat was lifted by the tail; 2, tilting and circling to the left side when walking; 3, falling to the left side; 4, failure of spontaneous walking and loss of consciousness. The total score was 4 points. The higher the score, the more severe impairment of the neurological function.

TTC staining

Two hours after the last administration of the drugs, the rats were decapitated and the brains were carefully taken out. The cerebellum, olfactory bulb, and brain stem were removed on the ice, and the remaining brain tissues were cut into 6 slices at a thickness of 2 mm. The brain slices were immediately stained with TTC in the dark at 37°C for 30 min. Next, the brain slices were fixed in 10% formaldehyde solution and stored away from light. The non-ischemic area was stained rose red, whereas the infarct area was white. The brain slices were scanned with a scanner, and the cerebral infarct volume was calculated and analyzed using Image-Pro Plus 6.0 software.

RT-PCR

The ischemic penumbra tissues were placed in the Eppendorf tubes containing Trizol. Total

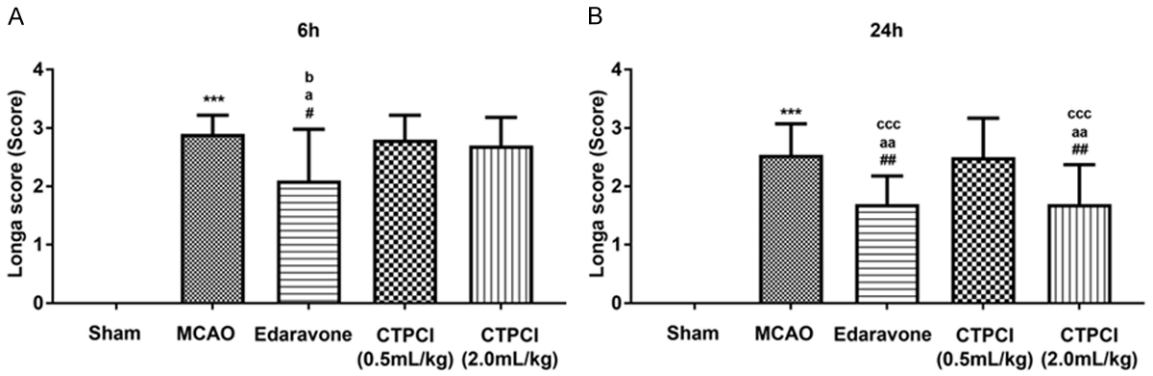

Figure 1. Long score in each group. A. Longa score of the rats at 6 h after modeling; B. Longa score of the rats at 24 h after modeling. ***P < 0.001 vs the sham group; #P < 0.05, ##P < 0.01 vs the MCAO group; aP < 0.05, aaP < 0.01 vs the CTPCI low-dose group; bP < 0.05 vs the CTPCI high-dose group; cccP < 0.001 vs the same group at 6 h after operation. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion.

Table 3. Cerebral infarct volume in each group (x ± sd)

Group	cerebral infarct volume (%)
Sham group	00.00±0.00
MCAO group	45.35±6.75***
Edaravone group	10.59±4.40###,aaa,bbb
CTPCI low-dose group	44.82±6.03
CTPCI high-dose group	32.78±7.86###,aa

Note: ***P < 0.001 vs the sham group; ###P < 0.001 vs the MCAO group; aaP < 0.01, aaaP < 0.001 vs the CTPCI low-dose group; bbbP < 0.001 vs the CTPI high-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion.

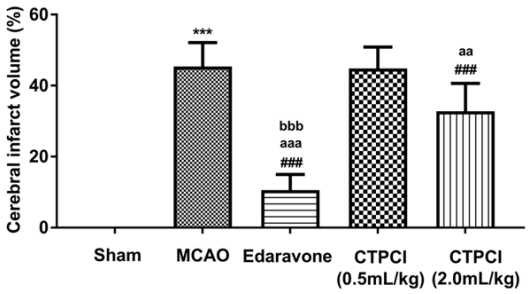

Figure 2. Cerebral infarct volume in each group. ***P < 0.001 vs the sham group; ###P < 0.001 vs the MCAO group; aaP < 0.01, aaaP < 0.001 vs the CTPCI low-dose group; bbbP < 0.001 vs the CTPCI high-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion.

RNA was extracted after ultrasonic disruption. The RNA has an A260/A280 ratio of 1.8-2.0 and the purity was over 90%. The mRNAs (2 μg) were reversely transcribed into cDNAs and amplified. The reaction conditions were as follows: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 30 s; the PCR steps were repeated for 40 cycles. β-actin was used as the internal reference, and the relative expression levels of each gene were calculated. The primer se-quences are listed in **Table 1**.

ELISA

At 2 h after the last administration of drugs, the rats were sacrificed by decapitation. The ischemic penumbra tissues were taken out and treated with pre-cooled normal saline in a mass-to-volume ratio of 1:9 (supplier of normal saline: Yichang Sanxia Pharmaceutical, China; specification: 100 mL, 0.9 g). The samples were then homogenized with an electric homogenizer in ice bath for 3 min followed by centrifugation at 10,000 rpm for 10 min to prepare 10% brain homogenate. The supernatant was stored at -20°C, and 50 μL of the supernatant of 10% brain homogenate was taken to determine the protein content using biuret method. The levels of ROS, MDA, SOD, and GSH-Px in the supernatant were detected according to the manufacturers' instructions of the test kits. The OD value at 450 nm was measured with an automatic microplate reader.

Western blot

The whole-cell, cytoplasm, and nucleoprotein were isolated from the rats' ischemic penumbra tissues according to the manufacturers' instructions of the test kits. The protein con-

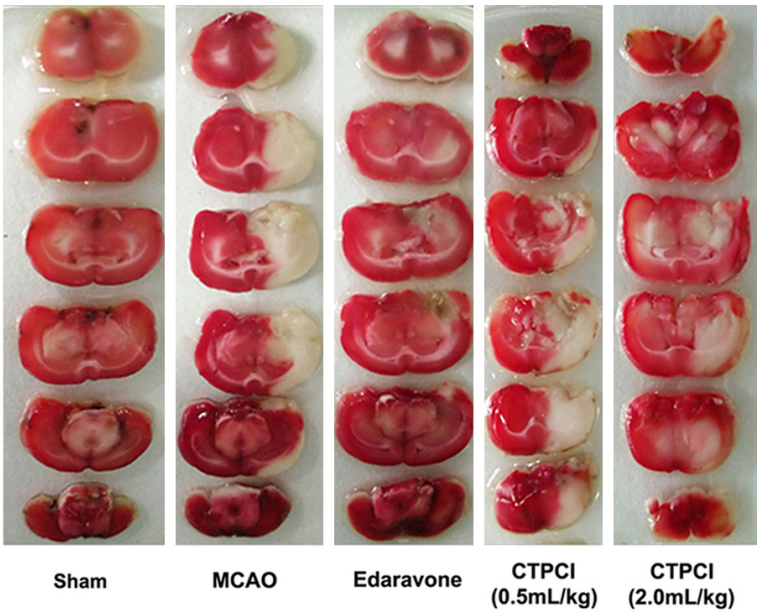

Figure 3. TTC staining of the brain slices in each group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; TTC: triphenyl tetrazolium chloride.

centration was determined by the modified bicinchoninic acid protein assay. The 12% gel was prepared and the electrophoresis was conducted to separate the proteins. After protein transfer and sealing, the membrane was placed at room temperature for 1 h. Next, the samples were incubated with antibodies of rabbit anti-rat HO-1 (1:1,000), anti-Nrf2 (1:1,000), anti-VEGF (1:1,000), anti-Ang-1 (1:1,000), anti-PI3K (1:1,000), anti-p-Akt (1:1,000), and anti-β-actin (1:5,000) at 4°C overnight. On the next day, the membrane was treated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies at room temperature for 2 h. The membranes were washed and stained with electrochemiluminescence solution in the darkroom for 1 min. After exposure, the image was visualized and photographed with a gel imaging system. β-actin was used as the internal reference, and the relative expression level of each protein (the ratio of target protein to internal reference) was analyzed using Quantity One software.

Statistical methods

SPSS 23.0 software was applied for data analysis. Count data were expressed as number or percentage and were examined by Chi-square test. Measurement data that were normally distributed were expressed as mean ± standard deviation (x ± sd). One-way analysis of variance was performed for comparison between multiple groups, and post hoc comparisons were performed using SNK-q and LSD t-tests. The test was two-sided at the $\alpha = 0.05$ level of significance. $P < 0.05$ indicated a statistically significant difference.

Results

Effect of CTPCI on the neurological function of the rats

No neurologic deficit was observed in the sham group (Longa score: 0). At 6 h after modeling, evident neurological deficit was observed in the MCAO group, and the Longa score in this group was higher than that in the sham group ($P < 0.001$), indicating successful creation of MCAO model. The Longa scores in the CTPCI low-dose and high-dose groups did not differ from that in the MCAO group at this time point (both $P > 0.05$) and the Longa score in the edaravone group was lower than those in the MCAO group and the two CTPCI groups at this time point (all $P < 0.05$). At 24 h after modeling, the Longa score in the MCAO group was still higher than that in the sham group ($P < 0.001$), and the Longa scores in the CTPCI high-dose group and the edaravone group decreased compared with their scores at 6 h after modeling (both $P < 0.001$) and the scores in these two groups were lower than that in the MCAO group ($P < 0.01$). At 24 h after modeling, there were no differences in the Long score between the CTPCI high-dose group and the edaravone group and between the CTPCI low-dose group and the MCAO group (both $P > 0.05$). See **Table 2** and **Figure 1**.

Effect of CTPCI on cerebral infarct volume in the rats

There was no cerebral infarct in the sham group (0%), whereas the other groups all had different degrees of cerebral infarct. The infarct volume in the MCAO group was larger than that in the sham group ($P < 0.001$). Compared with the MCAO group, the infarct volume in the edaravone group and the CTPCI high-dose group was smaller (both $P < 0.001$), while the infarct vol-

Table 4. Expression levels of Nrf2 and HO-1 in each group (x ± sd)

Group	Nrf2 mRNA	HO-1 mRNA	Nrf2	HO-1
Sham group	0.32±0.07	0.15±0.04	0.28±0.06	0.26±0.05
MCAO group	0.43±0.09**	0.25±0.08**	0.42±0.08***	0.45±0.07***
Edaravone group	0.53±0.10$^{\#,a}$	0.58±0.11$^{\#\#\#,aaa}$	0.70±0.15$^{\#\#\#,aaa}$	0.68±0.18$^{\#\#\#,aaa}$
CTPCI low-dose group	0.44±0.09	0.28±0.09	0.46±0.09	0.46±0.10
CTPCI high-dose group	0.54±0.10$^{\#,a}$	0.57±0.10$^{\#\#\#,aaa}$	0.67±0.12$^{\#\#\#,aaa}$	0.70±0.16$^{\#\#\#,aaa}$

Note: **P < 0.01, ***P < 0.001 vs the sham group; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs the MCAO group; ^{a}P < 0.05, ^{aaa}P < 0.001 vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; Nrf2: nuclear factor E2-related factor-2; HO-1: heme oxygenase-1.

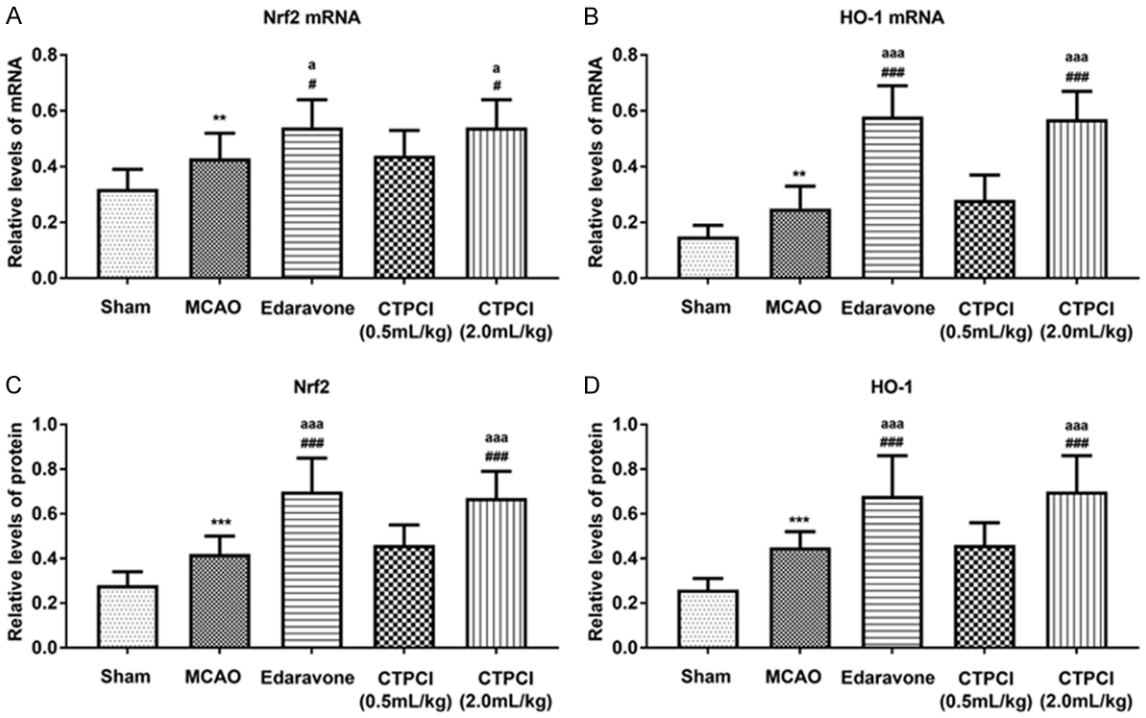

Figure 4. mRNA and protein expression levels of Nrf2 and HO-1 in each group. A. Nrf2 mRNA expression levels in rats' brain tissues; B. HO-1 mRNA expression levels in rats' brain tissues; C. Nrf2 protein expression levels in rats' brain tissues; D. HO-1 protein expression levels in rats' brain tissues. **P < 0.01, ***P < 0.001 vs the sham group; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs the MCAO group; ^{a}P < 0.05, ^{aaa}P < 0.001 vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; Nrf2: nuclear factor E2-related factor-2; HO-1: heme oxygenase-1.

ume in the CTPCI low-dose group was similar to that in the MCAO group (P > 0.05). Compared with the edaravone group, the infarct volume in the CTPCI high-dose was larger (P < 0.001). See **Table 3** and **Figures 2** and **3**.

Effect of CTPCI on the expression levels of Nrf2 and HO-1 in rats' brain tissues

Compared with the sham group, the mRNA and protein levels of Nrf2 and HO-1 in the MCAO group were higher (P < 0.01 or P < 0.001). Compared with the MCAO group, the mRNA and protein expression levels of Nrf2 and HO-1 in the edaravone group and the CTPCI high-dose group were higher (P < 0.05 or P < 0.001). There were no differences in the levels of these markers between the CTPCI low-dose group and the MCAO group and between the CTPCI high-dose group and the edaravone group (all P > 0.05). See **Table 4** and **Figures 4** and **5**.

Effects of CTPCI on the levels of MDA, ROS, SOD and GSH-Px in rats' brain tissues

Compared with the sham group, the MCAO group had higher levels of MDA and ROS and lower levels of SOD and GSH-Px (all P < 0.001).

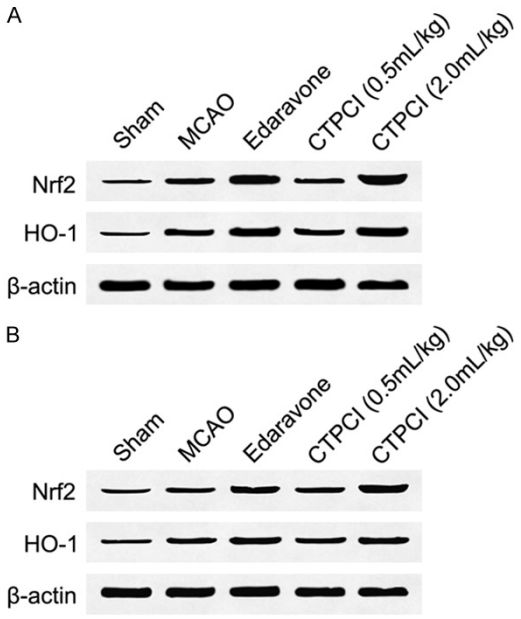

Figure 5. mRNA and protein expression levels of Nrf2 and HO-1 in each group. A. RT-PCR results of Nrf2 and HO-1 mRNA expression levels in rats' brain tissues; B. Western blot results of Nrf2 and HO-1 protein expression levels in rats' brain tissues. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; Nrf2: nuclear factor E2-related factor-2; HO-1: heme oxygenase-1.

Compared with the MCAO group, the edaravone group and the CTPCI high-dose group had lower MDA and ROS levels and higher SOD and GSH-Px levels (all $P < 0.001$); the MDA level in the CTPCI low-dose group was lower than that in the MCAO group ($P < 0.05$), but there were no differences in the levels of other markers between the CTPCI low-dose group and the MCAO group (all $P > 0.05$). Compared with the CTPCI low-dose group, the edaravone group and the CTPCI high-dose group had lower levels of MDA and ROS and higher levels of SOD and GSH-Px (all $P < 0.001$). There were no differences between the edaravone group and the CTPCI high-dose group in the levels of these markers (all $P > 0.05$). See **Table 5** and **Figure 6**.

Effects of CTPCI on the protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in rats' brain tissues

Compared with the sham group, the protein levels of PI3K and p-Akt in the ischemic penumbra of the rats in the MCAO group were lower ($P < 0.01$ or $P < 0.001$), whereas the protein levels of VEGF and Ang-1 in this group were higher (both $P < 0.001$). Compared with the MCAO group, the protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in the edaravone group and the CTPCI high-dose group were higher (all $P < 0.001$), while the protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in the CTPCI low-dose group were similar to those in the MCAO group (all $P > 0.05$). No differences were observed between the edaravone group and the CTPCI high-dose group in the levels of these markers (all $P > 0.05$). See **Table 6**; **Figures 7** and **8**.

Discussion

In the present study, we found that CTPCI significantly decreased the modified Longa score and the cerebral infarct volume of the MCAO rats, suggesting that CTPCI can achieve marked cerebral protective effects on the ischemic brain tissues of the MCAO rats.

The brain tissue injury following brain ischemia can greatly affect the neurological prognosis of patients. The process of ischemic brain tissue damage involves complex pathophysiological mechanisms, and the OS injury is clinically regarded as the key mechanism [5, 6, 14]. Ischemia, hypoxia, and especially ischemia-reperfusion injury, can result in the production and accumulation of large amounts of ROS in the brain tissue and continuous consumption of the antioxidant substances such as SOD and GSH-Px in the brain tissue, which can lead to the imbalance between oxidation and antioxidation in the brain and cause OS reaction [15]. ROS, a trigger of OS, can damage the cells by modifying various biomolecules of the cell and acting on specific signal molecules. These OS and oxidation products can activate the transient receptor potential channel, a non-selective cationic channel with high permeability to calcium and zinc ions, thus causing great influx of calcium and zinc ions and damaging the neurons [16, 17]. Therefore, reducing OS injury by inhibiting OS response serves an important role in protecting ischemic brain tissues. In this study, we found that CTPCI significantly reduced the levels of ROS and MDA and increased the activities of SOD and GSH-Px in the ischemic brain tissues of MCAO rats, indicating that CTPCI has marked antioxidant effect. Nrf2/

Table 5. Levels of MDA, ROS, SOD, and GSH-Px in each group (x ± sd)

Group	MDA (nmol/mgprot)	ROS (%)	SOD (U/gprot)	GSH-Px (μmol/L)
Sham group	0.99±0.10	100.02±10.82	10.47±0.84	70.75±6.24
MCAO group	1.64±0.14***	204.93±23.55***	6.74±0.61***	28.69±4.63***
Edaravone group	1.12±0.12$^{\#\#\#,aaa}$	158.92±15.38$^{\#\#\#,aaa}$	9.40±0.89$^{\#\#\#,aaa}$	65.34±5.09$^{\#\#\#,aaa}$
CTPCI low-dose group	1.50±0.18$^{\#}$	198.75±21.87	6.57±0.72	30.83±4.62
CTPCI high-dose group	1.15±0.08$^{\#\#\#,aaa}$	161.63±17.15$^{\#\#\#,aaa}$	9.03±0.95$^{\#\#\#,aaa}$	62.71±6.07$^{\#\#\#,aaa}$

Note: ***P < 0.001 vs the sham group; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs the MCAO group; ^{aaa}P < 0.001 vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase.

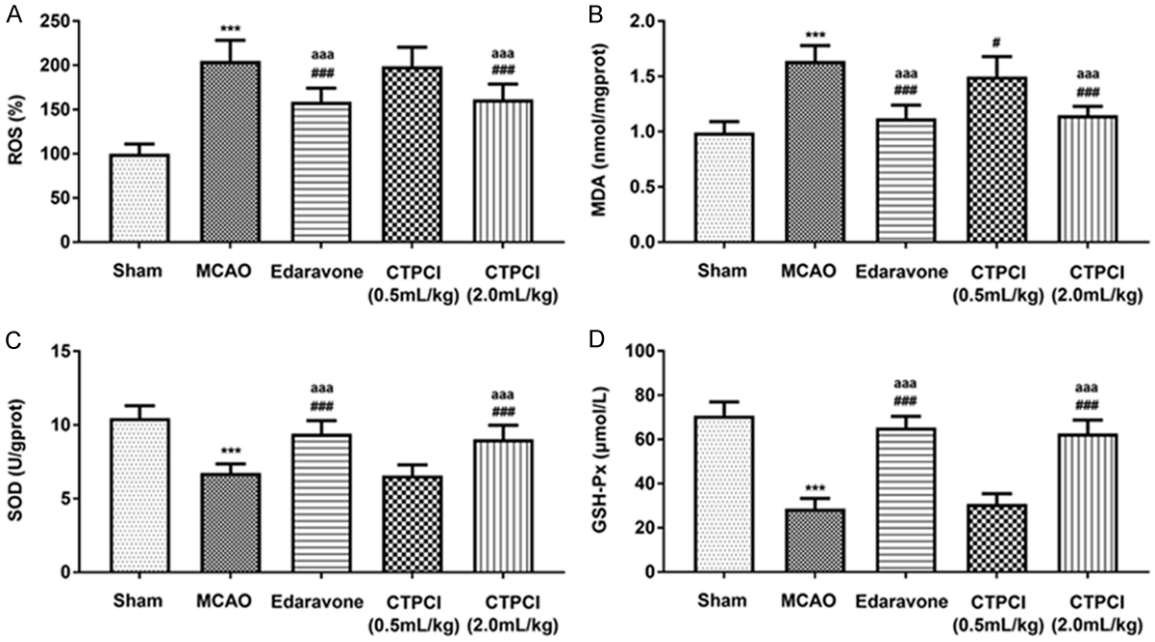

Figure 6. Levels of MDA, ROS, SOD, and GSH-Px in each group. A. MDA levels in rats' brain tissues; B. ROS levels in rats' brain tissues; C. SOD levels in rats' brain tissues; D. GSH-Px levels in rats' brain tissues. ***P < 0.001 vs the sham group; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs the MCAO group; ^{aaa}P < 0.001 vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase.

Table 6. Protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in each group (x ± sd)

Group	PI3K	p-Akt	VEGF	Ang-1
Sham group	1.40±0.23	1.25±0.22	1.01±0.07	1.03±0.10
MCAO group	1.15±0.13**	0.55±0.10***	1.49±0.16***	1.55±0.18***
Edaravone group	1.62±0.35$^{\#\#\#,aaa}$	1.12±0.20$^{\#\#\#,aaa}$	2.32±0.38$^{\#\#\#,aaa}$	1.98±0.30$^{\#\#\#,aaa}$
CTPCI low-dose group	1.18±0.15	0.57±0.13	1.56±0.19	1.60±0.21
CTPCI high-dose group	1.58±0.30$^{\#\#\#,aaa}$	1.13±0.22$^{\#\#\#,aaa}$	2.45±0.42$^{\#\#\#,aaa}$	2.13±0.42$^{\#\#\#,aaa}$

Note: **P < 0.01, ***P < 0.001 vs the sham group; $^{\#\#\#}$P < 0.001 vs the MCAO group; ^{aaa}P < 0.001 vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; PI3K: phosphatidylinositol-3 kinase; p-Akt: phospho-protein kinase B; VEGF: vascular endothelial growth factor; Ang-1: angiopoietin-1.

antioxidant response element signaling pathway has become a popular research topic in the studies of ischemic cerebral infarction in recent years. It has been reported that Nrf2 can interact with antioxidant response elements and regulate the expressions of various detoxifying

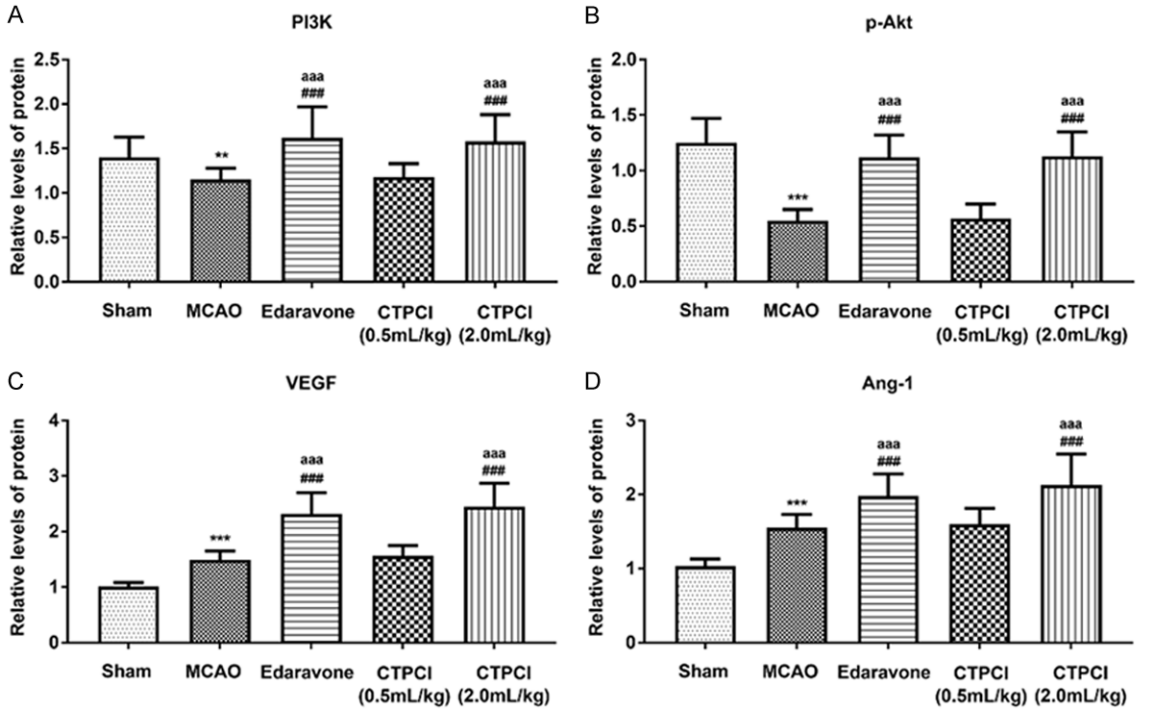

Figure 7. Protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in each group. A. PI3K protein expression levels in rats' brain tissues; B. p-Akt protein expression levels in rats' brain tissues; C. VEGF protein expression levels in rats' brain tissues; D. Ang-1 protein expression levels in rats' brain tissues. $^{**}P < 0.01$, $^{***}P < 0.001$ vs the sham group; $^{###}P < 0.001$ vs the MCAO group; $^{aaa}P < 0.001$ vs the CTPCI low-dose group. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; PI3K: phosphatidylinositol-3 kinase; p-Akt: phospho-protein kinase B; VEGF: vascular endothelial growth factor; Ang-1: angiopoietin-1.

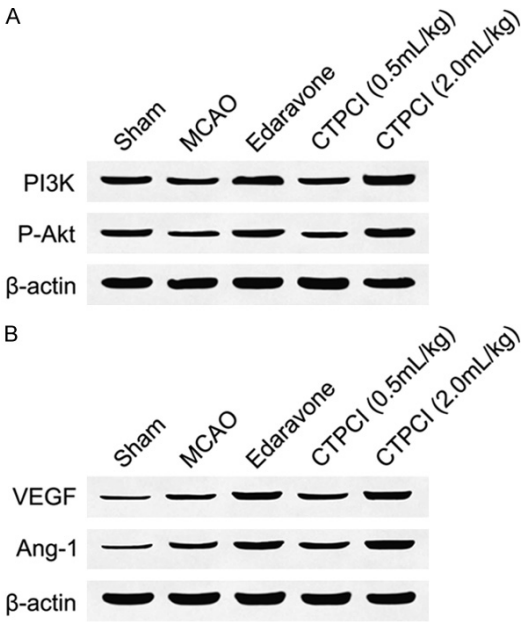

Figure 8. Western blot results of protein expression levels of PI3K, p-Akt, VEGF, and Ang-1 in each group. A. Western blot results of PI3K and p-Akt protein expression levels in rats' brain tissues; B. Western blot results of VEGF and Ang-1 protein expression levels in rats' brain tissues. CTPCI: compound troxerutin and porcine cerebroside injection; MCAO: middle cerebral artery occlusion; PI3K: phosphatidylinositol-3 kinase; p-Akt: phospho-protein kinase B; VEGF: vascular endothelial growth factor; Ang-1: angiopoietin-1.

enzymes, ROS scavengers, and electrophiles to improve the antioxidant capacity of the cells, which is one of the main mechanisms by which the cells resist OS or attack of electrophilic substances [18, 19]. Our results showed that CTPCI could up-regulate the expression levels of Nrf2 and HO-1 in the MCAO rats, which may explain the anti-OS effect of CTPCI in the ischemic brain tissues.

In recent years, many studies have documented that angiogenesis plays an important role in the treatment of cerebral ischemia [20, 21]. Studies have demonstrated that the prognosis of stroke patients with high vascular density is much better than those with low vascular density. The newly formed collateral vessels can improve the tissue perfusion around the isch-

emic area, revealing that the reconstruction of neurovascular network in the damaged area is essential for the recovery of neurological function after cerebral ischemia [22]. Via the regulation by signals that promote neovascularization, the vascular endothelia can change morphologically, proliferate, and migrate under the action of chemokines, which eventually lead to the formation of new blood vessels on the original vessels [23]. It has been reported that VEGF is a key factor in the process of angiogenesis. VEGF can promote angiogenesis, inhibit ischemic neuronal damage, and have anti-inflammatory effect [24]. Early studies by Sun et al. confirmed that intraventricular injection of exogenous VEGF in MCAO animals can promote neovascularization and reduce the cerebral infarct volume [25]. Ang-1 participates in the vascular development, and it can bind to the endothelium-specific tyrosine kinase receptor, Tie-2, to cause smooth muscle cells and pericytes to surround the new endothelial lumen so that the function of the new blood vessels can mature [26]. Ang-1 and VEGF can work synergistically to promote angiogenesis and maturity of the newly-formed vessels. Moreover, recent studies have found that PI3K/Akt pathway is a key signal pathway in the process of angiogenesis, which can stabilize vascular permeability by activating Tie-2 signal transduction pathway [27-29]. The results of our study showed that the protein levels of PI3K, p-Akt, Ang-1, and VEGF in the ischemic penumbra of MCAO rats significantly increased after the treatment with 2 mL/kg CTPCI, revealing that CTPCI has an evident angiogenesis-promoting effect, and this effect may be achieved by the activation of PI3K/Akt pathway via upregulating the expressions of VEGF and Ang-1.

However, it should be noted that the low dose of CTPCI (0.5 mL/kg) failed to markedly improve the neurological function and reduce the infarct volume in the MCAO rats, suggesting that the cerebral protective effect of CTPCI is dose-dependent. In addition, there were no significant differences in the levels of various markers between the CTPCI high-dose group (2 mL/kg) and the edaravone group. Therefore, whether a higher dose of CTPCI can further improve the therapeutic effect needs to be further investigated.

In conclusion, CTPCI can effectively improve the neurological function and reduce the cerebral infarct volume in the ACI rats. CTPCI may exert its cerebral protective effects by up-regulating the expressions of Nrf2 and HO-1 to reduce OS reaction and upregulating the expressions of VEGF and Ang1 and activating PI3K/Akt pathway to promote microangiogenesis.

Disclosure of conflict of interest

None.

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