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ORIGINAL RESEARCH

Administration of troxerutin and cerebroprotein hydrolysate injection alleviates cerebral ischemia/reperfusion injury by down-regulating caspase molecules

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Background: Cerebral ischemia/reperfusion injury (I/R injury) is an important pathological process for nervous system. The I/R injury usually causes cerebral hypoxia, infarct or stroke. This study aimed to evaluate effects of troxerutin and cerebroprotein hydrolysate injection (TC) on I/R injury in rat models.

Methods: Middle-cerebral artery occlusion/reperfusion (MCAO/R) rat models were established. Rats were divided into normal control (NC), MCAO/R rat model (injecting saline) and MCAO/R rats administrating with TC group (injecting with TC at concentration of 2 mL/100 g body weight). Neurological scores were evaluated with Garcia scale. Magnetic resonance imaging (MRI) was employed to observe infarct area, contralateral area and apparent diffusion coefficient (ADC) values. Cerebral infarct size was examined and visualized by staining with 2,3,5-triphenyltetrazolium chloride (TTC). Western blotting assay was used to determine caspase-1, caspase-3 and caspase-8 expression.

Results: The infarct size of mice in MCAO/R+TC group was smaller significantly compared to that in MCAO/R group ($p<0.05$). The infarct/contralateral area ratio of T2 and T2 Flair signals in MCAO/R+TC group were lower significantly compared to that in MCAO/R group ($p<0.05$). ADC values in MCAO/R+TC group were significantly enhanced compared to that in MCAO/R group ($p<0.05$). The troxerutin and cerebroprotein treatment significantly increased neurological scores compared to that in MCAO/R group ($p<0.05$). Troxerutin and cerebroprotein treatment significantly decreased expression of caspase-1, caspase-3, caspase-8 compared to that in MCAO/R group ($p<0.05$).

Conclusion: Troxerutin and cerebroprotein administration alleviated cerebral I/R injury by down-regulating caspase molecules.

Keywords: ischemia/reperfusion injury, troxerutin and cerebroprotein hydrolysate injections, apoptosis, caspase

Introduction

Cerebral ischemia usually defines the process where the blood flowing to cerebrum is insufficient in response to the metabolic demand.[1,2] Clinically, the cerebral ischemia is considered to be a leading reason for mortality and morbidity all over the world.[3] Cerebral ischemia always causes brain tissue death, cerebral hypoxia, ischemic stroke and cerebral infarction; therefore, it is related to cerebrovascular disorders or diseases.[4,5] Although the reperfusion is considered as a beneficial

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characteristic for the cerebral ischemia, the reperfusion might also cause detrimental damage, such as ischemia/reperfusion injury (I/R injury).[6] In recent years, many studies have proven the mechanisms for I/R injury; however, there are also a few controversies. The I/R injury leads to the over-load of free-radicals, which could cause the apoptosis via triggering lipid peroxidation and transmitting Ca^{2+} channel signals.[7] The apoptosis participating in processes of I/R injury is mediated by different signaling pathways, including mitochondrial signaling pathway, C-Jun N-terminal kinases (JNK) signaling pathway and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway.[8,9] Therefore, the drugs that target the pathological processes might be helpful for the treatment of I/R injury.

Troxerutin, as a naturally discovered flavonoid, plays critical roles in anti-oxidation, anti-inflammation, anti-apoptotic activities and anti-tumors.[10] Cerebroprotein could facilitate troxerutin distributing and associates to the effects of troxerutin on acute ischemic stroke or injury.[11] Meanwhile, the troxerutin and cerebroprotein hydrolysate injections (TC) could also promote the synthesis of brain proteins, improve metabolism of neurons and further protect against the neural damages.[12]

In this study, the middle-cerebral artery occlusion was employed to establish a middle-cerebral artery occlusion/reperfusion (MCAO/R) rat models. Therefore, the present study aimed to evaluate the effects of troxerutin and cerebroprotein hydrolysate injections (TC) on neurological behavior, infarct size and expressions of apoptotic markers.

Materials and methods

Animals

Male Sprague Dawley (SD) rats, weighing from 220 g to 250 g, were purchased from Animal center of China Medical University (Shenyang, China). SD rats were housed at 25±2°C, with a light/dark cycle of 12 hrs/12 hrs, and in pathogen-free conditions. The rats were free to the food and water.

This study was approved by the Ethical Committee of The First Hospital of Jinzhou Medical University, Jinzhou, China. The welfare of the animals complied with the Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892–2018) promulgated by the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China.

Establishment of MCAO/R rat model

The MCAO/R rat model was established according to the approach that studies the reversible regional I/R injury.[13] In brief, the rats were anesthetized by intraperitoneally injecting with 10% chloral hydrate (at final concentration of 0.4 mL/100 g body weight, Zhaohui Pharma. Co. Ltd., Shanghai, China). The internal-carotid artery, left external-carotid artery and left common-carotid artery were isolated and exposed using a neck incision. Then, the middle-cerebral artery was occluded using the embolus derived from the fishing-line with a small incision in the internal-carotid artery. The reperfusion was conducted for 23 hrs by removing fishing-line post 1 hr of cerebral ischemia. The rats' body temperatures were kept at 37°C in process of surgery.

Post-operative care

In order to prevent the dehydration, saline solution (2.5 mL) was subcutaneously injected. Then, Buprenex (0.05 mg/kg body weight) was subcutaneously injected post operation with interval of 6–12 hrs, as a need for relieving the pain. In order to stop the anesthesia, the rats were placed in a 37°C veterinary recovery chamber for 10 mins, and kept for observing. Finally, the rats were put into a sterilized cage and were free to the food and water.

Drug administration and trial grouping

The rats were divided into 3 groups (6 rats for each group), including normal control (NC) group (without any injections), MCAO/R rat model group (injecting with saline only) and MCAO/R rats administrating with TC group (injecting with TC at final concentration of 2 mL/100 g rat body weight). In this study, post the stroke surgery, the TC was intraperitoneally injected into rats at final concentration of 2 mL/100 g body weight. The TC was purchased from Buchang Pharma. Co. Ltd. (Changchun, China). From the total of 12 rats submitted to MCAO and involved in this study, 3 of them died before the designed protocols were completed (2 during the first 24 hrs and 1 during the first 12 hrs post occlusion). No rats died in NC group. Therefore, total of 9 MCAO rats were involved in this study and 3 MCAO rats were excluded. About 24 hrs post operation, rats were euthanized using sodium pentobarbital at a final dosage of 100 mg/kg body weight (IP injection).

Neurological scoring and evaluation

At 24 hrs post reperfusion, the neurological scores of rats were evaluated with Garcia scale in blinded fashion,

according to the previous study.[14] The neurological evaluation was conducted based on the Garcia scale with a few modifications as illustrated in Table 1. The used Garcia scale was divided into 6 subjects, including spontaneous activity, symmetry in movement of 4 limbs, forepaw outstretching, climbing, body proprioception and vibrissae touch. The rats demonstrated normal neurological functions were assigned as the highest score (18 scores) and the severe functional impaired rats were assigned as the lowest score (0 score).

Magnetic resonance imaging (MRI) inspection

Post neurological evaluation, rats were anesthetized by intraperitoneally injecting 10% chloral hydrate at final concentration of 0.4 mL/100 g body weight (Zhaohui Pharma. Co. Ltd., Shanghai, China) and fixed on board in supine position. The T2 images, T2 Flair images and apparent diffusion coefficient (ADC) images of rats undergoing 1 hr cerebral ischemia and 23 hrs reperfusion were evaluated using the 7-Tesla Bruker MRI scanner (Mode: Pharmascan 70/16, Bruker, Ettlingen, Germany). The infarct area/contralateral area ratios and ADC values were acquired and analyzed using the Medical Imaging Processing and Visualization software (National Institutes of Health, Bethesda, Maryland, USA).

TTC staining and infarct size measurement

Post neurological function evaluation, rats were sacrificed for infarct size analysis. The whole brain was quickly removed from the rats and frozen for 30 mins at −20°C. The cerebral infarct size was examined and visualized by staining with TTC (SolarBio. Life Sciences, Beijing, China). The brain tissues were sliced into sections with thickness of 2 mm. The sections were incubated with 2% TTC solution at 37°C for 10 mins in dark. Then, the sections were fixed using 4% paraformaldehyde (Beyotime Biotech. Shanghai, China) at 37°C for 30 mins. Finally, the infarct size or area was analyzed using image analysis software (NIH Image, version: 1.61, Scion Inc., Bethesda, USA) by integration of infarct areas. The infarct size data were expressed as the following equation: infarct size (mm^3) = infarct length (mm)×infarct width (mm)×infarct depth (mm). This measurement for the infarct size was measured on the 6 TTC stained sections using the image analysis software.

Western blotting assay

The rats were anesthetized by intraperitoneally injecting 10% chloral hydrate and sacrificed by cutting off the brain. Then the brain tissues were isolated immediately and brain tissues were lysed using radioimmunoprecipitation assay solution (RIPA, Beyotime Biotech. Shanghai, China) due to the instruction of the manufacturer. The concentration of isolated protein was examined using Pierce bicinchoninic acid (BCA) Protein Assay Kit (Cat. No. 23225, Thermo Scientific Pierce,

Table 1 Garcia scales for scoring the neurological scores

Garcia scales	0 score	1 score	2 scores	3 scores
Spontaneous activity	Does not move at all	Barely moves in cage	Moved around reluctantly, reaches at least one side of cage	Moved around, explored cage
Symmetry in movement of four limbs	Forelimb on contralateral side does not move at all	Limbs on contralateral side exhibit minimal movement	Limbs on contralateral side extend less than those on ipsilateral side	All four limbs extend symmetrically
Forepaw outstretching	Left forelimb does not move at all	Left forelimb has limited movement	Left side outstretched less than left, forepaw walking impaired	Forelimbs outstretched, walking symmetrically on forepaws
Climbing		Does not climb	Left side impaired, does not grip as tightly and releases before right	Climbs, grips tightly with both forepaws
Body proprioception		Does not react to stimulus on left side	Reacts slowly to stimulus on left side	Reacts by turning head, equally startled by stimulus on both sides
Vibrissae touch		Does not react to stimulus on left side	Reacts slowly to stimulus on left side	Reacts by turning head, equally startled by stimulus on both sides

Rockford, IL, USA). The protein lysates were separated using 10% separating sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Amresco Inc., Solon, OH, USA) and 5% concentrating SDS-PAGE. Then the proteins were electrotransferred onto polyvinylidene fluoride (PVDF, Amersham Biosciences, Piscataway, New Jersey, USA). PVDF membrane was blocked using 5% defatted milk (Beyotime Biotech. Shanghai, China) in phosphate-buffered saline (PBS, Beyotime Biotech) containing 0.05% Tween-20 (Beyotime Biotech). PVDF membranes were then incubated with rabbit anti-rat caspase 3 polyclonal antibody (Cat. No. D160009, BBI Life Sciences Corp., Shanghai, China), rabbit anti-rat caspase 8 polyclonal antibody (Cat. No. D155240, BBI Life Sciences Corp.), rabbit anti-rat caspase 1 polyclonal antibody (Cat. No. D220080, BBI Life Sciences Corp.) and mouse anti-rat β-actin monoclonal antibody (Cat. No. D190606, BBI Life Sciences Corp.) at 4°C overnight. The PVDF membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated Goat anti-Rabbit IgG (1:1000, Cat. No. BA1056, Boster Bio., Wuhan, China) and HRP-conjugated Goat-anti Mouse IgG (1:1000, Cat. No. BA1050, Boster Bio., Wuhan, China) at room temperature for 2 hrs. The Western blotting bands were visualized with Pierce chemiluminescence (ECL) Western Blotting Substrate (Cat. No. 32106, Thermo Scientific Pierce). Finally, the images for Western blotting bands were analyzed and captured using Image Pro-Plus software (Version: 6.0, Media Cybernetics Inc., Bethesda, MA, USA).

Statistical analysis

Data in this study were assigned as mean ± standard deviation (SD) and analyzed using SPSS software 17.0 (SPSS Inc., Chicago, Ull, USA). The differences of data between two groups were analyzed with Student's *t*-test. The differences of data among multiple groups were analyzed using validate analysis of variance (ANOVA) test, which was validated by Tukey's post hoc test. The $p<0.05$ was represented as the statistical significance.

Results

Troxerutin and cerebroprotein treatment improved cerebral infarction in MCAO/R rats

The infarct size could directly reflect the severity of cerebral infarction; therefore, the infarct size was evaluated in this study. The results showed that post 1 hr cerebral ischemia and 23 hrs reperfusion, the infarct size in MCAO/R+TC group was smaller significantly compared to that in MCAO/R group (Figure 1, $p<0.05$).

Troxerutin and cerebroprotein treatment reduced infarct/contralateral area ratio of MCAO/R rats

Post 1 hr cerebral ischemia and 23 hrs reperfusion, the MRI results demonstrated that there were high-signal areas in left brain of MCAO/R rats (Figure 2A). The high-signal areas were distinguished from the surrounding areas, which suggests that there were ischemic lesions in brain of MCAO/R rats (Figure 2A). The statistical analysis showed that the infarct/contralateral area ratio of T2 signals in MCAO/R+TC group was lower significantly compared to that in MCAO/R group (Figure 2B, $p<0.05$). The infarct/contralateral area ratio of T2 Flair signals in MCAO/R+TC group was also lower significantly compared to that in MCAO/R group (Figure 2C, $p<0.05$).

Troxerutin and cerebroprotein treatment enhanced ADC values of MCAO/R rats

The analysis of MRI also demonstrated that ADC values in MCAO/R+TC group were significantly enhanced compared to that in MCAO/R group (Figure 2D, $p<0.05$). Together with the infarct/contralateral area ratio of T2 and T2 Flair signals, our results suggested that troxerutin and cerebroprotein alleviate the I/R injury and play the protective effects.

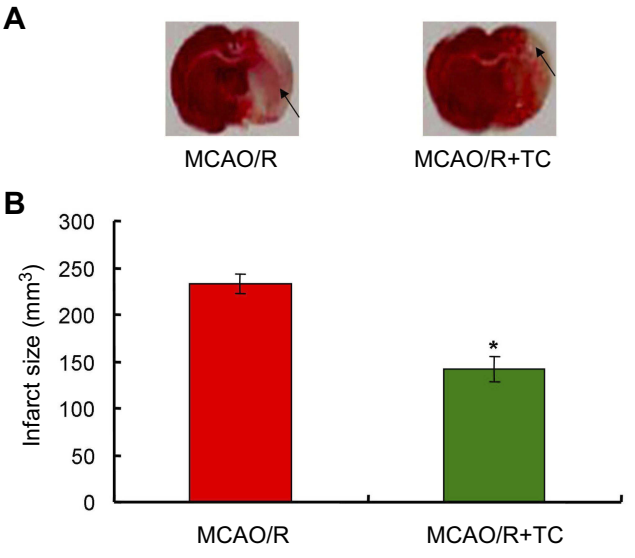

Figure 1 Comparison of the infarct sizes between MCAO/R group and MCAO/R +TC group. (**A**) The infarct images for both MCAO/R group and MCAO/R+TC group. (**B**) Statistical analysis for the infarct size in MCAO/R group and MCAO/R +TC group. *$p<0.05$ vs MCAO/R group.

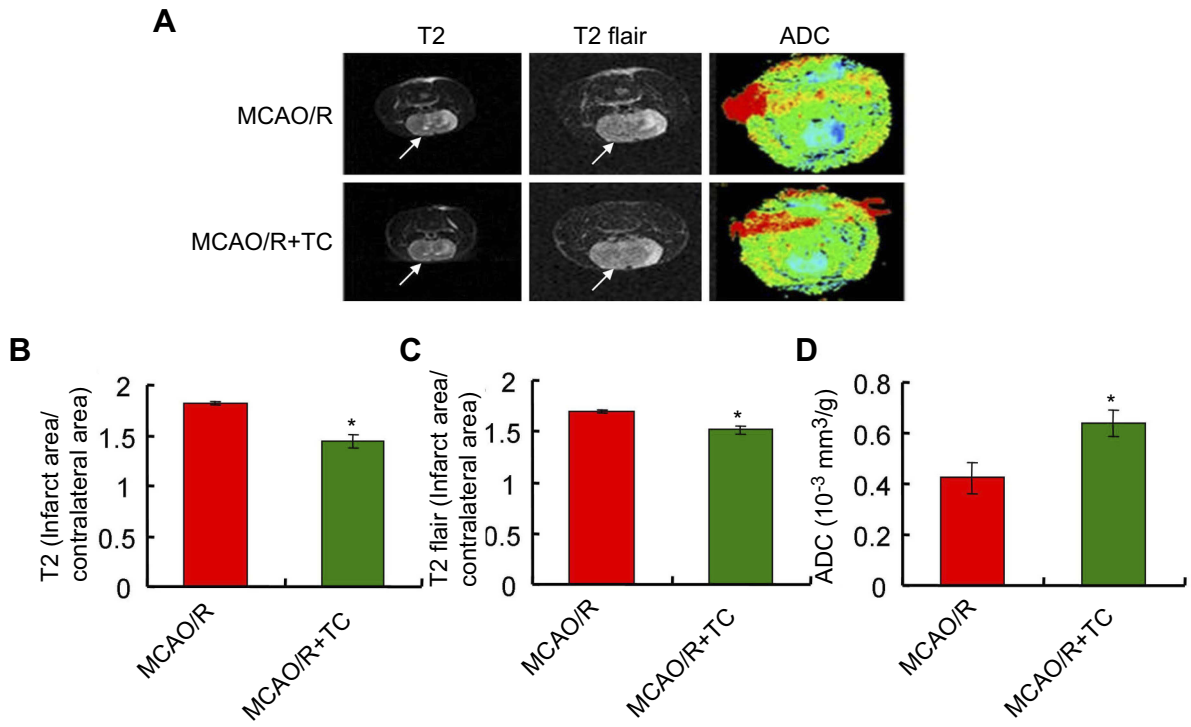

Figure 2 T2 and T2 infarct area/contralateral area ratio and ADC value evaluation according to the MRI inspection. (**A**) Images for the MRI inspection. (**B**) Statistical analyses for the T2 infarct area/contralateral are ratios. (**C**) Statistical analyses for the T2 flair infarct area/contralateral are ratios. (**C**) Statistical analysis for the ADC values. (**D**) The ADC values for both MCAO/R group and MCAO/R+TC group. *p<0.05 vs MCAO/R group. The white arrows represent the infarct areas.

Troxerutin and cerebroprotein treatment increased neurological scores of MCAO/R rats

The neurological evaluation findings showed that the neurological score in MCAO/R group was lower significantly compared to that in NC group (Figure 3, p<0.05). However, the troxerutin and cerebroprotein treatment (MCAO/R+TC group) significantly increased the neurological score in MCAO/R group (Figure 3, p<0.05).

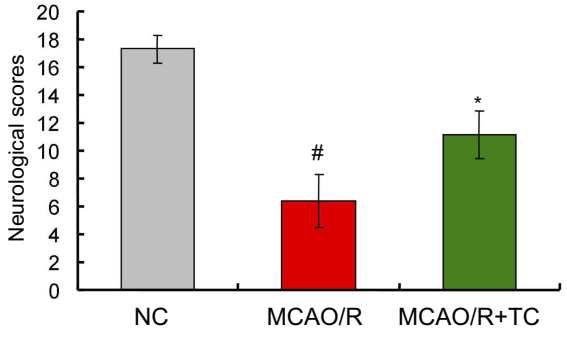

Figure 3 Evaluation for the neurological scores of the MCAO/R and MCAO/R+TC rats. #p<0.05 vs NC group, *p<0.05 vs MCAO/R group.

Troxerutin and cerebroprotein treatment decreased expression of caspase family molecules

In order to clarify the potential mechanism for the troxerutin and cerebroprotein-triggered anti-I/R injury effects, the caspase family molecules were detected using Western blotting assay (Figure 4A). The statistical analysis of Western blotting results indicated that caspase 1 expression was significantly increased in MCAO/R group compared to that in NC group (Figure 4B, p<0.05); however, this effect was remarkably blocked by the troxerutin and cerebroprotein treatment (MCAO/R+TC group, Figure 4B, p<0.05). Moreover, the caspase 3 (Figure 4C, p<0.05) and caspase 8 (Figure 4D, p<0.05) expressions were also increased in MCAO/R group compared to that in NC group. However, the effects in MCO/R group were also inhibited significantly by troxerutin and cerebroprotein treatment (MCAO/R+TC group, Figure 4C, D, p<0.05).

Discussion

The present study exhibited that the treatment of troxerutin combining cerebroprotein could alleviate I/R injury via decreasing infraction sizes and improving neurological

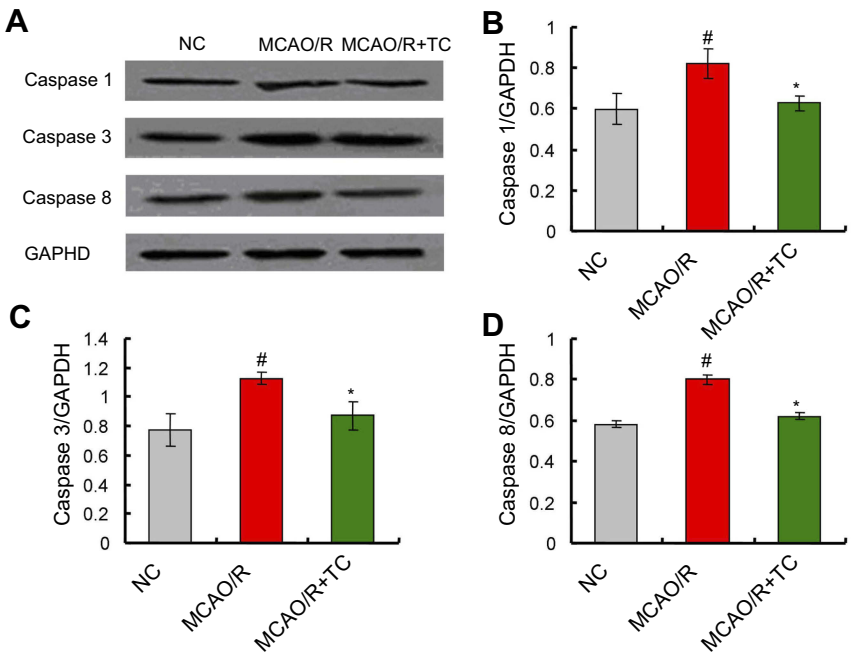

Figure 4 Determination for the caspase 1, caspase 3 and caspase 8 expressions in brain tissues of MCAO/R rats. (**A**) Western blotting images. (**B**) Statistical analysis for the caspase 1 expression. (**C**) Statistical analysis for the caspase 3 expression. (**D**) Statistical analysis for the caspase 8 expression. $^{\#}p<0.05$ vs NC group, $^{*}p<0.05$ vs MCAO/R group.

functions in MCAO/R rat models. The potential mechanism for the troxerutin and cerebroprotein treatment might be to inhibit or block caspase family molecules' mediated apoptosis.[15] All of the above results derived from the MCAO/R rats imply the promising therapeutic potential for troxerutin combining cerebroprotein protecting against I/R injury, which might be selected as the candidate clinical therapy.

In our study, the improvement of neurological scores and decreased infarct sizes were discovered in troxerutin and cerebroprotein-administrated MCAO/R rats, which might be associated with the regulatory effects on neuronal metabolism of the above drug.[16,17] The previous studies[18,19] reported that the improved neuronal metabolism could activate the activities of neurotransmitters and enzymes in brain, and further improved the status of cerebral ischemia. However, our findings showed that post 1 hr cerebral ischemia and 23 hrs reperfusion, the caspase 1, caspase 3 and caspase 8 expression in brain tissues of MCAO/R rats were significantly decreased, and were blocked or inhibited by the troxerutin and cerebroprotein administration. These results suggested that the I/R injury demonstrated obviously neuronal apoptosis, which could be inhibited by troxerutin combining cerebroprotein by suppressing the apoptosis of neurons.

The apoptosis is considered as a focus for pathogenesis of cerebral injuries.[20] The modulation of apoptosis is critical for maintaining balance between cell death and cell growth, and the balance of which is important for I/R injury.[21] The previous study[22] found that the neuronal apoptosis was caused by activation of caspase molecules, which indirectly mediate the release of cytochrome C, induce the apoptosis and damage the electron transport and oxidative phosphorylation. Wang et al[23] also reported that the inhibition of caspase 3 could prevent apoptosis of neurons and protect against cerebral ischemia. Shabanzadeh et al[24] also reported that suppressing the expression of caspase 8 could promote the neuronal survival post ischemic stroke. Moreover, in the study, caspase 1 levels were also activated in MCAO/R rats and suppressed by administration of troxerutin and cerebroprotein. The caspase 1 could activate the caspase 6, induce the apoptosis and finally cause the neuron injury. Therefore, the troxerutin and cerebroprotein administration could be an effective strategy against the neuronal apoptosis through activating caspase 1, caspase 3 and caspase 8 in MCAO/R rats. Certainly, the other caspases, such as caspase 9, are also involved in the stroke outcomes.[25] However, this study has not evaluated the other caspases, which is a limitation of this study. In the following study, we would also evaluate the changes of the other caspases in MCAO/R rats. Moreover, the previous study[26] also illustrated that the troxerutin could alleviate or even eliminate inflammatory responses, decrease release of inflammatory mediators and subsequently reduce expression of apoptotic factors.

Therefore, we speculated that the anti-apoptotic effects of troxerutin and cerebroprotein might be associated with the inflammatory responses in brain tissues of MCAO/R rats.

Although this study received some interesting findings, there are also a few limitations. First, except for the caspase 1, caspase 3 and caspase 8, the correlation between the other caspases-associated apoptosis and stroke outcomes has not been clarified. In the following study, we would further explore the mechanism for the stroke-caused injury. Second, the sample size of the MCAO/R mice is relatively small for confirming the findings. In the future study, we would enlarge the sample size to confirm our results in this study.

In conclusion, troxerutin and cerebroprotein administration reduced infarct size and enhanced neurological functions, and inhibited neuronal apoptosis of MCAO/R rats. Therefore, troxerutin and cerebroprotein administration alleviated the I/R injury caused neuronal apoptosis by down-regulating caspase molecules. The findings of this study would provide the theoretical basis for clinical therapy of I/R injury. Also, this study would also provide insight into the investigation for the pathological mechanism for I/R injury.

Disclosure

The authors report no conflicts of interest in this work.

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