Effects of thymosin β4 on neuronal apoptosis in a rat model of cerebral ischemia-reperfusion injury

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Abstract. The aim of the present study was to investigate the protective effects of thymosin β4 (Tβ4) on neuronal apoptosis in rat middle cerebral artery occlusion ischemia/reperfusion (MCAO I/R) injury, and determine the mechanisms involved in this process. Forty-eight adult male Sprague-Dawley rats were randomly divided into three groups (n=16 per group): A sham control group, an ischemia/reperfusion group (I/R group), and a Tβ4 group. The focal cerebral I/R model was established by blocking the right MCA for 2 h, followed by reperfusion for 24 h. The Zea-Longa method was used to assess neurological deficits. Cerebral infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride staining, and pathological changes were observed via hematoxylin and eosin staining. The terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was used to detect apoptosis. The expression of glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and caspase-12 (CASP12) protein was assessed using immunohistochemistry and western blotting 24 h after reperfusion. Infarct volume and neuronal damage in the I/R and Tβ4 groups were significantly greater than those observed in the sham group. The Zea-Longa score, neuronal apoptosis, and expression of GRP78, CHOP, and CASP12 in the I/R and Tβ4 groups were significantly higher than those reported in the sham group. However, the Longa score and neuronal apoptosis were lower in the Tβ4 group compared to the I/R group. The expression of GRP78 was significantly increased, whereas that of CHOP and CASP12 was significantly decreased in the Tβ4 group compared to the I/R group. The present data revealed that Tβ4 can inhibit neuronal apoptosis by upregulating GRP78 and downregulating CHOP and CASP12, thereby reducing cerebral I/R injury.

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Introduction

Cerebral infarction is the most common type of ischemic cerebrovascular disease, accounting for approximately 80% of all stroke cases (1,2). It is associated with high rates of morbidity, disability, and mortality. Following cerebral infarction, the blood flow to the brain is interrupted, and the intake of oxygen and glucose in the ischemic area is hindered (3,4). Therefore, timely recovery of blood supply to the brain tissue is crucial to reduce subsequent neuronal death. However, one study also revealed that reperfusion may lead to cerebral ischemia-reperfusion injury (CIRI) (5). The pathological mechanisms of CIRI involve inflammatory response, excitatory toxicity, calcium overloading, production of free radicals, mitochondrial dysfunction, and apoptosis (6-9). Therefore, the reduction of apoptosis is an important step in improving cerebral infarction (10). In recent years, there has been increasing research on the endoplasmic reticulum stress (ERS)-dependent apoptotic pathway, as an intervention for the reduction of I/R injury in cerebral infarction (11).

Thymosin β4 (Tβ4) is a polypeptide consisting of 43 amino acid residues, and belongs to the thymosin β family (12). Recent studies have confirmed that Tβ4 has various biological functions, such as promoting stem cell differentiation and angiogenesis, and enhancing cell proliferation, migration, and anti-apoptosis. Moreover, it is closely related to tissue regeneration, angiogenesis, and wound healing (13-15). Several studies have revealed that Tβ4 exerts a neuroprotective effect. For example, Tβ4 can reduce the death of motor neurons caused by staurosporine (16), reduce the damage caused by excitatory amino acids to cortical neurons, and improve symptoms of nerve injury in rats with cerebral ischemia (17). However, the specific neuroprotective mechanism of Tβ4 remains unclear. In the present study, a rat model of focal cerebral ischemia and reperfusion was established to evaluate the neuronal protection of thymosin β4. In conclusion, the present study provides an experimental basis for the clinical treatment of ischemic cerebrovascular disease using Tβ4.

Materials and methods

Reagents. Recombinant Tβ4 was purchased from Cloud-Clone Corp. The terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) apoptosis detection kit

was obtained from Beyotime Institute of Biotechnology. Rabbit anti-glucose-regulated protein 78 (GRP78), rabbit anti-caspase-12 (CASP12), and rabbit anti-C/EBP homologous protein (CHOP) monoclonal antibodies were obtained from Abclonal Biotech Co., Ltd.

Experimental animals. In total, 48 male Sprague-Dawley rats (clean grade) (male; body weight: 250-300 g; age: 7-8 weeks) were used to establish the focal cerebral I/R model. All animals were provided by the Experimental Animal Co., Ltd., and housed in a specific pathogen-free environment that was automatically maintained at a temperature of 23±2˚C, a relative humidity of 45-65%, and with a controlled 12 h light/dark cycle. The animals had free to access food and water. All animal procedures were approved by the Ethics Committee of Guangzhou Medical University.

According to the random number table method, 48 Sprague-Dawley rats were divided into three groups (n=16 per group): A sham operation group (sham group), an ischemia/reperfusion group (I/R group), and a Tβ4 group. In the sham group, the internal carotid artery was isolated but not tied, and the incision was subsequently sutured. Rats in the I/R and Tβ4 groups underwent a 2 h occlusion of the right middle cerebral artery (MCAO). Additionally, rats in the Tβ4 group were intraperitoneally injected with Tβ4 (8 mg/kg) 1 h after MCAO, whereas rats in the I/R group were intraperitoneally injected with normal saline.

Preparation of the cerebral I/R model. The Zea-Longa method was used to establish the rat MCAO-I/R model (18). All rats were anesthetized (4% isoflurane for induction and 1% for maintenance) and disinfected. An incision was performed in the middle of the neck to expose the external carotid, internal carotid, and common carotid arteries. The right MCA was blocked according to the method described by Longa (18). The length of the line inserted into the internal carotid artery was ~18 mm. The muscle and skin were sutured layer by layer, and the plug was removed to initiate reperfusion for 2 h after ischemia.

Neurological evaluation. Approximately 1.5 h after anesthesia, the rats had regained consciousness, and neurological scores were evaluated according to the Longa method as follows: 0 points, no symptoms of neurological deficits; 1 point, unable to fully extend the contralateral forepaw; 2 points, circle to the left (paralyzed) side when walking; 3 points, fall to the left (paralyzed) side when walking; and 4 points, unable to walk on their own, losing consciousness. Higher scores indicated more severe neurological impairments. Rats with a Zea-Longa score of 1-3 were included in the subsequent experiments.

2,3,5-Triphenyltetrazolium chloride (TTC) and hematoxylin and eosin (H&E) staining. The rats were subsequently anesthetized using isoflurane (4% isoflurane) and decapitated 3 days after the surgery. The brain tissue was washed with saline, and subsequently frozen at -20˚C for 20 min. Coronal slices (thickness, 2 mm) were collected, immediately placed in phosphate-buffered saline containing 2% TTC, and incubated for 20 min at 37˚C in the dark. The brain slices were analyzed using the ImageJ software version 1.48 (National Institutes of Health), and the percentage of infarct volume to total volume was calculated. The infarct volume was calculated based upon the formula: Infarct volume (%)=ischemic area/whole area of brain x100, using the ImageJ software as previously described (19). Brain sections were also used for histopathological analysis through H&E staining.

Assessment of apoptosis. The hippocampus was isolated on ice and fixed in 4% paraformaldehyde at 4˚C overnight and embedded in paraffin. The 3 μm sections were continuously sliced. Following dewaxing by xylene, the tissues were dehydrated in 70, 75, 80, 85 and 95% alcohol. To retrieve the antigen, 3% hydrogen peroxide was applied at 100˚C. The TUNEL assay was used to detect apoptosis in the hippocampus according to the instructions provided by the manufacturer (cat. no. C1088; Beyotime Institute of Biotechnology). Five different fields were randomly selected from each slice. The ImageJ software version 1.48 was used to calculate TUNEL-positive cells, 4',6-diamidino-2-phenylindole (DAPI)-positive cells, and apoptosis in the corresponding regions was calculated based on the formula: Apoptosis index=the number of TUNEL-positive cells/the number of DAPI-positive cells as previously described (20).

Immunohistochemical staining. The hippocampus tissues were fixed in 4% paraformaldehyde at 4˚C overnight. After dehydration in 30% sucrose, the hippocampi were sectioned into coronal slices. Following blocking in goat serum (2 h at room temperature; Hyclone; GE Healthcare Life Sciences), sections were incubated with rabbit anti-mouse monoclonal primary antibodies against GRP78 (1:200; cat. no. A11366; ABclonal Biotech Co., Ltd.), CASP12 (1:200; cat. no. A0217; ABclonal Biotech Co., Ltd.) and CHOP (1:200; cat. no. A0221; ABclonal Biotech Co., Ltd.) for 60 min at room temperature, followed by incubation with anti-rabbit IgG horseradish peroxidase-linked antibody (1:200; cat. no. 7074; ZB-2301, ZSGB-BIO; OriGene Technologies, Inc.) at 37˚C for 15 min. The number of GRP78-, CHOP-, and CASP12-positive cells in the corresponding region of the slice was calculated using the streptavidin-peroxidase method and imaged using the Image-Pro Plus software (National Institutes of Health). Immunohistochemical staining was visualized with 3,3'-diaminobenzidine chromogen for 3 min at room temperature. The nucleus was counterstained by hematoxylin at room temperature for 3 min.

Western blotting. After removal of the brain, brain tissues around the infarcted region were removed and cut into pieces. The brain tissue was subsequently homogenized, lysed using a protein isolation kit (GE Healthcare Life Sciences) and centrifuged (11,000 x g at 4˚C). The bicinchoninic acid (BCA) method was used to determine the total protein concentration. Western blotting was performed according to standard procedures (21). Briefly, protein samples (25 μg) obtained from each group were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and transferred onto nitrocellulose membranes for western blot analysis. Subsequently, the membranes were blocked in 5% skim milk for 2 h in room temperature. The membranes were subsequently incubated with the following primary antibodies: Rabbit anti-GRP78 (1:1,000), rabbit anti-CASP12 (1:1,000), and rabbit anti-CHOP

(1:1,000) for 60 min at room temperature. The nitrocellulose membranes were washed thrice, and incubated with secondary antibody (HRP-labeled goat anti-rabbit IgG; cat. no. A16104; Thermo Fisher Scientific, Inc.) at 4˚C for 2 h. The staining of the blots was enhanced using an electrochemiluminescence kit (Thermo Fisher Scientific, Inc.). The densities of the blots were quantified using the Quantity One software (v4.62; Bio-Rad Laboratories, Inc.).

Statistical data analysis. Data are presented as the mean and standard deviation. One-way analysis of variance with Newman-Keuls as the post-hoc test was performed using SPSS 17.0 (SPSS, Inc.) statistical software to assess the differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Tβ4 ameliorates the neurological deficit caused by I/R injury. Animal health and behaviors were monitored every day. The neurological behavior of the rats prior to the I/R injury was normal and the Zea-Longa score was 0, thereby indicating that the three groups exhibited similar baseline characteristics. Twenty-four hours after reperfusion, the Zea-Longa score was 0 in the sham group, 2.56±0.63 in the I/R group, and 1.94±0.68 in the Tβ4 group. The Longa score of the I/R group was significantly higher than that of the sham group (P<0.01), indicating that the rat model of focal cerebral I/R was successfully established. In contrast, the Longa score of the Tβ4 group was significantly lower than that of the I/R group (P<0.05) (Fig. 1).

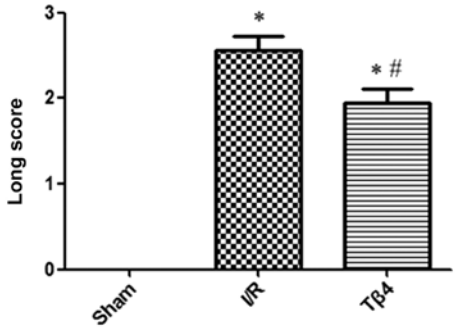

Figure 1. The Zea-Longa scores of rats in each group. Results are presented as the means ± standard deviation (n=16 animals in each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the I/R group (one-way ANOVA). I/R, ischemia-reperfusion; Tβ4, thymosin β4.

Tβ4 ameliorates cerebral infarction caused by I/R injury. Twenty-four hours after cerebral I/R, TTC staining was performed in brain tissues obtained from each group. In the sham group, the brain slices were stained red and there was no infarct observed. However, areas of white infarct regions were observed in the I/R and Tβ4 groups, and were consistent with the range of arterial embolization. The infarction volume in the I/R and Tβ4 groups was significantly increased (P<0.05) compared with that measured in the sham group. The cerebral infarction volume was 0 in the sham group, 44.05±3.54 in the I/R group, and 33.75±3.44 in the Tβ4 group. Moreover, the volume of cerebral infarction in the Tβ4 group was significantly lower than that measured in the I/R group (P<0.05) (Fig. 2).

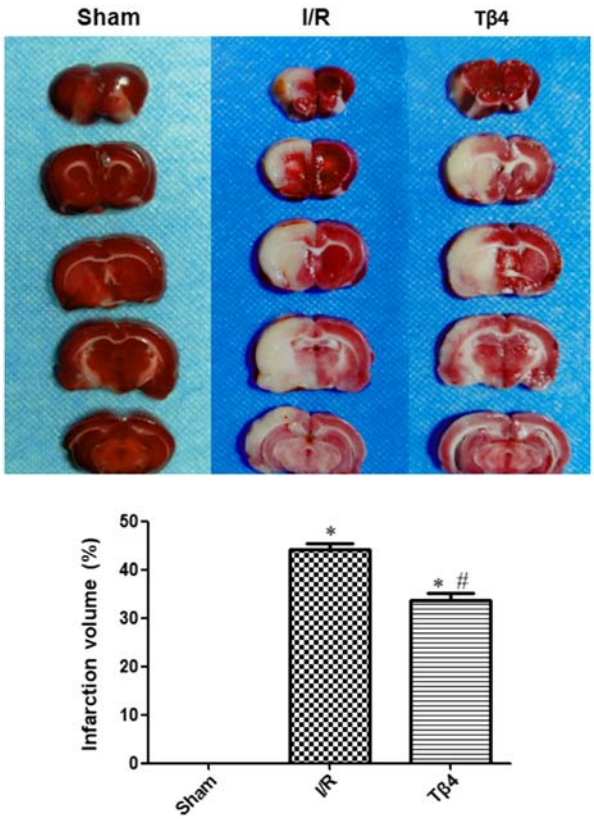

Figure 2. TTC staining of rat brain tissue revealing cerebral infarction in each group. Upper panel: Representative images. All images for each group were obtained from one rat of each group; Lower panel: Quantitative data. Results are presented as the means ± standard deviation (n=16 animals in each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the I/R group (one-way ANOVA). TTC, 2,3,5-Triphenyltetrazolium chloride; I/R, ischemia-reperfusion; Tβ4, thymosin β4.

Tβ4 ameliorates the pathological changes caused by I/R injury. Pathological examination revealed that brain tissues obtained from the I/R and Tβ4 groups exhibited different degrees of damage. However, this damage was not observed in the sham group. The pathological manifestations included interstitial edema, vacuolation, nuclear condensation, and dissolution. The pathological manifestations were also mild in the Tβ4 group (Fig. 3).

Tβ4 ameliorates the neuronal apoptosis caused by I/R injury. The results of the TUNEL assay revealed that the rate of apoptosis in hippocampus was 0.13±0.03 in the sham group, 0.50±0.05 in the I/R group, and 0.32±0.04 in the Tβ4 group (P<0.05). The rate of apoptosis in the Tβ4 group was lower compared with that reported in the I/R group (P<0.05) (Fig. 4).

Tβ4 promotes the expression of GRP78 and reduces that of CHOP and CASP12. Immunohistochemical analysis revealed that the number of GRP78-, CHOP-, and CASP12-positive cells in the I/R and Tβ4 groups was significantly higher than that observed in the sham group 24 h after reperfusion (P<0.05). The number of GRP78-positive cells in the Tβ4 group was higher than that recorded in the I/R group (P<0.05).

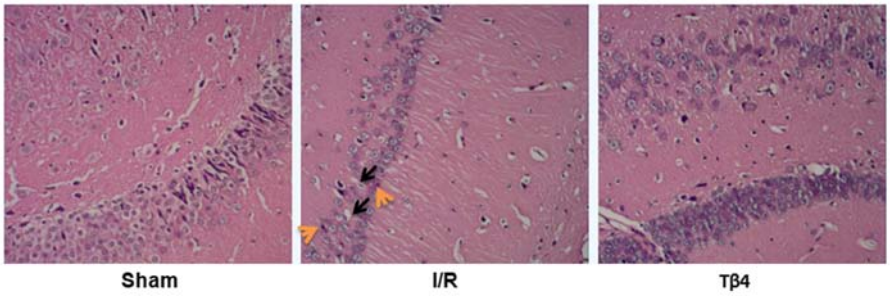

Figure 3. H&E staining of rat brain tissue revealing cerebral infarction (magnification x100). Pathological examination revealed that brain tissues of the I/R and Tβ4 groups exhibited different degrees of damage. However, this damage was not observed in the sham group. Both the I/R and Tβ4 groups exhibited obvious areas of infarction and tissue loss. In addition, the degree of tissue edema, neuronal vacuolization (black arrows), and nuclear pyknosis (orange arrows) were also mild compared with the Sham group. I/R, ischemia-reperfusion; Tβ4, thymosin β4.

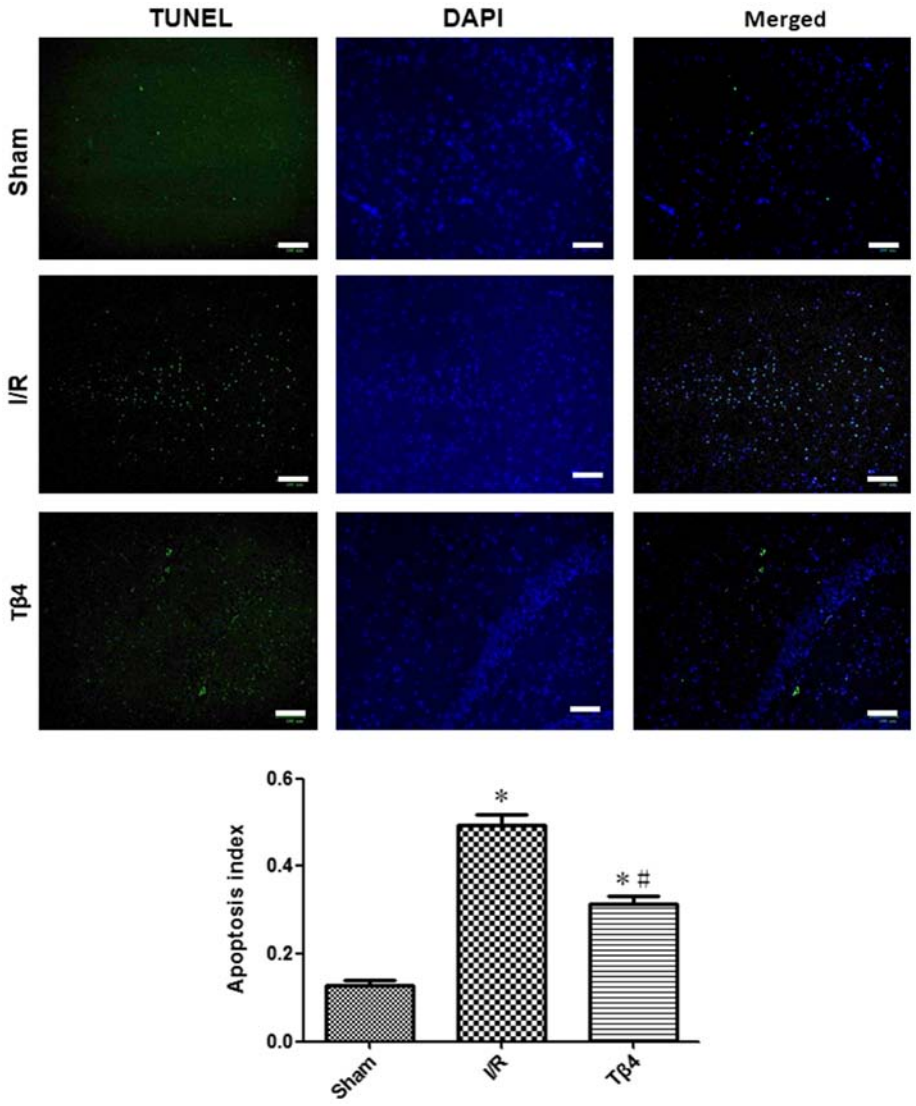

Figure 4. Apoptosis in the hippocampus is induced by cerebral ischemia in rats after cerebral ischemia/reperfusion for 24 h. Upper panel: Representative images (Scale bar, 100 μM); Lower panel: Quantitative data. Results are presented as the means ± standard deviation (n=5 animals in each group). *P<0.05 vs. the Sham group, $^{\#}$P<0.05 vs. the I/R group (one-way ANOVA). I/R, ischemia-reperfusion; Tβ4, thymosin β4.

In contrast, the number of CHOP- and CASP12-positive cells in the Tβ4 group was lower than those reported in the I/R group (P<0.05) (Fig. 5).

Moreover, western blotting revealed that the GRP78, CHOP, and CASP12 proteins were highly expressed in the I/R and Tβ4 groups compared with the sham group. The expression of GRP78 was higher in the Tβ4 group versus the I/R group. In contrast, the expression of CHOP and CASP12 was lower in the Tβ4 group compared to the I/R group (P<0.05) (Fig. 6).

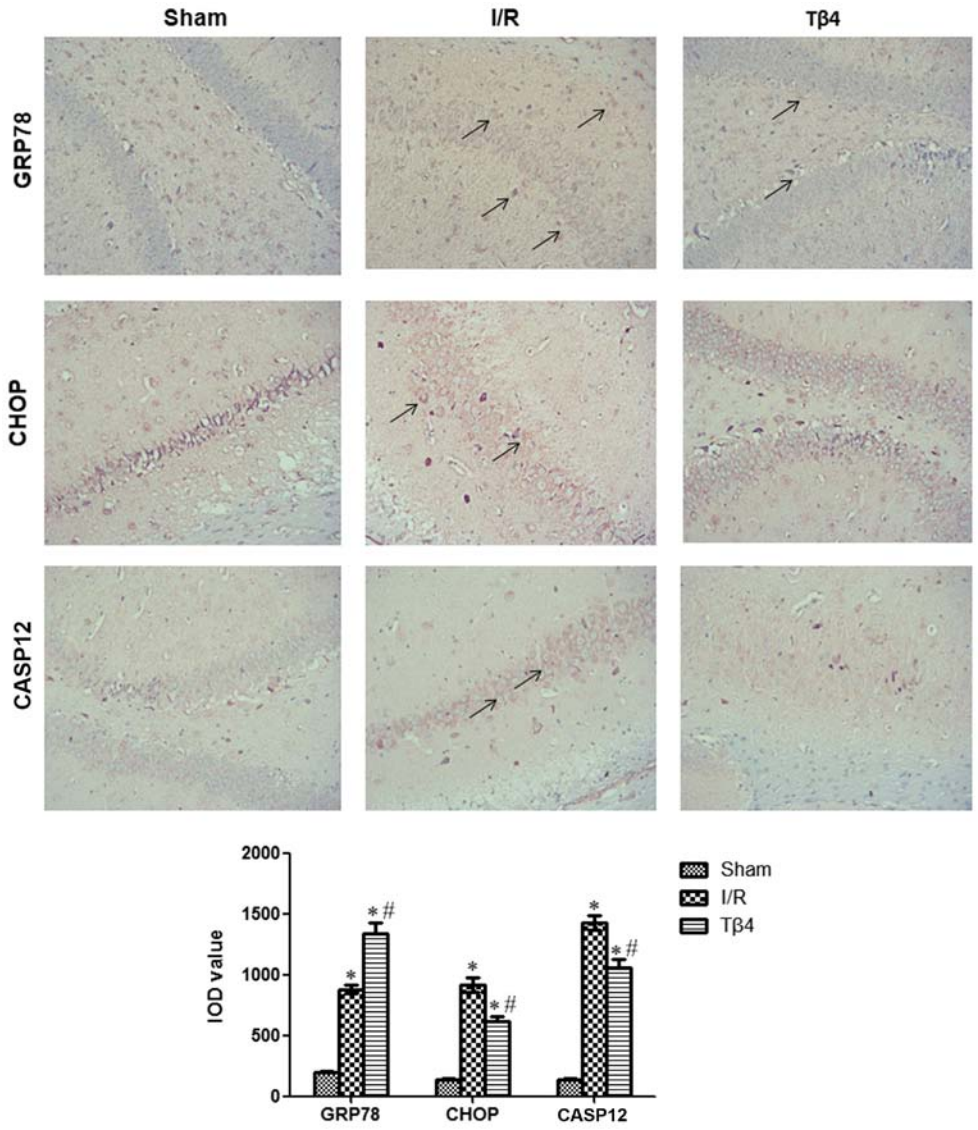

Figure 5. Tβ4 promotes the hippocampal expression of GRP78 and reduces that of CHOP and CASP12. Upper panel: Representative images (magnification x100); Positive expression of the proteins were indicated by arrows. Lower panel: Quantitative data. The results are presented as the means ± standard deviation (n=5 animals in each group). *P<0.05 vs. the Sham group, #P<0.05 vs. the I/R group (one-way ANOVA). Tβ4, thymosin β4; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; CASP12, caspase-12; I/R, ischemia-reperfusion.

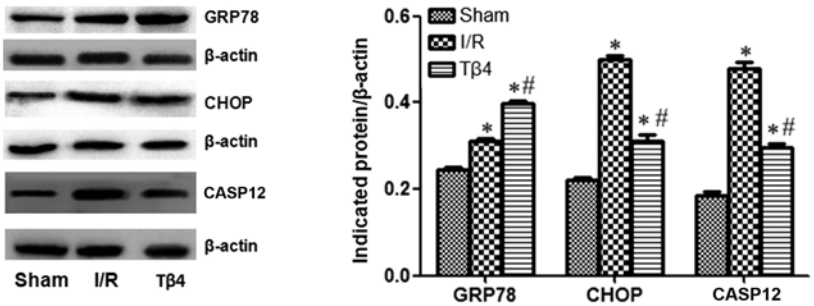

Figure 6. Expression of GRP78, CHOP, and CASP12 24 h after cerebral I/R in each group. Left panel: Representative images; Right panel: Quantitative data. Results are presented as the means ± standard deviation (n=5 animals in each group). *P<0.05 vs. the Sham group, #P<0.05 vs. the I/R group (one-way ANOVA). GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; CASP12, caspase-12; I/R, ischemia-reperfusion; Tβ4, thymosin β4.

Discussion

Considering that brain tissue has limited energy for self-sustenance, energy metabolism in the brain is almost entirely dependent on blood circulation (22). Therefore, brain tissue is extremely sensitive to ischemia and hypoxia. The occurrence of cerebral ischemia results in rapid depletion of the energy stores of the brain, followed by initiation of

neuronal programmed cell death (23). In the present study, it was demonstrated that intervention with Tβ4 after ischemia can reduce the neurological deficits in rats. The mechanism involved in this process was inhibition of neuronal apoptosis.

Tβ4 is a small molecule composed of 43 amino acid residues. It is involved in multiple responses, such as wound healing, tissue development, angiogenesis, and myocardial repair (24-27). Tβ4 can inhibit apoptosis and exerts neuroprotective effects (28). In the present study, it was demonstrated that treatment with Tβ4 reduced the neurological deficits in rats. It also revealed different degrees of apoptosis in the ischemic region of the rat brain 24 h after reperfusion. This finding was consistent with the results of previous research (29,30). The rate of apoptosis in the Tβ4 group was significantly lower than that observed in the I/R group, suggesting that Tβ4 can reduce apoptosis induced by cerebral I/R. Cerebral infarction volume is one of the most intuitive indicators for the evaluation of the degree of brain tissue damage. In this experiment, brain infarction in the Tβ4 group rats was significantly lower than those in the I/R group, indicating that Tβ4 reduced the injury caused by I/R. Compared with the Tβ4 group, the I/R group exhibited greater infarct area and brain tissue loss, interstitial edema, neuronal vacuolar degeneration, and nucleus pyrolysis. These results indicated that Tβ4 exerted neuroprotective effects on neurological behavior, cerebral infarct volume, microscopic pathology, and apoptosis in rats with cerebral I/R injury.

The ER is one of important organelles of eukaryotic cells. It is present in all cells, except the red blood cells. Furthermore, ERS refers to the disruption of ER homeostasis by harmful factors, such as ischemia, hypoxia, and glucose deprivation. This process interferes with the function of the ER, and can cause the accumulation of misfolded and unfolded proteins. The unfolded protein response (UPR) induced by the accumulation of unfolded/misfolded proteins in the ER is the most important signaling mechanism of ERS (31,32).

GRP78 is a molecular chaperone located in the ER, and plays an important role in maintaining the stability of the ER. Studies have revealed that the upregulation of GRP78 is an important marker of ERS, and can protect cells from stress. Cerebral ischemia impairs energy metabolism in the cell, and leads to the accumulation of unfolded or misfolded proteins. GRP78 is rapidly upregulated to relieve ERS injury (33,34). A previous study demonstrated that, under ERS, the upregulation of GRP78 promoted the expression of pro-survival proteins, reduced neuronal death, and exerted anti-apoptotic effects (35). The increase in GRP78 after ischemic injury indicated the presence of a protective mechanism of the ER in response to stress. Moreover, intervention with Tβ4 further increased the expression of the GRP78 protein to reduce the production of unfolded or misfolded proteins, maintain ER homeostasis, and inhibit apoptosis. The mechanism of Tβ4 for the reduction of apoptosis may involve the upregulation of GRP78 and the UPR pathway to relieve ERS after cerebral I/R.

Under severe ERS, the function of the ER is impaired, and the ER apoptotic signaling pathway is activated to induce apoptosis. CHOP is an ERS-specific transcription factor. Under normal conditions, CHOP is rarely expressed; hence, a marked increase in its expression may be indicative of ERS (36). The activation of CASP12 is another signal transduction pathway involved in ERS-induced apoptosis. Studies have revealed that increased expression of CASP12 is an important marker of ERS-induced apoptosis (37,38).

The present data revealed that, in the I/R rat model, the expression of CHOP and CASP12 was significantly increased, along with an increased rate of apoptosis. These findings indicated that excessive ERS may initiate the apoptosis pathway by upregulating the expression of CHOP and CASP12. Treatment with Tβ4 reduced the expression of CHOP and CASP12 proteins in rat ischemic brain tissue. It is speculated that the downregulation of the expression of CHOP and CASP12 may be one of the mechanisms involved in the anti-apoptotic effect of Tβ4.

The present study had some limitations. Firstly, although the expression of GRP78, CHOP, and CASP12 was detected in the present study, and GRP78 and CHOP are typical markers of ERS, the potential function of GRP78 and CHOP in the protection induced by Tβ4 needs to be assessed. Secondly, other UPR pathways were not investigated in the present study.

In conclusion, treatment with Tβ4 can reduce cerebral I/R injury in rats through inhibition of neuronal apoptosis, promoting the recovery of the normal physiological functions of damaged cells.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZZ, SL and SH performed the experiments and analyzed the data. ZZ designed the study and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal procedures were approved by the Ethics Committee of Guangzhou Medical University (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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