Naoluo Xintong capsule ameliorates apoptosis induced by endoplasmic reticulum stress in rats with cerebral ischemia/reperfusion injury

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Background: Naoluo Xintong (NLXT) capsuleis a newly developed drug recorded in the Chinese Pharmacopoeia. It is derived from traditional Chinese medicine (TCM) NLXT decoction, and has been widely used to treat cerebrovascular diseases in clinic. However, it is currently unknown whether it improve cerebral ischemia reperfusion (I/R) injury.

Methods: The effect of NLXT on regional cerebral blood flow (rCBF) was examined using Laser Doppler flower. The Terminal deoxyribonucleotide transferase-mediated Nick end labeling (Tunel) assay was performed to determine the effects of NLXT on apoptosis. Subsequently, cerebral water content and TTC staining were measured to assess cerebral edema and infarct volume, respectively. The protein expression levels were analyzed with Immunofluorescence and western blot assays.

Results: The results indicated that NLXT ameliorated MCAO-induced cerebral I/R injury by decreasing infract volume, inhibition of apoptosis, and upregulation rCBF. In addition, it decreased the expression of key protein involved endoplasmic reticulum (ER)-stress, including glucose-regulated protein 78 (GRP78), C/EBP-homologous protein (CHOP) and Caspase-12 at 24 h following reperfusion. This was accompanied reduced degradation level of TRPC6 and increased phosphorylation of cAMP/Ca^{2+} response element-binding protein (p-CREB), and decreased calpain-specific αII-spectrin breakdown product (SBDP145) activity. Interestingly, inhibition of mitogen-activated protein kinase (MEK) activity abolished the effect of NLXT on CREB activity.

Conclusions: Collectively, the results indicated that NLXT can improve I/R injury therapy by activating TRPC6/MEK/CREB signaling pathway to attenuate ER-stress related neuronal apoptosis.

Keywords: Naoluo Xintong capsule; cerebral ischemia reperfusion injury; endoplasmic reticulum stress; apoptosis

Introduction

Stroke is characterized by high morbidity, mortality and disability rates, with World Health Organization (WHO) surveys showing that 15 million new stroke cases occur per year worldwide (1-3). Effective protection against ischemic brain damage is a worldwide conundrum, necessitating research into novel approaches for control of ischemia-reperfusion (I/R) injury.

Cerebral I/R injury comprises various complex cascade reactions, such as calcium overload and neuron apoptosis. Key among them is the destruction of endoplasmic reticulum (ER) function, mediated by calcium overload, which can further induce ER-stress during ischemic (4). This process triggers expression of a series of associated factors or proteins, and initiates apoptosis, ultimately leading to neuronal death (5). Glucose-regulated protein 78 (GRP78), CHOP and caspase-12, comprise the key elements in the entire process of ER-stress. Generally, C/EBP homologous protein (CHOP) inhibits expression of antiapoptotic protein Bcl-2 during ER-stress. On the other hand, direct activation of Caspase-12, an endoplasmic omentum protein, induces apoptosis only under the condition of ER-stress promotes its translocation to the cytoplasm, and eventually cleaves Caspase-3 to cause apoptosis (6,7). To date, research evidence has proven that regulating ER stress-related proteins inhibits apoptosis and exerts a remarkable protective effect on I/R injury (8). These studies further suggest that effectively controlling apoptosis-induced ER-stress might be a key approach for developing treatment therapies against ischemic stroke.

Transient receptor potential cation channels 6 (TRPC6) comprises a subfamily of non-selective calcium channels, which are widely expressed cell types, including neurons (9). Previous studies have shown that calpain activation leads to degradation of TRPC6 and neuronal damage in ischemia (10). Similarly, *in vitro* and *in vivo* studies have shown that CREB activation is a key downstream target for the neuronal protective effect of the TRPC6 channel (11). Consequently, it evident that inhibiting TRPC6 degradation may be a key therapeutic target for developing protection against ischemic brain injury.

To date, numerous basic experiments and clinic reports have reported the importance of traditional Chinese medicine (TCM) in conferring protection against cerebral I/R injury (12,13). For example, Naoluo Xintong (NLXT) capsule, a modern hospital preparation created by Xin'an Medical master Letao Wang following years of clinical practice, at the First Affiliated Hospital of Anhui University of Chinese Medicine, has been found to increase drug concentration in the brain (Chinese national patent number: CN103182033A). Summarily, NLXT is prepared from six herbs namely, *Astragalus membranaceus* (Fisch.) Bge (Astragali Radix) 30 g, *Ligusticum chuanxiong* Hort. (Rhizoma Chuanxiong) 10 g, *Parmx notoginseng* (Burk.) F. H. Chen (Notoginseng Radix Et Rhizome) 6 g, *Gastrodia elata* Bl. (Gastrodiae Rhizoma) 10 g, *Angelica sinensis* (Oliv.) Diels (Radix Angelicae Sinensis) 10 g, *Carthamus tinctorius* L. (Carthami Flos) 10 g and one animal medicine *Scolopendra subspinipes mutilans* L.Koch (Scolopendra) 4 g. A combination of 3D-UPLC-DAD and a mixture of standard compounds have enabled determination of fingerprints and eight active components in NLXT, as well as flavones, glucosides, polysaccharides and alkaloids, that are hypothesized to be the key biologically active components of the drug (14-17). Consequently, NLXT has been widely used to treat ischemic cerebrovascular diseases in clinics owing to its ability to enhance blood circulation and regulate meridians. Previous studies have demonstrated that NLXT remarkably improves survival rates and hemorheology in acute ischemic stroke patient (18-20). In addition, animal studies have affirmed NLXT's ability to control cerebral I/R injury in rats, owing to its Ca^{2+} content, ability to inhibit apoptosis, increase bFGF levels as well as regulate the coagulation and fibrinolytic system (21-26). However, to date, little is known regarding its specific anti-apoptosis mechanisms. Given that ER stress-mediated neuronal apoptosis plays a key role in ischemic stroke, the present study sought to explore the underlying mechanism employed by NLXT to manage I/R injury, targeting the ER stress signaling pathway in rats induced by MCAO.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/apm-20-387).

Methods

Animal

Sprague-Dawley (SD) rats of SPF grade, weighing 240–280 g, were purchased from the Animal Experimental Center of Anhui Medical University and used in the present study. License No: SCXY (Anhui Province 2014-0009). All rats were allowed free access to standard diet and water. All animal handling and experimental procedures were approved by the ethics committee on Animal Care and

Use of Anhui University of Chinese Medicine, China, and conducted in accordance with the guidelines provided by the National Institutes of Health on the Care and Use of Animals (NIH Publication number 80-23, revised 1986).

Drug and reagents

NLXT were prepared by the Department of Pharmacy, at the First Affiliated Hospital of Anhui University of Chinese Medicine (Batch number: 140701, 140802). Nimodipine tablets were purchased from Bayer Ltd., (batch No: 141207). Whereas PD98059 (MEK inhibitor) was acquired from Sigma-Aldrich Inc. DMSO was used at a concentration of 1.5 mg/mL, and was prepared prior to application.

The TUNEL reagent kit was purchased from Roche (batch number: 10279600), BCA kit was from Beyotime (P0012), SDS-PAGE gel preparation kit from Biyotime (batch number: P0012A), and Nuclear/cytoplasmic protein extraction kit from Shanghai Yisheng Biotechnology Co., Ltd. Additionally, rat primary antibodies, against Caspase-12, CHOP, GRP78, Phospho-CREB, TRPC6, SBDP145, LaminB1, β-actin and horseradish peroxidase (HRP)-conjugated goat anti-rat IgG were purchased from Abcam (Cambridge, MA, USA), whereas Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Establishment of I/R model

In vivo simulation of a cerebral I/R model was performed according the method described by Longa with minor modification (27). Summarily, rats were anesthetized with 4% isoflurane (3 mL/kg) period, then their right sternocleidomastoid muscle separated from the sternoglossal muscle to expose the common carotid artery (CCA). Thereafter, the external carotid artery (ECA) was ligated by electrocauterization, and the CCA clamped. A small incision was then made, at the free end of the ECA, and a loose knot tied under the orifice. The prepared filament was inserted through the incision, along the ECA, then gently and slowly moved along the internal carotid artery (ICA), until there was slight resistance. Finally, an 18 mm embolus thread was inserted into the ICA, from the bifurcation of ICA and ECA, then the filament was removed to complete the reperfusion of the middle cerebral artery (MCA), after 2 h of occlusion. Rats in the sham group were subjected to a similar operation, but without inserting filament occlusion and reperfusion. Neurological deficits were evaluated using the Garcia JH score, whereas the neurological deficits and the regional cerebral blood flow (rCBF) was monitored by laser Doppler, during the operation.

Grouping and dosages

The whole experiment was divided into three batches. The first batch comprised 72 healthy male SD rats, randomly divided into six groups with 12 rats per group. There were the Sham group (S), Middle cerebral artery occlusion (MCAO) group (M), MCAO + positive drug group (0.02 g/kg, Nimodipine, NM), MCAO + NLXT group [1.125 g/kg (N1), 2.25 g/kg (N2), 4.50 g/kg (N3), respectively]. NLXT (1.125, 2.25 and 4.50 g/kg) and Nimodipine (0.02 g/kg) doses were converted according to clinical adult dose. Rats in the MCAO + NLXT or MCAO + Positive drug groups had intragastrical administration NLXT or Nimodipine once a day, for 14 days following MCAO induction, and at onset of reperfusion. Rats in the Sham and MCAO groups had an intragastrical administration of normal saline.

In the second batch, 40 SD rats were randomly divided into four groups of 10 rats each. These were Sham, MCAO, MCAO + Positive drug (0.02 g/kg, Nimodipine 0.02 g/kg) and MCAO + NLXT drug (2.25 g/kg). After reperfusion, the MCAO + Positive drug and MCAO + NLXT drug groups were given their respective doses by intragastric administration, once a day for 7 days, whereas the Sham and MCAO groups were given normal saline for 7 days.

The third batch comprised 20 SD rats, randomly divided into five groups of 4 rats each. These included the Sham, MCAO, MCAO + NLXT drug (2.25 g/kg), MCAO + MEK inhibitor (PD98059) and MCAO + NLXT + PD98059 co-administration groups. After reperfusion, the MCAO + NLXT + PD98059 and MCAO + NLXT drugs were given their respective doses by intragastric administration of their respective dose, once a day for 7 days, whereas the Sham and MCAO groups were given physiological saline for 7 days. Rats were administered with PD98059, dissolved in 1% DMSO [final dose of 2.5 mg/kg (28)], by caudal vein injection 30 min prior to MCAO.

Neurological impairment score

Neurological function was evaluated using a blind method at 1, 7 and 14 after drug administration. Autonomic

movement, symmetry of posture, extending function of forelimbs, and screen test were assessed using the Garcia JH score (29). In addition, the degree of neurological deficits was assessed based on six aspects, including bilateral tactile sensation and bilateral beard reflex. The highest score was 18 points, for a normal function, whereas the lowest score was 3 points for the most serious neurological deficits.

Brain water content

Rats were anesthetized with 4 % isoflurane (3 mL/kg) and their brain immediately removed, 30 minutes after the last drug administration. Residual blood, on the brain surface, was cleaned off with filter paper, and the wet brain mass determined using an electronic weight balance. Thereafter, the brain tissue was quickly dried in a dryer at a constant temperature of 115 ℃, and the dry weight determined. Brain water content was calculated as follows; Brain water content = (wet weight – dry weight)/wet weight × 100.

Assessment of cerebral blood flow

Laser Doppler instrument was used to monitor rCBF at 7 and 14 days after ischemia and reperfusion process. The blood flow was monitored in infarcted area and its surrounding tissues. Rats were anesthetized and disinfected after per routine protocols. An incision was made on the skin, subcutaneous tissue along the midline and the right side of the skull. The fascia was separated to expose the skull. The location of rCBF was assessed in relation to Bregma: ML: +2.0 mm, AP: +1.0 mm (12). The infarct site in the cortex was considered as the ischemic peripheral area (the depth was within 1 mm of the cerebral cortex, that is, the ischemic penumbra area). The probe pedestal of the laser Doppler instrument was fixed with biological gum. Analysis and calibration were performed using routine debugging instrument.

Terminal deoxyribonucleotide transferase-mediated Nick end labeling (Tunel)

Brain sections were dewaxed and dehydrated as stated above, followed by blocking of the endogenous peroxidase. The sections were rinsed 3 times in PBS, and then incubated with the membrane breaking liquid for 10 min at room temperature. They were washed 3 times with PBS, and incubated with a mixture of reagent 1 and reagent 2 of the apoptosis detection kit (1:9) in a water bath with 37 ℃

for 60 min. After washing with PBS, DAB color was developed. A brown precipitate was observed in the nucleus caused by the reaction between horseradish peroxidase and its substrate DAB, indicating a positive TUNEL signal. Hematoxylin counterstaining was also performed, followed by dehydration, clearing, and sealing with tablets. The number of positive cells in three different visual fields on the ischemic side under a 400× objective were counted.

TTC staining

After 7 days of drug administration, the rats were anesthetized with 4% chloral hydrate, and decapitated immediately. The brain was immediately removed from the olfactory bulb to the cerebellum and placed on an ice bath platform. Coronal sections were cut from front to back, sectioned continuously into 5 slices. Brain slices were soaked in 4% TTC phosphate buffer solution at 37 ℃ for 10 min. They were then fixed in 10% formalin solution for 24 hours. The brain slices were arranged in the order of anterior and posterior. Positive and negative sides of the brain slices were photographed by the pathological image collection and analysis system. Areas appearing red and white were measured, and the percentage of infarcted area was calculated with Image J software.

Immunofluorescence

Paraffin zed sections were dewaxed and dehydrated as described above. Sections were washed in PBS and placed in a box filled with EDTA solution (antigen retrieval buffer PH8.0) for antigen retrieval in a microwave. They were washed thrice with PBS and treat with BSA solution to block non-specific binding sites, followed by incubation in the primary antibody against TRPC6 at 4 ℃ overnight. Sections were washed and then incubated with secondary antibody in the fluorescein isothiocyanate (FITC) solution at room temperature for 50 min. After washing with PBS, sections were dried slightly and sealed with an anti-fading tablet (Solarbio Technology Co., Ltd.). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Finally, the sections were examined under a Nikon inverted fluorescence microscope and images were captured and saved.

Western blot analysis

Hippocampus tissues were homogenized with pre-cold

RIPA lysis buffer and immediately mixed with 10ul of Phenylmethanesulfonyl fluoride (PMSF, Beyotime Institute of Biotechnology, Nanjing, China) and phosphatase inhibitor (1 mM) (Calbiochem, Merck, Germany), centrifuged at 12,000 rpm at 4 ℃ for 15 min. The concentration of the proteins was determined with the BCA assay. Protein were mixed with loading buffer and boiled for 10 min to denature the proteins. About 20 μg of protein was loaded on the SDS-PAGE gel and separated by electrophoresis was completed. Proteins were transferred from the gel to a PVDF membrane by wet transfer method. The membrane was blocked using 5% skim milk powder for 2 hours, followed by incubation in the primary antibody (Caspase-12, 1:1,000; CHOP, 1:1,000; GRP78, 1:800; p-CREB, 1:500; TRPC6, 1:500; αII-spectrin, 1:500; β-actin, 1:1,000) overnight at 4 ℃. After washing with TBST, the membrane was incubated in the HRP conjugated secondary antibody (dilution: 1:10,000) for 2 h at room temperature. Finally, the membrane was emerged in the ECL reagent for 2 min to visualize the protein bands.

Statistical analysis

All data are expressed as mean ± standard deviation (x±s). Mean value between groups were compared with one-way analysis of variance (ANOVA) followed by Dunnett's test. All analyses were performed with SPSS 17.0 software package. $P<0.05$ indicated a significant difference.

Results

Effect of NLXT on neurological score, cerebral water content, cerebral blood flow (rCBF) and apoptosis

The rCBF of the model group was significantly lower than that of the sham-operated group at time points (*, $P<0.05$; **, $P<0.01$) (*Figure 1A*). Moreover, the medium dose of NLXT (2.25 g/kg) significantly increased rCBF of the rats on day 7 and 14 ($P<0.01$). Interestingly, both low and high NLXT doses (1.125 and 4.5g/kg) did not significantly increase rCBF.

Cerebral I/R injury led to a significant deterioration in neurobehavioural score across days 1, 7 and 14 ($P<0.01$), relative to the Sham group. However, both low and medium NLXT doses significantly restored neurological deficits, relative to the MCAO group ($P<0.05$, $P<0.01$) on days 7 and 14. Subsequently, we found a significantly higher brain water content in the MCAO group than in the Sham group ($P<0.01$). Conversely, both medium ($P<0.05$) and high ($P<0.01$) NLXT doses significantly reduced water content (*Figure 1B,C*).

Results from Tunel analysis revealed a significantly higher number of apoptotic cells in the hippocampus of MCAO rat relative to those in the Sham group ($P<0.01$). However, medium ($P<0.05$) and high ($P<0.01$) NLXT doses significantly reduced apoptosis of hippocampal neurons. Overall, these results indicate that NLXT mediates protection against cerebral I/R injury in rats (*Figure 1D*).

Effect of NLXT on the volume of cerebral infraction after MCAO

TTC staining is the key method for determining the success of the infarction model, therefore an important indicator during evaluation of a drug's protective effect. In the present study, we selected the 2.25 g/kg for further evaluation of NXLT's effect on infraction volume. Consequently, brain slices of rat across each group were stained with TTC (*Figure 2*), photographed and the red and white areas measured. Determination of the percentage infarction areas, using Image J revealed that NLXT significantly reduced the white area compared to rats in the MCAO group ($P<0.01$).

Effect of NLXT on ER stress-related proteins in MCAO-injured rats

We analyzed expression profiles of ER stress-related marker proteins using western blot assay. Results revealed significantly higher ($P<0.01$) levels of ER-stress marker caspase-12, GRP78, and CHOP in the model than the sham groups (*Figure 3*). Upon NLXT administration (2.25 g/kg), significantly lower levels of caspase-12, GRP78, and CHOP were observed in the model group. Taken together, these results indicate that NLXT protects against I/R injury thereby alleviating ER-stress induced cell apoptosis.

Effect of NLXT on TRPC6/MEK/CREB pathways in MCAO-mediated rat

During cerebral ischemia, Calpain is activated, then decomposed into a calpain-specific αII-spectrin protein product (SBDP145). Therefore, changes in patterns of SBDP145 protein expression can reflect the activity level of cerebral ischemia-reperfusion injury in rats. In the present study, we found a significant increase in the level of SBDP145 in rats with ischemia reperfusion ($P<0.01$)
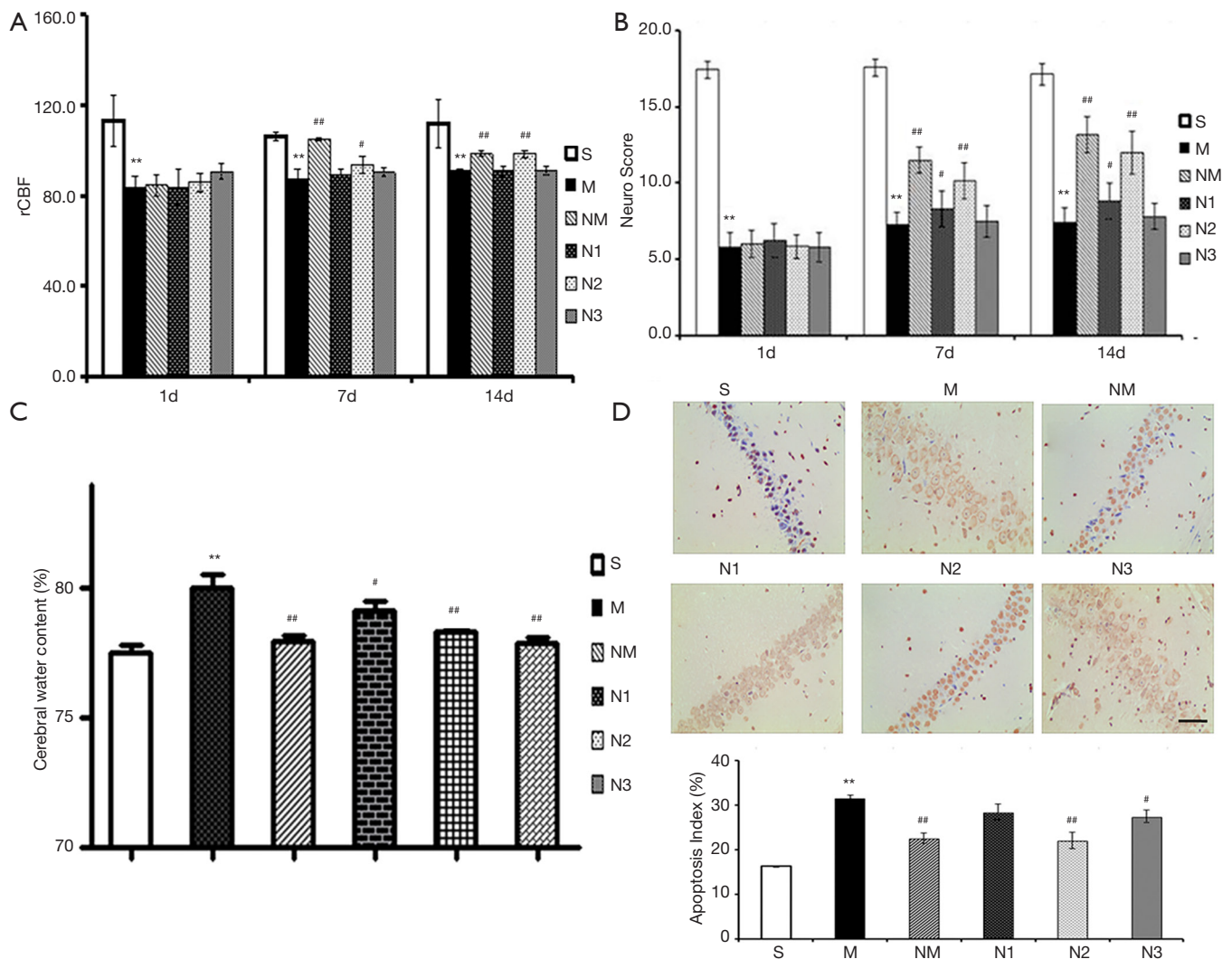
Figure 1 Effect of NLXT on rCBF, neuron score, cerebral water content and apoptosis in rats induced by MCAO. (A) The changes of rCBF in all group at 1, 7 and 14 days; (B) quantitative analysis of neurologic deficit scores in different groups at 1, 7 and 14 days; (C) quantitative analysis of cerebral water content at 14 days; (D) representative images of neuron stained with the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) (top) and Quantitative analysis of apoptosis (bottom). TUNEL-positive cells per field were counted. Scale bar =50 μm (×200 magnification). S: Sham group; M: MCAO group; NM: Nimodipine group; N1: NLXT (1.125 g/kg); N2: NLXT (2.25 g/kg) and N3: NLXT (4.5 g/kg). Data were presented as mean ± SD. **, P<0.01 versus Sham group; #P<0.05,##P<0.01 versus MCAO group (n=6). NLXT, Naoluo Xintong; rCBF, regional cerebral blood flow; MCAO, middle cerebral artery occlusion.

relative to those in the sham operation group (*Figure 4A*). In addition, NLXT significantly reduced SBDP145 expression (P<0.05), whereas significantly lower TRPC6 levels were observed in the model compared to the sham operation group (P<0.01). NLXT also significantly increased levels of TRPC6 in rats with I/R injury (P<0.01). Similarly, images from immunofluorescence staining showed that NLXT improved TRPC6 changes in the

hippocampal neuron membrane, consistent with western blots (*Figure 4B,C*). In addition, significantly lower p-CREB levels were observed in the MCAO relative to the Sham group (P<0.01). However, NLXT administration led to a significantly increase in p-CREB expression. Co-administration of PD98059 reversed NLXT-mediated p-CREB up-regulation (P<0.05) (*Figure 4D*). Overall, these results indicate that NLXT mediates protection
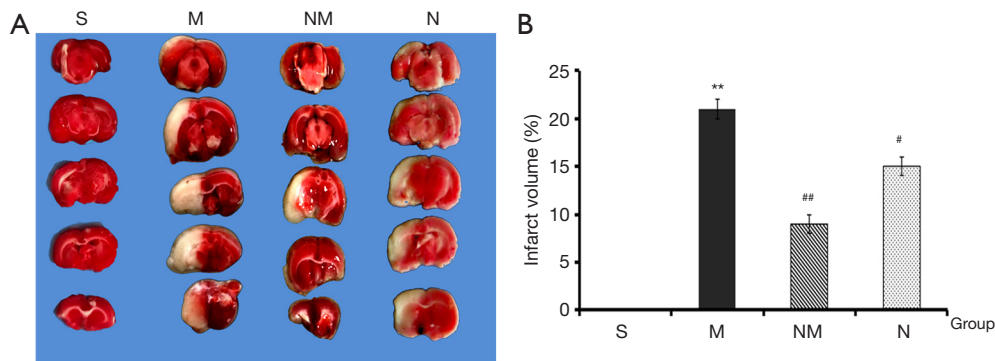
Figure 2 Effect of NLXT on infarct volume in rats induced by MCAO. Representative TTC-stained (left) brain slices and quantitative analysis (right) of infarct volume in all group. S: Sham group; M: MCAO group; NM: Nimodipine group and N: NLXT (2.25 g/kg), Data were presented as mean ± SD. **, $P<0.01$ versus Sham group; #$P<0.05$, ##$P<0.01$ versus MCAO group (n=6). NLXT, Naoluo Xintong; MCAO, middle cerebral artery occlusion.
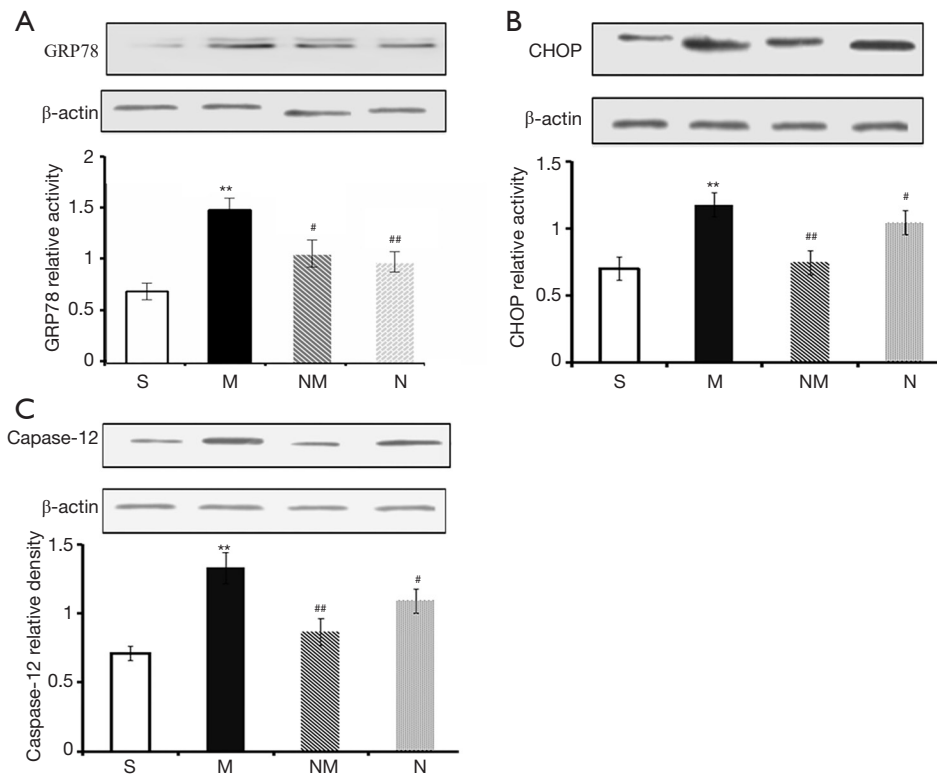

Figure 3 Effect of NLXT on ER stress-related proteins in MCAO-injured rat. Rats were subjected to 24 h of reperfusion, and NLXT (2.25 g/kg) was given by intragastric administration for 7 days after reperfusion. Representative Western blot band (top) and the quantitative analysis (bottom) of the ratio of (A) GRP78, (B) CHOP and (C) caspase-12. S: Sham group; M: MCAO group; NM: Nimodipine group and N: NLXT (2.25 g/kg). Data were presented as mean ± SD. **, $P<0.01$ versus Sham group; #$P<0.05$, ##$P<0.01$ versus MCAO group (n=3). NLXT, Naoluo Xintong; ER, endoplasmic reticulum; MCAO, middle cerebral artery occlusion.

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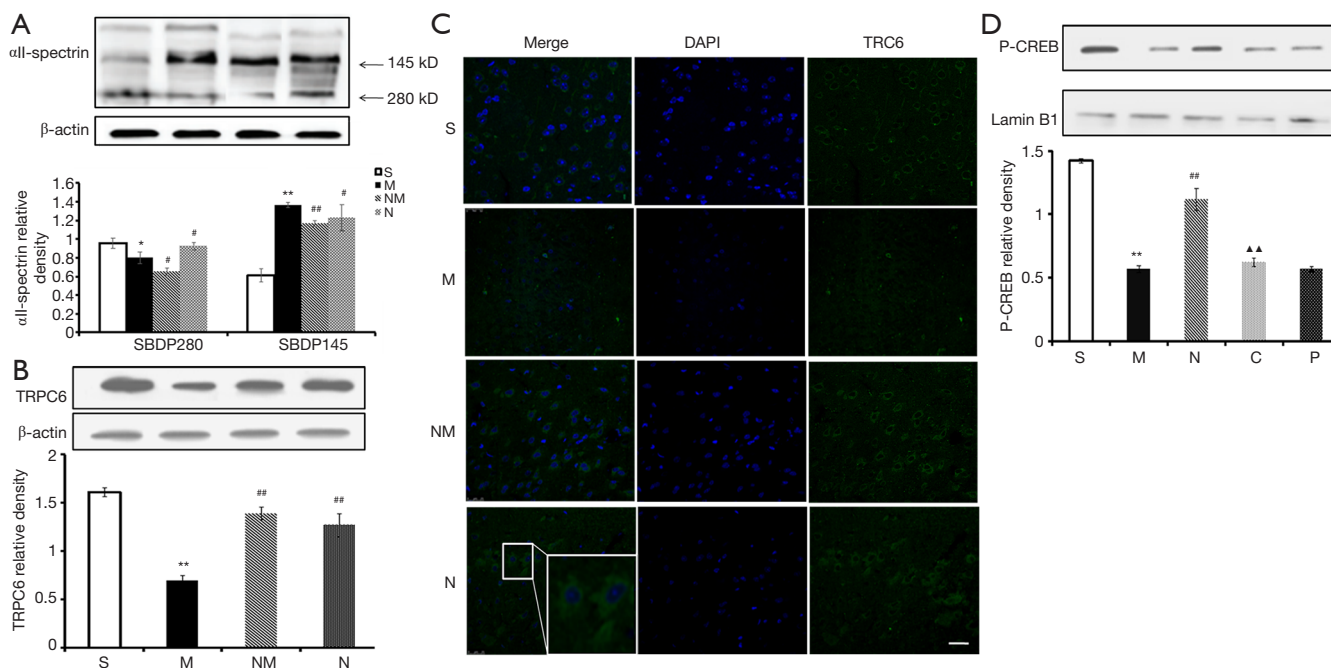
Figure 4 Effect of NLXT on TRPC6/MEK/CREB pathways in MCAO-injured rat. Rats were subjected to 24 h of reperfusion, and NLXT (2.25 g/kg) was given by intragastric administration for 7 days after reperfusion. Representative Western blot band (top) and the quantitative analysis (bottom) of the ratio of (A) SBDP 145, (B) TRPC6 and (D) p-CREB. (C) Immunostaining labeled with TRPC6 (green) and co-staining with 4, 6-diamidino-2-phenylindole (blue) Scale bar =50 μm (×200 magnification). S: Sham group; M: MCAO group; NM: Nimodipine group; N: NLXT (2.25 g/kg); C: combine group (NLXT + PD98059) and P: PD98059 group. Data were presented as mean ± SD. *, P<0.05; **, P<0.01 versus Sham group; #P<0.05, and ##P<0.01 versus MCAO group; ▲▲, P<0.05 versus NLXT (2.25 g/kg) group (n=3). NLXT, Naoluo Xintong; MCAO, middle cerebral artery occlusion.

against I/R injury by regulating the TRPC6/CREB pathway, in a MEK-dependent.

Discussion

In the present study, we have demonstrated NLXT's protective effect against I/R injury and elucidated its role on ER stress-induced neuronal apoptosis in MCAO-injured rats. Specifically, results from *in vivo* experiments demonstrated that NLXT treatment improves neurological defects and increases rCBF. Consequently, this effect reduces brain infarct size, and regulates expression of ER stress-related proteins in the brain of MCAO-treated rats.

Previous studies have implicated lack of energy supply to neuronal, following cerebral ischemic injury, in ineffective transport of Ca^{2+} from the cytoplasm. In fact, a release of calcium ions in the ER aggravates Ca^{2+} overload (30,31). Consequently, an influx of Ca^{2+} impels development of I/R injury. Calpains are intracellular calcium-dependent cysteine endopeptidases that get activated by cytosolic Ca^{2+} overload. Numerous studies have demonstrated that activation of calcium activator calpain, after I/R injury, leads to degradation of αII-spectrin (32,33) and TRPC6 (34,35), which in turn increases extracellular calcium influx. In the present study, rats in the sham group exhibited significantly lower αII-spectrin protein decomposition product (SBDP145), whereas those in the NLXT group had high levels SBDP145 mediated by MCAO, but low calpain activity, as evidenced by low levels of calpain specific αII-SBDP145.

Previous studies have shown that the TRPC subfamily is an essential channel in the regulation of calcium concentration, and plays a key role in the MCAO injury (36). In addition, TRPC6 promotes survival and differentiation of neurons induced by brain-derived nerve growth factor (BDNF) and has been directly associated with neuronal apoptosis in rats with cerebral ischemia (36,37). Furthermore, numerous studies have shown that

p-CREB not only effectively inhibits expansion of the infarction area, but also stimulates nerve re-generation (38,39). These studies affirm that CREB activation is a critical target for neuroprotection from ischemic injury. However, appropriate concentrations of Ca^{2+} are needed to stimulate downstream CREB, through the TRPC6 channel, to promote neuronal survival (11). Therefore, modulating degradation of the TRPC6 channel maintains p-CREB, thereby exerting a remarkable protective effect on ischemic brain damage. In the present study, we investigated the effect of NLXT, and its underlying mechanisms, on TRPC6 signaling. Interestingly, immunofluorescence and SDS-PAGE results indicated that NLXT treatment mediated a significant reduction of MCAO-induced TRPC6 degradation. Particularly, significantly higher p-CREB levels were evident in the NLXT group, after ischemia, relative to the MCAO group. Conversely, significantly lower p-CREB levels were observed, following blocking of MEK activity, with a similar trend observed in either NLXT or co-administration (NLXT + PD98059) groups. Western blots demonstrated that NLXT, administered immediately following ischemia, stimulated the MEK signaling pathway and ultimately induced CREB activation. Overall, these findings indicated that NLXT blocks degradation of the calpain-mediated TRPC6 channel, which in turn activates CREB via the MEK signaling pathway.

Generally, UPR is a self-cytoprotective response that helps adaption, to re-establish ER homeostasis, although its continuous or prolonged activation can lead to cell death. In addition, an influx of calcium ions is an important signal for apoptosis, which also plays an important role in ER storage (40,41). Generally, ER stress-induced apoptosis requires action of various related molecules, with previous evidence showing that an individual's GRP78 is directly correlated to the degree of ER-stress (42,43). In addition, CHOP involvement in ER stress apoptotic pathway also represents a marker of ER stress. In fact, development of ER-stress results in an increase in CHOP, which subsequently inhibits anti-apoptosis by blocking Bcl-2expression. Similarly, Caspase-12 is present on ER's cytoplasmic surface, with previous studies showing that Ca^{2+} release activates procaspase-12 into Caspase-12, a special pathway for apoptosis induced by ER-stress (44,45), and ER-stress mediated cell apoptosis plays a key role incidence of

in stroke (46). In the present study, our results showed that NLXT intervention mediated a gradual decrease in I/R-induced neuron apoptosis in rats. In addition, electrophoretic bands revealed that ischemic reperfusion injury initiated ER-stress, which increased expression of GRP78, CHOP and Caspase-12. Conversely, NLXT intervention significantly reduced expression of these proteins. ER stress is a critical intracellular apoptosis signal. Results from Tunel staining indicated that NLXT administration mediated a reduction of apoptosis. Overall, these results suggested that NLXT plays a potential role in I/R mediated apoptosis linked with ER stress related pathway, providing useful insights for its use in treatment of cerebral I/R injury (*Figure 5*).

Taken together, our results confirmed that NLXT confers protection against cerebral I/R injury, by inhibiting ER-stress induced apoptosis and the TRPC6/MEK/CREB signal pathway. Nevertheless, the following further studies are needed to fully elucidate the complex mechanisms of ischemia-induced apoptosis; Firstly, it will be interesting to ascertain whether activated TRPC6 can activate CREB via the CaM/CaMKIV signaling pathway and confer protection against I/R injury (47). It is also not known whether NLXT can modulate apoptosis through other pathways. Moreover, NLXT's effect on the dsRNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol-Requiring Enzyme 1(IRE1) and Activating Transcription Factor 6 (ATF6) signaling pathways, during ER stress-induced apoptosis, remains unclear. Therefore, future studies are expected to assess whether NLXT may be beneficial in these pathways. Finally, further studies are expected to expound on the effect of various inhibitors, such as dantrolene and edaravone, which have been proven to be effective in attenuating ER stress after experimental cerebral ischemic injury (48). Future studies using ER-stress inhibitor are needed to enlarge our findings.

Conclusions

Summarily, our findings provide novel insights into NLXT's protective effects against I/R-induced brain injury. In addition, we have demonstrated the underlying mechanisms of NLXT action, including diminution of ER stress-induced neuronal apoptosis, inhibition of calpain proteolysis of TRPC6 as well as subsequent activation of CREB via the MEK signaling pathway.
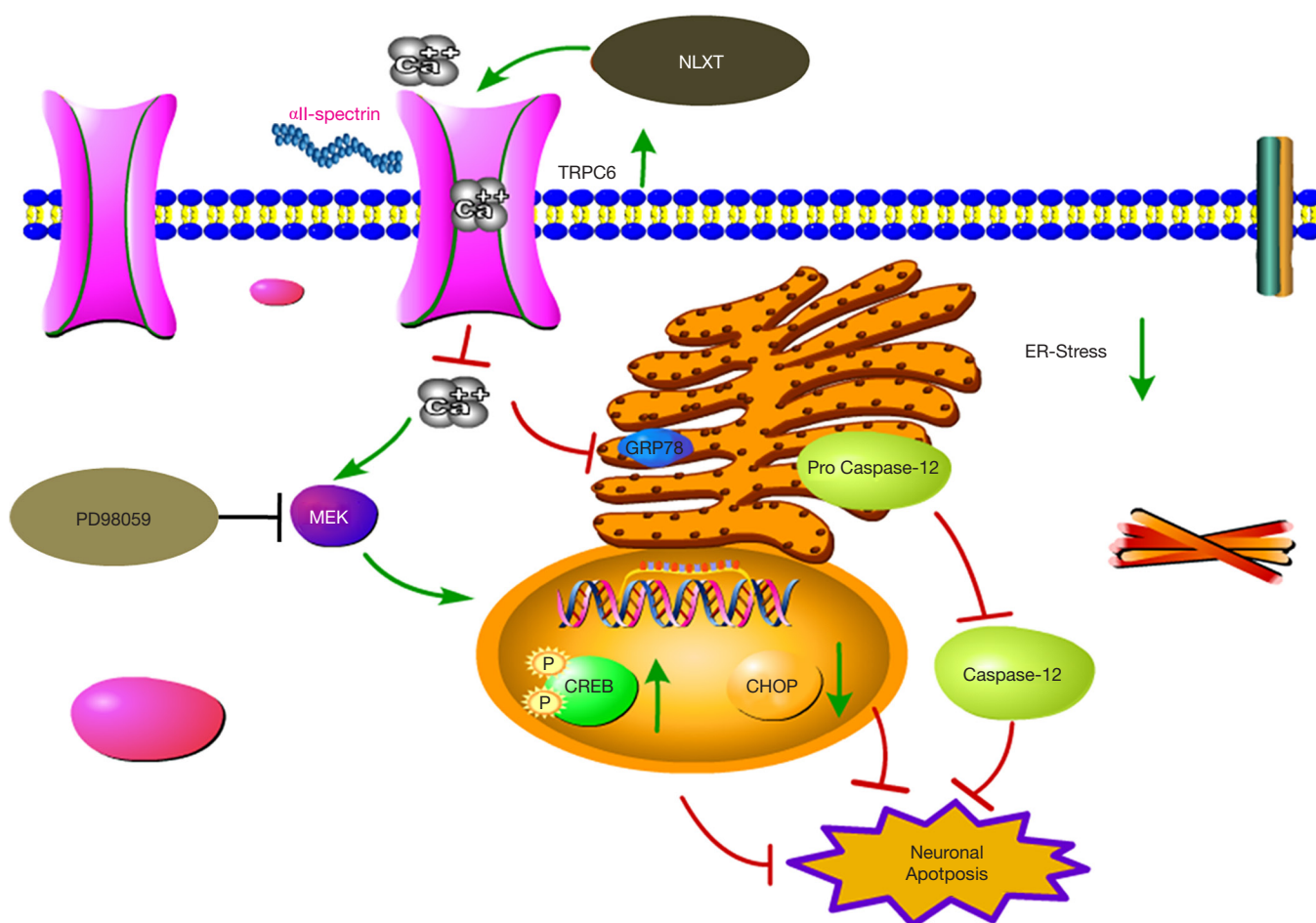
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Figure 5 A schematic diagram illustrating the underlying molecular mechanism of NLXT on ER-stress induced apoptosis in rats with I/R injury. NLXT mitigated I/R induced rats injury by suppressing over-activated ER-stress induced apoptosis, and such inhibition may be associated with the reduction of degradation of TRPC6, regulation of concentration of calcium. Finally, the activation of the TRPC6/MEK/CREB signaling pathway involved in this protection process. NLXT, Naoluo Xintong; I/R, ischemia-reperfusion.

of Chinese Medicine, China, and conducted in accordance with the guidelines provided by the National Institutes of Health on the Care and Use of Animals (NIH Publication number 80-23, revised 1986).

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