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Novel scorpion venom peptide HsTx2 ameliorates cerebral ischemic brain injury in rats via the MAPK signaling pathway

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ABSTRACT

Ischemic stroke is a severe threat to human health due to its high recurrence, mortality, and disability rates. As such, how to prevent and treat ischemic stroke effectively has become a research hotspot in recent years. Here, we identified a novel peptide, named HsTx2 (AGKKERAGSRRTKIVMLKCIREHGH, 2 861.855 Da), derived from the scorpion *Heterometrus spinifer*, which showed obvious anti-apoplectic effects in rats with ischemic stroke. Results further demonstrated that HsTx2 significantly reduced formation of infarct area and improved behavioral abnormalities in ischemic stroke rats. These protective effects were likely exerted via activation of the mitogen-activated protein kinase (MAPK) signaling pathway, i.e., up-regulation of phosphorylated ERK1/2 in both rat cerebral cortex and activated microglia (AM); up-regulation of phosphorylated p38 (p-p38) in the cerebral cortex; and inhibition of phosphorylated JNK and p-p38 levels in the AM. In conclusion, this study highlights HsTx2 as a potential neuroprotective agent for stroke.

1. Introduction

Ischemic stroke, which accounts for about 75% of all strokes, demonstrates high incidence and mortality rates [1]. So far, the only drug approved for the treatment of ischemic stroke is recombinant human tissue plasminogen activator (rt-PA), however, due to its narrow thrombolytic time window (4.5 h), considerable contraindications (such as age limit and active internal bleeding), and delayed diagnosis, the utilization rate of rt-PA is less than 10%, implying that the development of new effective anti-ischemic stroke drugs is an important research direction [2,3,4].

Based on modern pharmacology, animal toxins have been shown as molecular probes in the analysis of human disease mechanisms [5,6]. Scorpions are among the oldest and widely distributed extant species on Earth [7]. As reported in the 'Compendium of Materia Medica' (a traditional Chinese medicine book), scorpion toxin, as an active compound, exhibits anti-epileptic, analgesic, and antispasmodic effects [8]. Modern pharmacological studies have shown that scorpion toxins can not only affect ion channels but also reflect in the regulation of muscle contraction and nerve secretion [7,9]. Meucin-24 and -25 from the scorpion *Mesobuthus eupeus* are considered ideal candidate drugs for malaria due to their ability to specifically kill *Plasmodium* species [10]. As an effective selective Kv1.3 channel-blocking peptide, HsTX1 from *Heterometrus spinifer* has been applied in the treatment of rodent autoimmune diseases such as rheumatoid arthritis and

Abbreviations: MAPK, mitogen-activated protein kinase; rt-PA, recombinant human tissue plasminogen activator; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; MCAO, middle cerebral artery occlusion; TTC, 2, 3, 5-Triphenyltetrazolium chloride; PVDF, polyvinylidene difluoride; OMM, observed molecular mass; TMM, theoretical molecular mass; AM/BM, activated microglia/brain macrophages.

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multiple sclerosis [11]. Other active peptides derived from *H. spinifer*, such as HsAp family, Heterin family and Spiniferin, exhibit antibacterial activity [12,13]. Thus, peptides derived from toxins, especially bioactive peptides derived from scorpions, exhibit great potential in the development of drugs to treat various types of human diseases and consequently warrant further pursuit and exploration.

In the current study, we identified a novel active peptide derived from *H. spinifer* toxin, named HsTx2 (amino acid sequence: AGKKERAGSRRTKIVMLKCIREHGH, 2 861.855 Da). Using a rat ischemic stroke model, HsTx2 showed strong neuroprotective effects by effectively reducing the area of cerebral infarction caused by ischemia and improving behavioral abnormalities and motor disorders in rats. We further verified the neuroprotective mechanism using western blotting and immunofluorescence. Results indicated that HsTx2 activated the mitogen-activated protein kinase (MAPK) signal transduction pathway by increasing phosphorylated-p38 (p-p38) and -Erk1/2 (p-Erk1/2) expression in brain tissue and inhibiting phosphorylated-JNK (p-JNK) and p-p38 expression but up-regulating p-Erk1/2 in activated microglia (AM). The purpose of this study was to explore the neuroprotective effects of HsTx2 and the specific underlying mechanism, ultimately providing a potential candidate drug precursor for the clinical application of novel anti-apoplexy peptides.

2. Materials and methods

2.1. Collection of H. spinifer venom and animal ethics

In total, 40 *H. spinifer* scorpions were collected in Xishuangbanna County, Yunnan Province, China. The venom gland was stimulated by a 3-V alternating current, with venom then secreted from the sting. The venom was collected in a 1.5-mL centrifuge tube and stored at −80 °C until use.

2.2. Purification of venom peptide

Crude venom was ultrafiltered using an ultrafiltration device with a molecular weight cutoff of 10 kDa (1.5 × 31 cm, superfine, GE Healthcare, Sweden). The ultrafiltered samples were then applied to a C18 reverse-phase high-performance liquid chromatography (RP-HPLC) column (Hypersil BDS C18, 4.0 × 300 mm; Elite, Dalian, China) pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. Elution was achieved by a linear gradient of 0.1% (v/v) TFA in acetonitrile at a flow rate of 1 mL/min, with monitoring at 220 nm. Peaks were collected and lyophilized for further analysis.

2.3. Peptide synthesis

The peptide (purity >95%) was commercially synthesized and provided by Wuhan Bioyeargene Biotechnology Co., Ltd. (China).

2.4. Determination of amino acid sequence of peptide

Average molecular weight and purity of the peptide were analyzed on an UltraFlex I mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ion mode. The complete amino acid sequence of the peptide was determined by Edman degradation on a PPSQ-31A protein sequencer (Shimadzu, Tokyo, Japan) according to the standard protocols of the manufacturer. Both MS and MS/MS analyses were performed in positive charge mode on a mass spectrometer (Autoflex speed TOF/TOF, Bruker Daltonik GmbH, Leipzig, Germany).

2.5. Surgical procedures

In total, 40 male Sprague-Dawley (SD) rats (250−300 g) obtained from Hunan SJA Laboratory Animal Co., Ltd. (China) were used in the surgical procedures. The rats were randomly divided into sham-operated group (control), middle cerebral artery occlusion group (MCAO) and MCAO + HsTx2 group. After the rats were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg), the right MCA was permanently occluded at its origin according to Tamura's surgical procedures, as used in the previous study [14]. Rats in the treatment groups were given an i. p. injection of HsTx2 (1, 2, and 5 nmol/kg) 2 h before and 24, 36, and 72 h after MCAO. Then all the rats were sacrificed with a narcotic overdose on the third day after MCAO. In the control group, the same surgical procedure was carried out, but the MCA was not cauterized.

All animal care and handling protocols were conducted in accordance with the requirements of the Ethics Committee of Kunming Medical University.

2.6. 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining and evaluation of infarction volume after MCAO

Viability of brain ischemia was evaluated using TTC (Sigma-Aldrich, St. Louis, MO, USA) staining 72 h after MCAO. After sacrificed, rat brains were rapidly removed, frozen at −20 °C for 5 min, and then sliced coronally into 10 slices. Sets of serial slices from each brain were incubated for 30 min at 37 °C in 2% TTC in the dark. The infarction and hemisphere areas of each section were traced and measured using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., MD, USA). Infarct volume was expressed as a percentage of the contralateral hemisphere.

2.7. Postoperative behavioral evaluation (Zea-longa score)

After recovered from anesthesia, the rats were scored according to the Zea-Longa scoring system, as reported previously [15]. The scoring was as follows: 0 points, no symptoms of neurological impairment; 1 point, side front paw could not stretch completely; 2 points, paw rotated inwards when walking; 3 points, paw tilted inwards when walking; and 4 points, failed to spontaneously walk or experienced loss of consciousness. According to the experimental requirements, rats that scored 0 or 4 were excluded from the study; scores of 1, 2, and 3 indicated that the model was successful.

2.8. Balance beam walking test (BBT)

Neurobehavioral outcomes were evaluated using the balance beam walking test at 24 h, 48 h, and 72 h after surgery. The beam was 2.5 cm wide and 150 cm long, also at a 70 cm height above the sawdust cushion. A box was placed at the end of the beam for rats to rest between tests. Before the first test, rats were trained to walk on the beam from one end to the other, and rest in the box after walking. We recorded the time each rat took to cross the balance beam and the number of missteps.

2.9. Western blot analysis of MCAO tissues

A total of 15 rats were used for Western blot analysis. The control, MCAO, and MCAO + HsTx2 group rats were sacrificed at 72 h, and the infarct area of the cortex was obtained and homogenized with protein extraction reagent containing protease inhibitors. Samples of supernatants containing 50 μg of protein from tissues were separated using 10% sodium dodecyl sulfate-

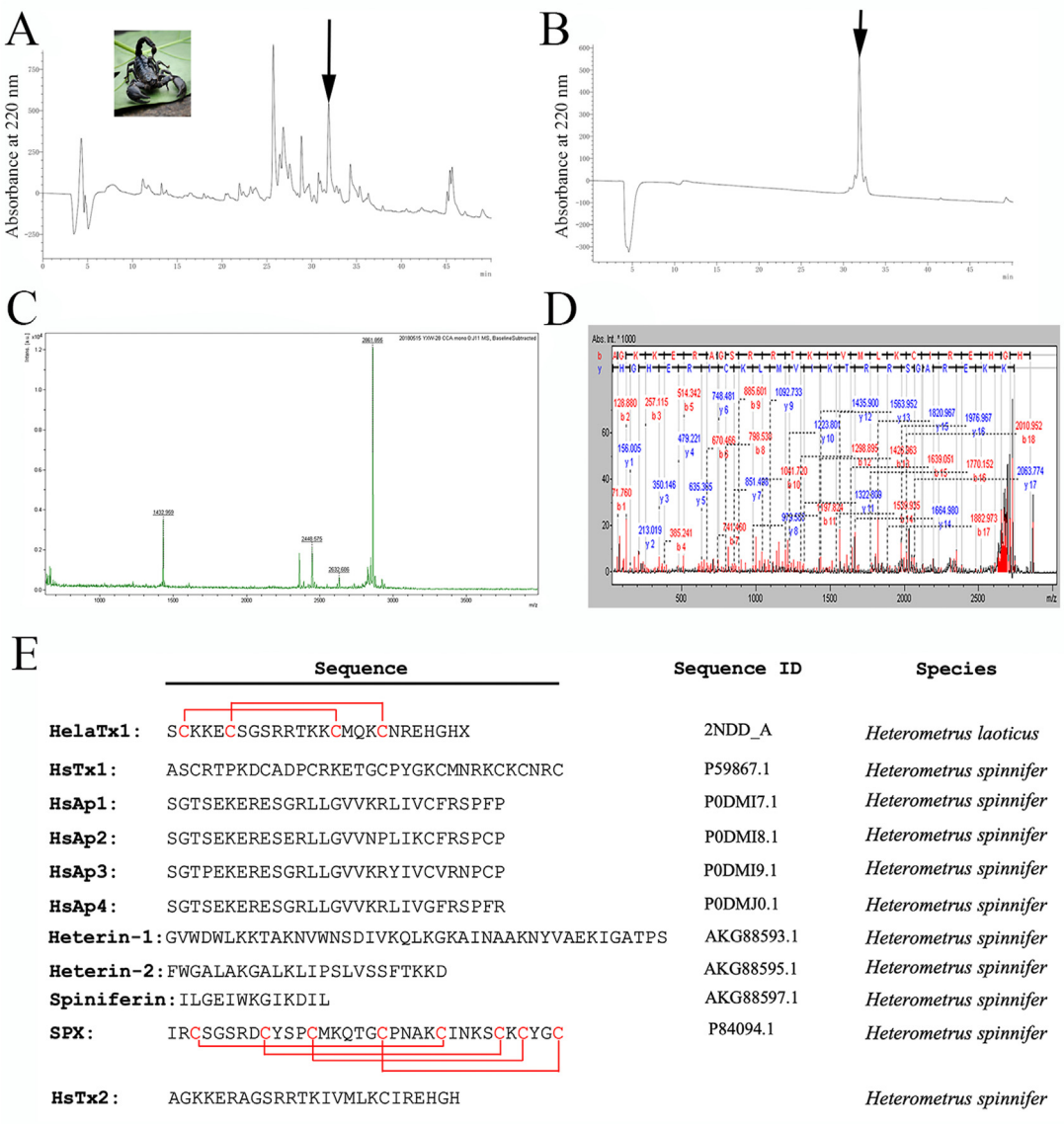

Fig 1. Purification and structural determination of HsTx2. **A**. *H. spinifer* photo and first HPLC chromatogram (HsTx2 is indicated by an arrow). **B**. Second HPLC chromatogram (HsTx2 is indicated by an arrow). **C**. Observed molecular mass of HsTx2. **D**. The peptide sequence showed by Mass spectrometry. **E**. Sequence alignment showing that HsTx2 is a new peptide. Intramolecular disulfide bond is marked in red.

polyacrylamide gel electrophoresis in a Mini-Protein II apparatus (Bio-Rad, CA, USA). Protein bands were electroblotted onto polyvinylidene difluoride (PVDF) membranes and blocked with non-fat dried milk for 1 h. The membranes were incubated with p-Erk1/2, Erk1/2 (1:2 000); p-JNK, JNK (1:2 000); p-p38, p38 (1:1 000) (Cell Signaling Technology, CA, UK); and β-actin (1:10 000) (Sigma-Aldrich, St. Louis, Missouri, USA) primary antibodies overnight at 4 °C. They were then incubated with secondary antibodies for 1 h in room temperature. Proteins were detected using a chemiluminescence kit (GE Healthcare UK Limited, Bucks, UK) following the manufacturer's instructions. Band intensity was quantified using ImageJ software (National Institutes of Health, NIH, USA). All experiments were repeated in triplicate.

2.10. Double immunofluorescence labeling in cerebrum

A total of 15 rats from the various experimental groups were used for double immunofluorescence labeling. After rats were sacrificed, the brains were removed and embedded in paraffin. Coronal sections (7-μm thick) were cut on a microtome (Leica, Bensheim, Germany) and rinsed with PBS. The tissue sections were incubated in 5% normal goat serum for 1 h at room temperature. After discarding the serum, the sections were incubated overnight at 4 °C with primary polyclonal antibodies p-Erk1/2, Erk1/2 (1:200), p-JNK, JNK (1:50), p-p38, p38 (1:200) (Cell Signaling Technology, CA, UK), and lectin (1:200) (Sigma-Aldrich, St. Louis, Missouri, USA). After washing in PBS, the sections were separately incubated for 1 h at room temperature with fluorescent secondary antibodies. Finally, the sections were mounted using 4',6-diamidino-2-phenylindole (DAPI) (Sigma, MO, USA). Colocalization was observed by confocal microscopy (FluoView 1000, Olympus Company Pte. Ltd., Tokyo, Japan).

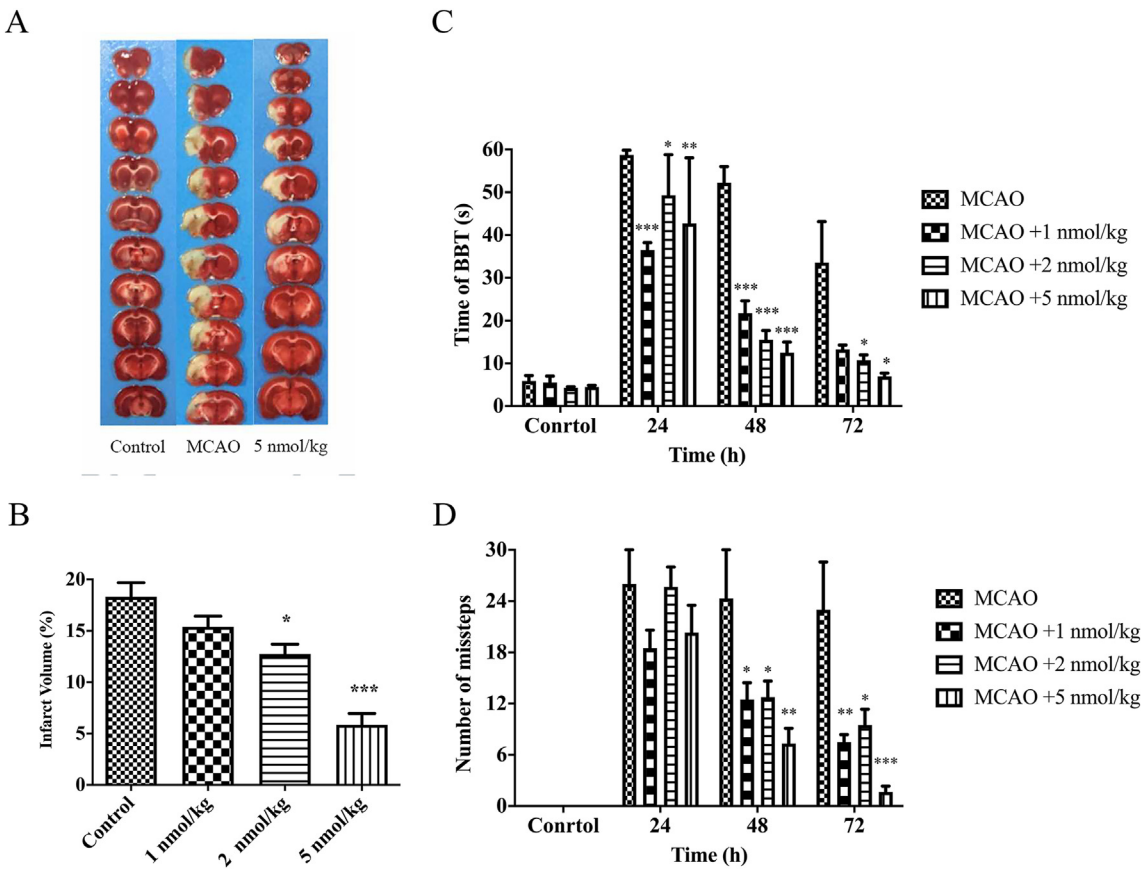

Fig. 2. HsTx2 reduced infarct volume and improved neurological deficits in MCAO rats. **A**. Representative images of TTC-stained brain slices at 72 h after MCAO. **B**. Infarct volume percentages in control and HsTx2-treated groups. **C**. Time required to cross balance beam at 24 h, 48 h, and 72 h post-surgery in MCAO and HsTx2-treated rats. **D**. Number of missteps when crossing the beam in MCAO and HsTx2-treated rats. Data are mean ± SEM. Compared with control group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

2.11. Statistical analysis

Data were depicted as means ± standard errors of the mean (SEM). Results were analyzed using GraphPad Prism 8.0 (La Jolla, CA, USA) and pairwise comparisons were conducted using one-way analysis of variance (ANOVA) with the Bonferroni *post-hoc* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification and structural determination of peptide

As shown in Fig. 1A, following HPLC purification, 29 peaks were obtained, with one showing the required activity (as indicated by the arrow). This peak was further purified by HPLC again, and one peak with an identical elution time and ideal shape was obtained (Fig. 1B). Mass spectrometry revealed that the observed molecular mass (OMM) of this peptide was 2 161.855 Da (Fig. 1C) and the theoretical molecular mass (TMM) (calculated at http://web.expasy.org/compute_pi/) was 2 162.41 Da. Thus, the OMM and TMM values were basically consistent, indicating that there was no post-translation modification. Using an Edman sequencer, the amino acid sequence of the peptide was determined to be 'AGKKERAGSRRTKIVMLKCIREHGH' from the N-terminus to C-terminus (Fig. 1D). By Blast searching the NCBI database, the peptide and HelaTx1, both from *Heterometrus*, showed a sequence similarity of 73.9% (Fig. 1E), indicating that HsTx2 is a new peptide.

3.2. HsTx2 application significantly improved cerebral infarction volume

At 72 h after operation, TTC staining showed considerable cerebral tissue infarction in the control group (Fig. 2A). Treatment with 5 nmol/kg HsTx2 also resulted in a significant decrease compared with that in the MCAO group ($P < 0.001$) (Fig. 2B).

3.3. HsTx2 application significantly improved behavior in rats with cerebral infarction

Pre-operative rats in each group showed no significant differences in time required and foot slip numbers whilst crossing the balance beam. After 48 h and 72 h, however, the HsTx2-pretreated rats required significantly less time crossing the beam and experienced a significantly decrease in the number of foot slips compared with the MCAO group. These results suggest that the peptide can improve motor dysfunction and physical coordination decline after cerebral ischemia (Fig. 2C and D).

3.4. HsTx2 application suppressed p-JNK expression but promoted p-p38 and p-Erk1/2 expression in cerebral cortex of MCAO rats

As seen in Fig. 3, western blotting showed the protein expression changes in MAPKs and p-MAPKs in MCAO rats. The JNK and p-JNK protein expression levels were up-regulated in the MCAO group but down-regulated in the MCAO + HsTx2 pretreatment group. In addition, p-Erk1/2 and p-p38 protein expression levels

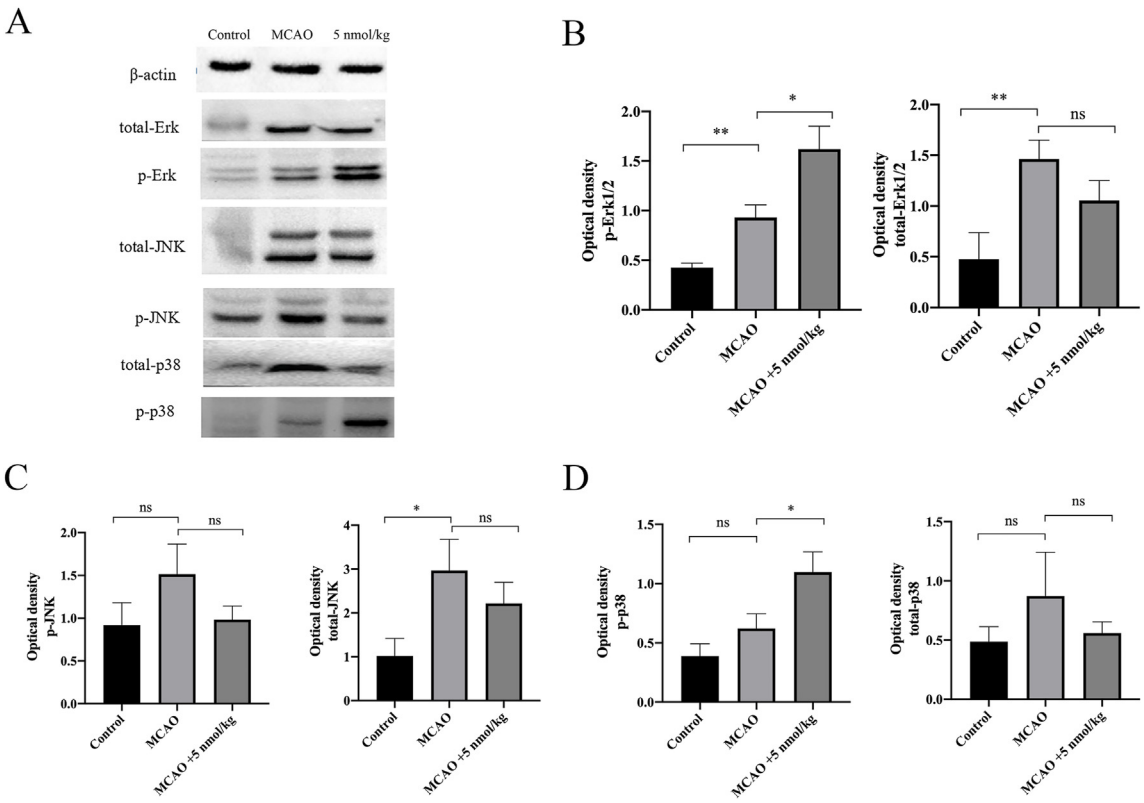

Fig 3. HsTx2 activated the MAPK signaling pathway. **A**. Representative western blots of MAPK protein levels in rat cerebral cortex. **B-D**. Protein expression levels of p-MAPK in MCAO group were increased compared with control group. HsTx1 promoted p-Erk1/2, p-JNK and p-p38 expression in MCAO rats. Data are means ± SEM. *significant differences in protein levels, $P < 0.05$; **significant differences in protein levels, $P < 0.01$.

were up-regulated in the MCAO group and MCAO + HsTx2 pre-treatment group.

3.5. HsTx2 application suppressed p-JNK and p-p38 expression but promoted p-Erk1/2 expression in MCAO-activated microglia

As shown in Fig. 4, immunofluorescence labeling of p-p38 and p-JNK in MCAO rats decreased in activated microglia/brain macrophages (AM/BM) double-labeled with lectin in MCAO rats pretreated with HsTx2. Strikingly, p-Erk1/2 immunofluorescence in AM/BM was further enhanced in MCAO rats pretreated with HsTx2 (Fig. 4A).

4. Discussion

Stroke is the most common cause of permanent disability in the world and one of the main causes of death [3,16]. By evaluating cerebral infarction area and behavioral scores in the MCAO and HsTx2 rats, we found that the while administration of the active peptide reduced the infarction area by almost half (Fig. 2A and B). For behavioral evaluation, with the extension of time post-surgery (24–72 h), the time required to cross the balance beam and the number of missteps in MCAO rats were decreased. Although we could not exclude the partial adaptation of rats to the behavioral experiments through learning, HsTx2 pretreatment (especially 5 nmol/kg) partially resisted the above behavioral changes (Fig. 2C and D). These results indicate that the active peptide can alleviate neurological impairment and neurobehavioral changes caused by cerebral ischemia in rats.

An inflammatory reaction occurs followed by ischemia, with rapid activation of glial cells (especially microglia), infiltration of peripheral inflammatory cells and release of pro-inflammatory mediators from the damaged tissues, which further aggravates the damage to brain tissue [2]. The activation of microglia induced by cerebral ischemia precedes the occurrence of neuronal injury, so it is very important to explore the specific mechanism of microglia in the cerevral ischemia inflammatory stage. MAPKs are important signaling pathways in the body, and are closely related to cell differentiation, apoptosis, and proliferation [17,18]. Studies have shown that ischemia, hypoxia, stress, and other stimuli can activate the MAPK signaling pathways and mediate physiological and biochemical reactions [19,20]. In the rat model of cerebral ischemia, the MAPK signaling pathway can activate and mediate partial neuro-inflammation [21]. The Erk1/2 pathway, a MAPK subfamily, is mainly related to cell proliferation and growth, while the JNK and p38 families are related to cell injury and apoptosis [14,17]. In this study, we found that cerebral ischemia increased the levels of p-MAPKs in the cerebral cortex of rats, whereas application of HsTx2 further increased the levels of p-Erk1/2 and p-p38, but inhibited the level of p-JNK (Fig. 3). Our results are consistent with previous studies, i.e., cerebral ischemia and hypoxia increase the levels of p-MAPK in the cerebral cortex, but application of neuroprotective drugs, such as scutellarin, can further increases the level of p-Erk1/2 and decreases the levels of p-JNK and p-p38 [14,22,23]. However, in this research, after HsTx2 application, the levels of p-Erk1/2 and p-p38 increased. The increase in p-p38 level in the HsTx2 group suggests that other molecules or signaling pathways may be influencing the level of p-p38 in the whole signal transduction network in rat brains, not just activation of the MAPK signaling pathway (Fig. 3A, D).

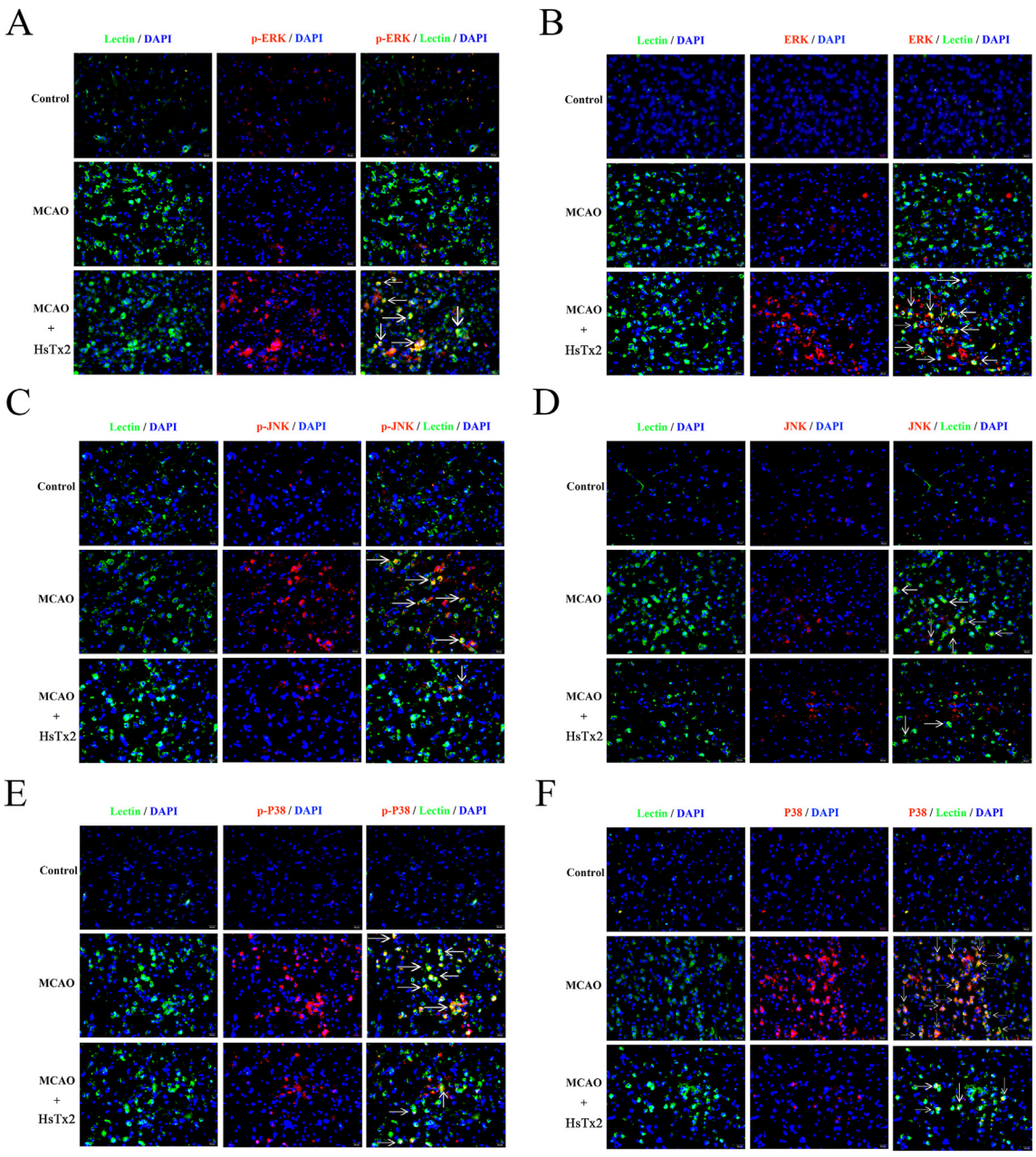

Fig 4. Fluorescence images of MAPK signaling pathway activation in cerebral cortex of MCAO and HsTx2 pretreated rats. **A**. Confocal images showing increased p-Erk1/2 and Erk1/2 immunofluorescence (red) in lectin-positive AM/BM (green) of MCAO rats pretreated with HsTx2 compared with MCAO rats. **B**. p-JNK (red) expression increased in AM/BM of MCAO rat brains but was attenuated by HsTx2 treatment. **C**. p-p38 and p38 (red) expression increased in AM/BM of MCAO rat brains but was attenuated by HsTx2 treatment. DAPI—blue. Scale bar = 20 μm.

To further explore the neuroprotective mechanisms of the MAPK signaling pathway in the HsTx2 model of cerebral ischemia, we studied the microglia. Immunofluorescence double-labeling was used to label microglia, p-MAPK and MAPK, respectively. As shown in Fig. 4, the number of AM/BM in the normal rat cerebral cortex was limited. Following cerebral ischemia, however, a large number of AM/BM were observed, indicating that cerebral ischemia could activate microglia in the rat cerebral cortex. The expression of p-MAPK in microglia increased after cerebral ischemia, but the level of p-p38 decreased after HsTx2 treatment, which differed from the western blotting results (Fig. 4E and F). This indicates that the protective effect of HsTx2 against cerebral ischemia may not only be carried out by microglia, but also be coordinated by multiple cells and factors. Many signal transduction mechanisms are involved in cerebral ischemia, and in addition the signal transduction pathway network is a complex whole, and operation depends on the cascade reaction of each signaling pathway in the bodies [24,25]. Therefore, it is necessary to explore the activation of signaling pathways at the cellular and organizational levels to reflect the whole process of disease occurrence and development.

Comparing HsTx2 and HelaTx1, we found that although both are composed of 25 amino acids and show 73.9% sequence similarity, HelaTx1 has two disulfide bonds, while HsTx2 does not (Fig. 1E). Furthermore, despite HsTX1 and HsTx2 come from the same

species, but HsTX1 is comprised of 34 amino acids and plays a therapeutic role in autoimmune diseases, mainly by regulating K^+ ion channel [11]. So, the structures and functions of HsTX1, HelaTx1 and HsTx2 may be completely different. Otherwise, the HsAp family, Heterin family, and Spiniferin peptides though also from *H. spinifer*, they were only reported to have antibacterial activity or the Kv1.3 channel inhibitors anyway [12,13,26].

Our study emphasized that application of the active peptide HsTx2, derived from scorpion venom, can reduce cerebral infarction area, improve neurobehavioral deficits, and play a protective role in a rat cerebral ischemia model by regulating activation of the MAPK signaling pathway in brain tissue and microglia. Moreover, the neuroprotective mechanism of HsTx2 may be achieved by increasing the expression of p-Erk1/2 and decreasing the expression of p-JNK in brain tissue and activated microglia, as well as regulating the level of p-p38. Therefore, HsTx2 has the potential to become a new candidate molecule for anti-cerebral ischemia protection drugs. Further exploration should consolidate its neuroprotective mechanisms.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.11.062.

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