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Original Article

Tongluoxingnao effervescent tablets ameliorate learning and memory impairment in a rat model of vascular dementia via the regulation of the p38 and ERK MAPK signaling pathways

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Abstract: Tongluoxingnao (TLXN) effervescent tablets consist of *Scutellaria baicalensis* Georgi, *Radix Angelicae Sinensis*, and *Ligusticum chuanxiong* Hort that can improve cognitive dysfunction in dementia. This study aimed to evaluate the effect of TLXN on learning and memory impairment in vascular dementia (VD) rats' model, explore the effect of the MAPK signaling pathway on chronic ischemia, and determine the underlying mechanism. VD was induced in rats by permanent occlusion of the bilateral common carotid arteries. The Morris water maze and histopathological examinations were used to evaluate the effect of TLXN on learning and memory impairment, whereas real-time PCR and Western blot analysis were used to determine the levels of mRNA and phosphorylation of members of the MAPK signaling pathway, respectively. Compared to rats in the sham group, VD rats exhibited learning and memory impairments such as escape latency prolongation and decreases in the times of entrance into the first quadrant, valid region and escape platform; however, these conditions improved following TLXN administration. Moreover, TLXN may regulate the p38 MAPK and extracellular signal-regulated kinase (ERK) signaling pathways via the inhibition of the phosphorylation of p38 MAPK (p38), ERK1/2, MAP kinase kinase 6 (MKK6), and c-jun and a reduction in the mRNA levels of p38, ERK1/2, and c-jun. These results suggest that TLXN demonstrates the potential for development as a drug for vascular dementia and that the underlying mechanism involves the regulation of the p38 and ERK MAPK signaling pathways.

Keywords: Tongluoxingnao effervescent tablet, vascular dementia, p38, ERK1/2, MKK6, c-jun

Introduction

Vascular dementia (VD) is a disease with cognitive dysfunction induced by cerebral injury following ischemia, hypoperfusion and hemorrhage [1]. VD is the second-most-common cause of dementia in the elderly after Alzheimer's disease (AD) [2, 3]. In developing countries, the prevalences of VD and AD are 30% and 60%, respectively [4]. Several studies have shown that AD and VD may not represent two independent disorders but may overlap or interact with each other [5-7]; therefore, VD has attracted increasing attention in recent years.

All members of the mitogen-activated protein kinase (MAPK) signaling pathway are phosphorylated and activated to a certain extent by ischemia, and they participate in the regulation of cellular injury and repair [8-10]. Numerous studies have indicated that p38 MAPK (p38) can be activated by inflammatory cytokines, environmental stresses such as hypoxia and ischemia [11] or MAP kinase kinase 6 (MKK6) [12]. p38 contributes to hippocampal synaptic plasticity [13] and tau protein hyperphosphorylation [14, 15], and its activation may result in apoptosis. Additionally, the inhibition of p38 MAPK activation is thought to protect the brain in focal cerebral ischemia models [8, 16] or global cerebral ischemia models [17, 18]. Extracellular signal-regulated kinases (ERKs), including ERK1 and ERK2, play a role in cell proliferation, and their levels of phosphorylation have been reported

to increase in a VD rat model induced by chronic ischemia [19-22]. ERK1/2 activation activates transcription factors involved in the expression of immediate early genes such as c-jun, ultimately resulting in irreversible damage. Collectively, studies have shown that alterations in the MAPK signaling pathway, particularly the p38 and ERK signaling pathways, may be responsible for the cognitive dysfunction in VD or AD [23]. Therefore, targeting the MAPK signaling pathway may represent a promising approach for the prevention of VD, as in other diseases such as melanoma [24], arthritis [25], gynecologic malignancies [26], and cancer [27, 28].

Traditional Chinese medicines (TCMs) have often been used to treat VD, with few adverse effects [29, 30]. Tongluoxingnao (TLXN) is a patent formula based on Xionggui soup documented in *Pu Ji Fang*, which consists of *Radix Scutellariae* (*Scutellaria baicalensis* Georgi), *Radix Angelicae Sinensis* and *Rhizoma Ligusticum* (*Ligusticum chuanxiong* Hort). We have previously shown that TLXN improves learning and memory in a scopolamine mouse model of dementia. The underlying mechanism may be associated with the enhancement of cerebral cholinergic function and the promotion of acetylcholine synthesis, the level of which may be influenced by the ERK signaling pathway [31]. TLXN also inhibited the expression of cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase-3 (GSK3), which are key proteins that regulate tau protein phosphorylation, in AD rat hippocampus [32]. Moreover, TLXN promoted synaptophysin remodeling and the expression of insulin-degrading enzyme or the Na^+-K^+-ATPase to alleviate energy failure, ultimately improving neuropathological damage [33-35]. As a positive drug of TCM, Fufang Congrong Yizhi Capsule (FCYC) has also recently been shown to improve learning and memory [36] to a similar extent as dihydroergotoxine mesylate (Hydergine) [37, 38]. The safety and efficacy of FCYC in VD were evaluated in Phase II and III clinical trials in China in 2002. The production of FCYC was approved by the China State Food and Drug Administration in 2008.

Considering the key roles of p38 and ERK in chronic ischemia and hypotension, we hypothesized that TLXN ameliorates ischemic injury via the regulation of the p38 and ERK signaling pathways. To evaluate our hypothesis, we assessed the therapeutic effects of TLXN in a VD rat model induced by permanent occlusion of the bilateral common carotid arteries (2-VO) and explored the underling mechanism using real-time PCR and Western blot analysis.

Materials and methods

Ethics statement

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the Animal Ethical Committee of Chengdu University of Traditional Chinese Medicine (CDUTCM). All surgery was performed under sodium pentobarbital anesthesia. Damage induced by experiments was kept at a minimum.

TLXN extract preparation and quality analysis

In the present study, *Radix Scutellariae* (*S.baicalensis* Georgi), *Radix Angelicae Sinensis* and *Rhizoma Ligusticum* (*L.chuanxiong* Hort) were obtained from the Chengdu branch of Beijing Tongrentang Pharmaceutical Co. Ltd., China, and identified by Dr. Y.T. Ma, CDUTCM. Voucher specimens were deposited in the Science and Technology Building of the College of Pharmacy of CDUTCM. *Radix Scutellariae*, *Radix Angelicae Sinensis*, and *Rhizoma Ligusticum* were mixed at a weight ratio of 3:1:5. These components were soaked in 15-fold water for 30 min prior to boiling for 15 min and then filtered with gauze. Finally, all filtrations were freeze dried.

Samples were prepared from freeze-dried powder (100 mg) dissolved in water, which was mixed with acetonitrile at a volume ratio of 0.5:3. The samples were sonicated for 3 min followed by centrifugation at 13,000 rpm for 10 min to obtain the supernatant for a fingerprint test. A liquid chromatography (LC) assay was performed using ferulic acid, baicalin, baicalein and wogonin as standards for quality control analysis of the TLXN extract in each experiment. An LC assay was performed using an LC-30A (Shimadzu, Japan) liquid chromatography system, with aLabSolutions 5.41.20 chromatography workstation and an Agilent Poroshell 120 EC-C_{18} column (2.1×100 mm, 2.7 μm). The mobile phase consisted of acetonitrile

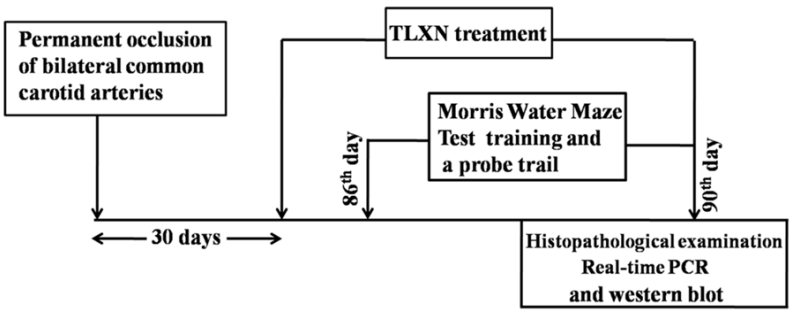

Figure 1. Experimental design. Sprague-Dawley rats were used to establish a vascular dementia (VD) model via the permanent occlusion of the bilateral common carotid arteries. Thirty days post-surgery, TLXN (7.56 g/kg, 3.78 g/kg, or 1.89 g/kg) was administered for 90 days. All rats were subjected to daily MWM tests from Day 86 of administration. Training was provided per day for five consecutive days. Rats were sacrificed on the 90th day, and brains were removed for histopathological examination, real-time PCR and Western blot analysis.

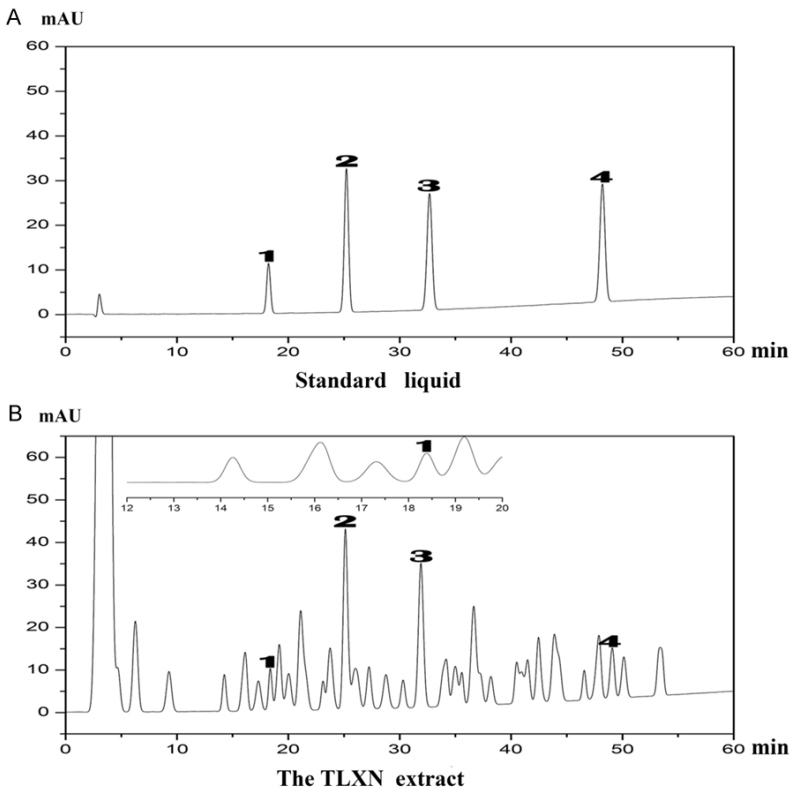

Figure 2. LC analysis of the TLXN extract. A. Standards: ferulic acid (18.5 min, peak (1)), baicalin (25.2 min, peak (2)), baicalein (33.4 min, peak (3)), and wogonin (48.9 min, peak (4)). B. The TLXN extract.

(1) and 0.01% phosphoric acid (2), and the following gradient elution was used: (1)/(2)=7/93 (0 min), (1)/(2)=22/78 (20 min), and (1)/(2)=70/30 (60 min). The flow rate was 0.5 mL/min, and the injection volume was 5 μL. Ferulic acid, baicalin, baicalein and wogonin were detected at a wavelength of 278 nm. The TLXN extract was analyzed in triplicate, and the ferulic acid content was determined to be approximately 1.92 mg/g; the contents of baicalin, baicalein, and wogonin were 45.27 mg/g, 19.37 mg/g, and 4.04 mg/g, respectively.

Animal model and drug administration

One hundred and fifty Sprague-Dawley rats (of either sex, 3-4 months old, 200±20 g) were purchased from the Experimental Animal Center of the Sichuan Academy of Medical Sciences (China, Certificate NO.SCXK Chuan 2008-24). The rats were housed under controlled temperature (22-24°C) and humidity (50%±5%) with access to food and water ad libitum and a regular 12-hour light-dark cycle for three days prior to experiments in the animal observation room in the College of Pharmacy of CDUTCM (Certificate NO. SYXK Chuan 2009-124). All conditions conform to the laboratory animal care guidelines issued by National Institutes of Health (NIH), USA.

One hundred twenty rats were used to establish the VD model as previously described [19, 39], and all of the procedures were conducted under sodium pentobarbital anaesthesia with minimal or transient pain after atropine sulfate (0.1 mg/kg) was used to prevent dyspnea by intramuscular injection.

During recovery from anesthesia, gentamicin was given intramuscularly for three days to avoid complications such as infection.

Additionally for rats which lose weight without water drinking or food or which were shivering buprenorphine (0.05 mg/kg) were used to alleviate pain or suffering from neck surgery by intraperitoneal injection, meanwhile it is wise to take warmth retention measures. One month following surgery, nearly 40% of the rats died due to serious damage caused by ischemia. Sixty VD rats were randomly divided into 6 groups: a model group, a Hydergine group (0.7 mg/kg, Huajin Pharmaceutical Co., Ltd., China), a FCYC group (3.6 g/kg, Liaoyuan Yulongyadong Pharmaceutical Co., Ltd.), and TLXN high-dose [TLXN(H)], medium-dose [TLXN(M)] and low-dose [TLXN(L)] groups (7.56 g/kg, 3.78 g/kg, and 1.89 g/kg, respectively, CDUTCM). Another ten rats in the sham group were randomly selected as the parallel control group. The volume of drug administration for each group was 10 ml/kg/day for 90 days (**Figure 1**). During drug administration, eight rats died in the treatment groups due to injury from intragastric administration or the stress induced by long-term oral feeding (n=7-10 rats in each group).

Morris water maze test

Spatial learning and memory performance were evaluated using the Morris water maze (MWM), as previously described [39]. All rats were subjected to daily MWM tests from Day 86 of administration. Four trails were provided per day for five consecutive days (**Figure 1**). Rats were gently placed in the water in a random quadrant facing the wall of the pool. The average escape latencies of each day were recorded by an automatic image acquisition and analysis system. Spatial memory was evaluated on the fifth day using a probe trail in which the platform was removed and times of entrance into the first quadrant, times of entrance into escape platform and times of entrance into valid region were recorded. All tests were performed at night.

Histopathological examination

After behavioral tests, the rats were made euthanasia via an excess of sodium pentobarbital by intravenous injection at the end of the trails on the 90th day. Brains were removed under sterile conditions, the left brains of which were fixed with 4% paraformaldehyde and embedded in paraffin. Coronal sections were cut and stained with hematoxylin and eosin (HE). Additionally, the hippocampus and cortex were isolated from the right brain and were stored at -80°C.

Real-time PCR

One hundred milligrams of hippocampal tissue was homogenized, and total RNAs were isolated using TRIzol® reagent (Invitrogen, USA). The cDNA was produced by reverse transcription using the PrimeScript™ RT Reagent Kit (Takara, Japan). PCR was performed using the SYBR® Premix Ex Taq™ Kit (Takara, Japan) in an ABI 7300 Real-Time PCR System (USA). The following primers were used for real-time PCR analysis: ERK1, 5'-CCAGAGTGGCTATCAAGAAG-3' (forward) and 5'-TCCATGAGGTCCTGAACAA-3' (reverse); ERK2, 5'-TGCCGTGGAACAGGTTGT-3' (forward) and 5'-TGGGCTCATCACTTGGGT-3' (reverse); c-jun, 5'-ATGGGCACATCACCACTACACC-3' (forward) and 5'-TGAAGTTGCTGAGGTTGGCGTA-3' (reverse); p38, 5'-AGCCAATTCCAGTGTTGGAC-3' (forward) and 5'-TTCTGGGCTCCAAATGATGC-3' (reverse); and β-actin, 5'-CTATCGGCAATGAGCGGTTCC-3' (forward) and 5'-GCACTGTGTTGGCATAGAGGTC-3' (reverse). The expression of the genes of ERK1, ERK2, p38, and c-jun was evaluated. β-Actin was used as the endogenous control to normalize the levels of the target genes. All of the products of the target assays and endogenous control assays were amplified to detect the expression of specific RNA sequences. Analysis was performed in triplicate.

Western blot analysis

Thirty milligrams of hippocampal tissue and cortex was isolated and homogenized in RIPA lysis buffer (Puli Lai Gene Technology Co., Ltd.) in an ice bath. The homogenate was centrifuged for 20 min at 12,000×g (4°C) to obtain the supernatant, of which the total protein was measured using a BCA assay kit (Puli Lai Gene Technology Co., Ltd.). Equal amounts of proteins per gel lane were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were blocked and incubated with primary antibodies (1:1000 dilution) against p38, phospho-p38, ERK1, ERK2, phospho-ERK1/2, MKK6, phospho-MKK6, c-jun, and phospho-c-jun (Cell Signaling Technology, USA) overnight at 4°C, followed by incubation with a secondary antibody (HRP-conjugated goat anti-rabbit IgG; Santa Cruz Biotechnology, CA, USA) at room temperature for 2 h. Immunolabeling was visualized using Enhanced Chemiluminescence Plus (GE

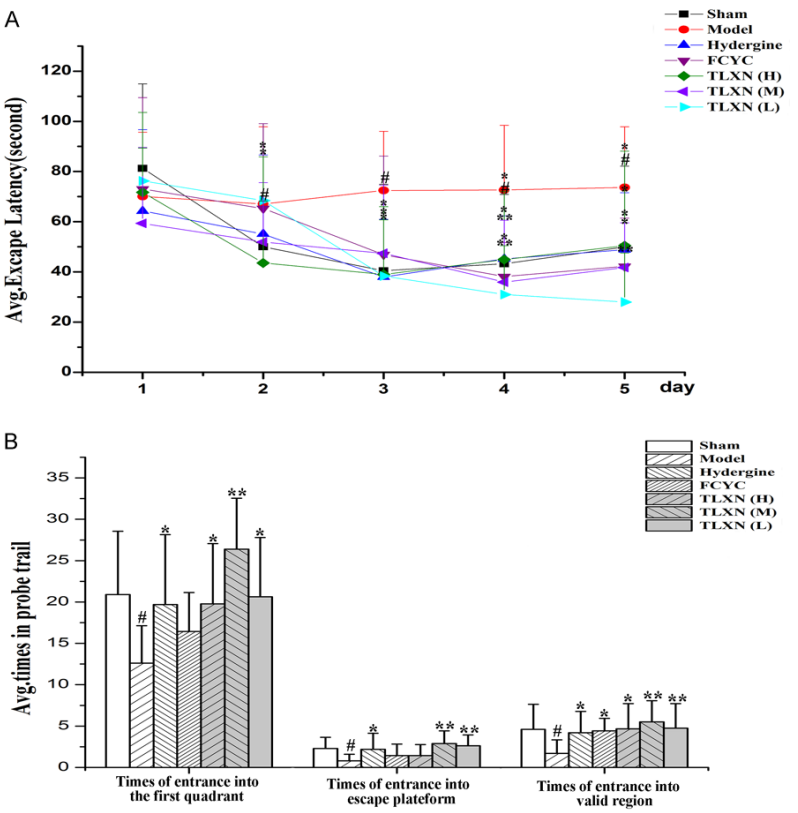

Figure 3. TLXN can alleviate spatial memory impairment induced by permanent ischemic insults. A. The escape latency for five days during training. Rats in the sham group exhibited reduced escape latency during the training cycle. The rats in the model group exhibited little improvement; however, TLXN administration clearly rescued learning and memory deficits induced by permanent ischemia. B. Times in the probe trail. Rats in the model group exhibited spatial memory deficits, whereas TLXN administration increased the times of entrance into a particular space or location (n=7-10 in each group; #P<0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

Healthcare, USA). The relative intensity of each protein band was measured using Image J software.

Statistical analysis

All experimental data underwent statistical analysis using SPSS17.0 software, and the values are expressed as the means ± SD. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) with Tukey's test for post hoc analysis. A level of P<0.05 was considered significant.

Results

LC analysis of the aqueous TLXN extract

The quality of TLXN was primarily evaluated using ferulic acid, baicalin, baicalein and wogonin, which have previously been isolated from *S. baicalensis* Georgi [40] *Radix Angelicae Sinensis* and *L.chuanxiong* Hort [41], as standards. As shown in **Figure 2**, four peaks were observed at the retention times of approximately 18.5 min, 25.2 min, 33.4 min, and 48.9 min, respectively.

TLXN improves spatial learning and memory in VD rats

VD is the second-most-common cause of dementia in the elderly, manifesting as spatial and memory deficits [2, 3]. The MWM test was used to assess the effect of TLXN on learning and memory in VD rats. As shown in **Figure 3A**, compared to the sham group, the escape latency in the four days following training was significantly prolonged in the model group (P<0.05 or P<0.01). Administration of three doses of TLXN, particularly TLXN (L), shortened the escape latency at different time points (P<0.05 or P<0.01). In probe trials, TLXN treatment resulted in an increase in the times of entrance into the first quadrant and valid region compared with the model group, and the times of entrance into the escape platform in the TLXN (M) and TLXN (L) groups significantly increased (P<0.05 or P<0.01, **Figure 3B**). Therefore, TLXN played a callback role, indicating that TLXN could alleviate spatial and memory deficits.

TLXN ameliorates histopathological damage in VD rats

Brains may exhibit histopathological damage following ischemia. To determine the extent of damage, coronal sections of brains were stained with routine HE and observed under a microscope. The standard of pathological grading is provided in **Table 1**. In the sham group,

Table 1. The pathological grading standard

Grade	Microscopic description
-	No lesion.
	It was graded as '-' when conformed to this description above, score of which was 0.
+	1. Focal edema of gray and white matter, cells dissolved;
	2. Focal atrophy of gray and white matter, hyperchromatic nuclei and cytoplasm;
	3. Focal slight cellular proliferation (mainly gliacyte);
	4. Slight perivascular edema;
	5. Slight vascular engorgement and hemorrhage;
	6. Slight inflammatory cell infiltration;
	7. Slight gray matter atrophy;
	8. Slight ependymal cells proliferation.
	It was graded as '+' when conformed to one of all above, score of which was 1.
++	1. Multifocal edema of gray and white matter, cells dissolved;
	2. Focal atrophy of gray and white matter, hyperchromatic nuclei and cytoplasm;
	3. Focal cellular proliferation (mainly gliacyte);
	4. Medium vascular edema and hemorrhage;
	5. Medium inflammatory cells infiltration;
	6. Medium perivascular edema.
	It was graded as '++' when conformed to one of all above, score of which was 2.
+++	1. diffuse edema of gray and white matter, cells dissolved;
	2. Multifocal atrophy of gray and white matter, hyperchromatic nuclei and cytoplasm;
	3. Focal mass cellular proliferation (mainly gliacyte);
	4. Severe vascular edema and hemorrhage;
	5. Severe inflammatory cell infiltration.
	It was graded as '+++' when conformed to one of all above, score of which was 3.

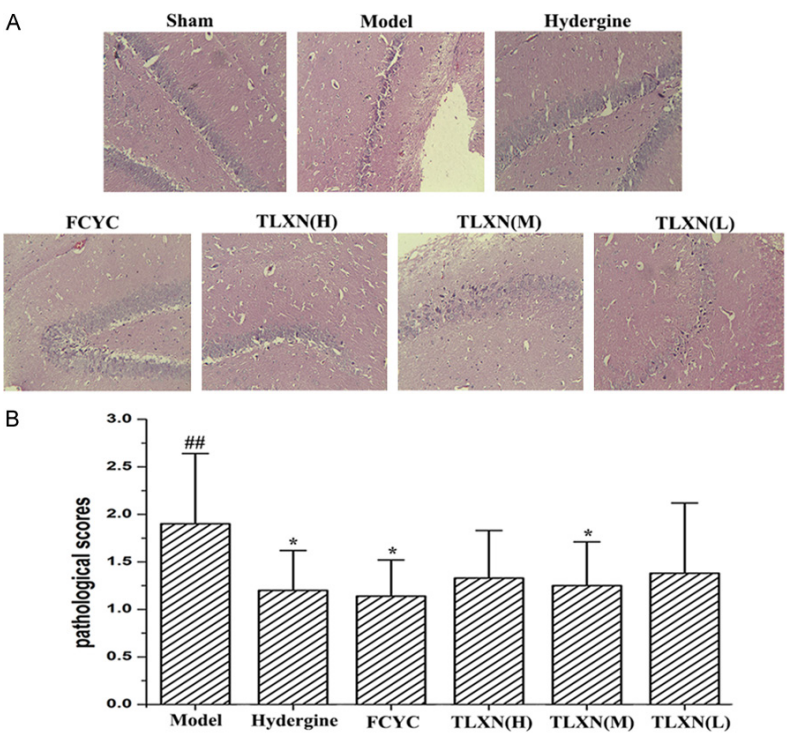

Figure 4. TLXN ameliorated the histopathological damage induced by chronic ischemia in VD rats. A. Pathological section. B. Pathological scores. The pathological scores in the model group were higher than those in the sham group; only TLXN (M) reduced these scores (n=7-10 in each group; #P< 0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

normal structures of gray and white matter and clear cellular gradations in the gray matter were observed. Moreover, pyramidal cells and granulosa cells in the hippocampal CA1 region were regularly arranged with normal sizes, numbers, shapes and distributions. The cells were evenly stained with cytoplasmic processes, blue and clear nuclei and pink plasma. No edema, necrosis, or fracture of nerve fibers was

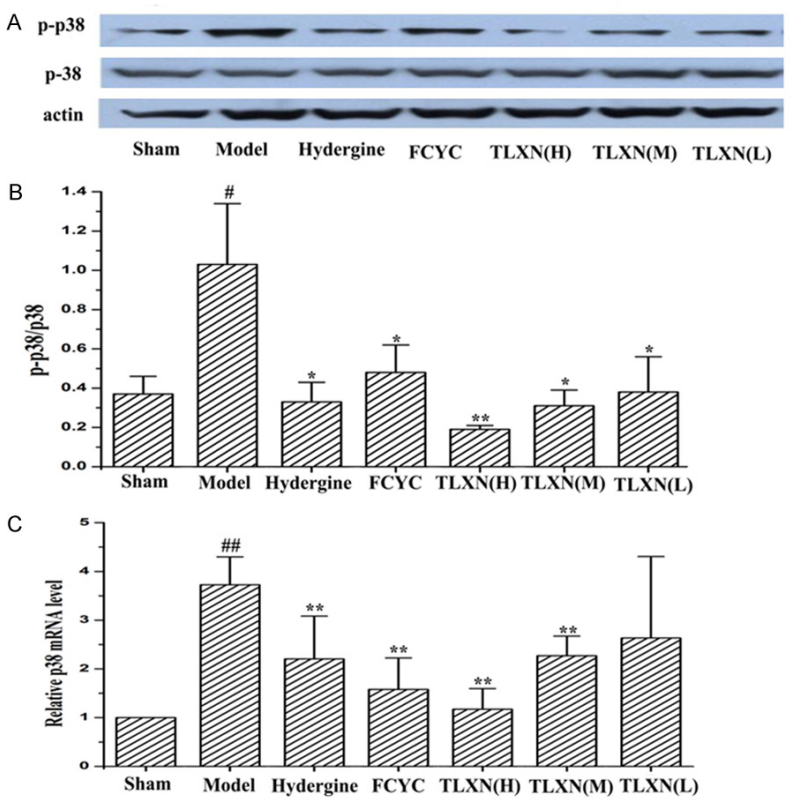

Figure 5. TLXN reduced p38 phosphorylation induced by chronic ischemia. A. Representative Western blot analysis of p-p38 (top), p38 (middle), and actin (bottom). B. The level of p-p38 increased following chronic ischemia, and TLXN reduced the level of p-p38 compared with that in the model group (n=3 in each group). C. The level of p38 mRNA increased following ischemia, whereas TLXN (H) and TLXN (M) reduced the expression of p38 mRNA (n=4-7 in each group; #P<0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

observed in the white matter. The interstitium was scattered with gliocytes. In the control group, cell layers were not clear in the gray matter and decreased in the hippocampus with a sparse and disordered distribution. Neurons were partially retrograded to hyacinth bodies, whose structure was dense, basophilous, trachychromatic and irregular. The degeneration of neurofibers may result in dense and erythrocyte-staining white matter. Visible degenerative lesions, including lipofuscin granules, neurofibrillary tangles and retrogression, occurred in neurons. The pathological damage in each treatment group was smaller than that in the control group. Brain pathological changes were significant in the model group compared with the sham group (P<0.01, **Figure 4A**). TLXN (M) ameliorated the histopathological damage (P<0.05). Although the TLXN (H) and TLXN (L) groups exhibited a trend toward an improvement in the pathological scores, this improvement was not significant (**Figure 4B**).

The effect of TLXN on the levels of p38 phosphorylation and mRNA in the hippocampus of VD rats

To determine whether p38 is phosphorylated following permanent ischemia, whether p38 mRNA levels correspond to p38 protein levels, and whether these levels are reduced following TLXN administration, the levels of phosphorylated p38 (p-p38) and p38 mRNA were determined using Western blot analysis and real-time PCR, respectively. As shown in **Figure 5A**, **5B**, the level of p-p38 clearly increased in the VD model group compared with that in the sham group (P<0.01). However, TLXN administration effectively inhibited p38 phosphorylation (P< 0.05 or P<0.01). This result is consistent with the p38 mRNA levels (**Figure 5C**).

The effect of TLXN on the level of MKK6 phosphorylation in the hippocampus of VD rats

Similarly, to determine whether MKK6 is phosphorylated following permanent ischemia and whether MKK6 phosphorylation is reduced following TLXN administration, the level of phosphorylated MKK6 (p-MKK6), as an upstream activator of p38 [42], was determined using Western blot analysis. As shown in **Figure 6**, the level of p-MKK6 was reduced following TLXN (M) and TLXN (H) treatment (P<0.01 or P<0.05).

The effect of TLXN on the levels of ERK phosphorylation and mRNA in the hippocampus of VD rats

To determine whether ERK (ERK1, ERK2) is phosphorylated following permanent ischemia, whether ERK mRNA levels correspond to ERK

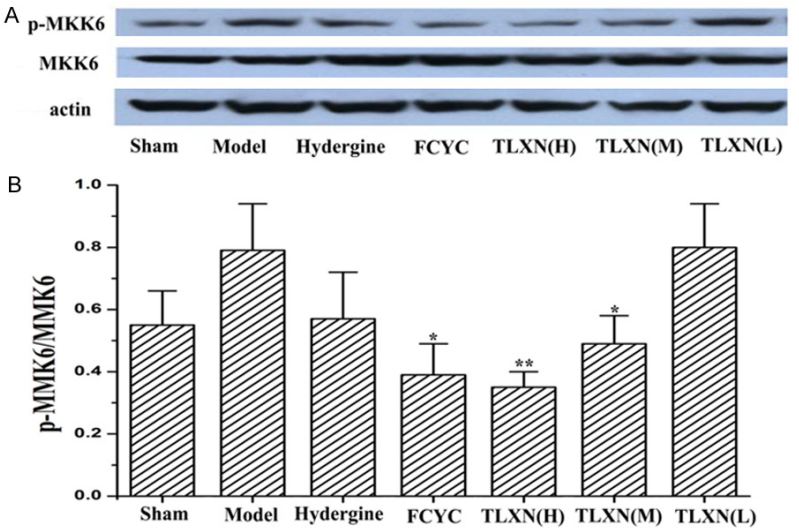

Figure 6. TLXN inhibited MKK6 phosphorylation induced by chronic ischemia. A. Representative Western blot analysis of p-MKK6 (top), MKK6 (middle), and actin (bottom). B. The level of p-MKK6 increased following chronic ischemia, whereas TLXN (H) and TLXN (M) reduced the level of p-MKK6 (n=3 in each group; P<0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

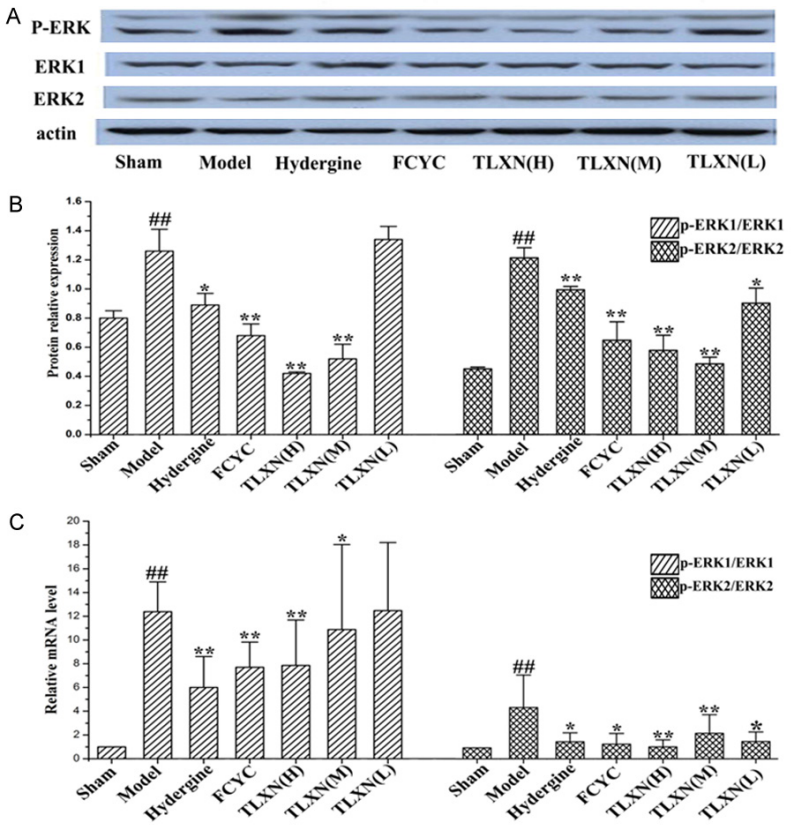

Figure 7. TLXN reduced ERK1/2 phosphorylation induced by chronic ischemia. A. Representative Western blot analysis of p-ERK (top), ERK1and ERK2 (middle), and actin (bottom). B. The level of p-ERK1/2 increased following chronic ischemia, and TLXN inhibited the phosphorylation of ERK1/2, particularly that of ERK2 (n=3 in each group). C. The level of ERK1/2 mRNA increased following ischemia, and TLXN reduced the expression of ERK1/2 mRNA, particularly that of ERK2 (n=4-7 in each group; #P<0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

protein levels, and whether these levels are reduced following TLXN administration, phosphorylated ERK (p-ERK1 and p-ERK2) and ERK mRNA levels were determined using Western blot analysis and real-time PCR, respectively. As shown in **Figure 7A**, **7B**, the levels of p-ERK1 and p-ERK2 were significantly higher in the VD model group than those in the sham group (P<0.01). However, ERK1/2 phosphorylation was reduced following TLXN treatment (P<0.05 or P<0.01). These results are consistent with the ERK1/2 mRNA levels (**Figure 7C**).

The effect of TLXN on the levels of c-jun phosphorylation and mRNA in the hippocampus of VD rats

To explore whether c-jun is phosphorylated following permanent ischemia, whether c-jun mRNA levels correspond to c-jun protein levels, and whether these levels are reduced following TLXN administration, the levels of phosphorylated c-jun (p-c-jun) and c-jun mRNA were determined using Western blot analysis and real-time PCR, respectively. As shown in **Figure 8A**, **8B**, the level of p-c-jun clearly increased in the VD model group compared with that in the sham group

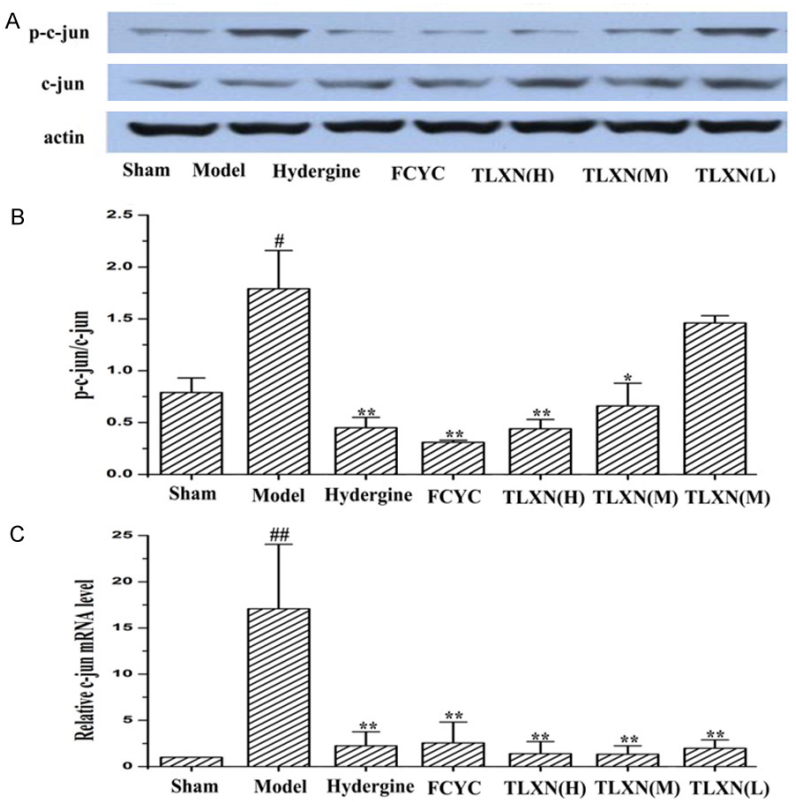

Figure 8. TLXN reduced c-jun phosphorylation induced by chronic ischemia. A. Representative Western blot analysis of p-c-jun (top), c-jun (middle), and actin (bottom). B. The level of p-c-jun increased following chronic ischemia; however, TLXN (H) and TLXN (M) inhibited the phosphorylation of c-jun compared with that in the model group (n=3 in each group). C. The level of c-jun mRNA increased following ischemia, and TLXN reduced the expression of c-jun mRNA (n=4-7 in each group; #P<0.05 or ##P<0.01 versus the sham group; *P<0.05 or **P<0.01 versus the model group).

(P<0.01). However, treatment with TLXN effectively reduced c-jun phosphorylation (P<0.05 or P<0.01). These results are consistent with the c-jun mRNA levels (**Figure 8C**).

Discussion

Chronic cerebral ischemia is a common pathological condition of the nervous system and is the primary cause of cognitive dysfunction in individuals with cardiovascular disease [5, 43]. Studies on VD in cells, organs or tissues have not been reported, so the animal model is the only way to the experimental study for VD. Meanwhile replacing higher animals with lower animals (rats) to establish an animal model comply with 3R principle of substitution in the animal experiments. Bilateral common carotid artery occlusion (2-VO) is a valid way to simulate the pathogenesis of humane VD perfectly; therefore, rats with 2-VO represent a "vascular cognitive impairment rat model" [44, 45]. In this study, 2-VO was used to establish a VD model in rats [35]. However, the underlying mechanisms of the neural insult mediated by 2-VO that result in VD are complex. Several reports have shown that MAPK signaling pathways can be activated and play a vital role in this neural insult [19-22].

We have previously shown that TLXN improves cholinergic nerve function and regulates tau protein phosphorylation [32] and synaptophysin remodeling [33] in a rat AD model. TLXN also promotes the expression of the Na^+-K^+-ATPase to maintain and balance the energy supply to certain important brain regions in rats with cognitive dysfunction induced by 2-VO [35]. However, the underlying signaling pathways have not been investigated. Several studies have shown that members of the MAPK signaling pathway are activated to varying extents by phosphorylation following transient or persistent ischemia and focal or global cerebral ischemia, demonstrating that cerebral ischemia can rapidly trigger phosphorylation and that MAPK signaling pathways are involved in the mechanism of cerebral ischemia [15-18]. In this study, we further demonstrated that TLXN ameliorates learning and memory impairment by targeting the MAPK signaling pathway, and members of this signaling pathway were assessed in VD rats.

Consistent with previous studies [35], our results revealed that TLXN protects the brain from chronic ischemic injury. Our results suggested that TLXN shortened the escape latency at different time points, increased the times of entrance into the first quadrant, valid region and the escape platform, and ameliorated the

histopathological damage in the hippocampus following intragastric administration at doses of 7.56 g/kg, 3.78 g/kg, and 1.89 g/kg TLXN. Thus, TLXN may represent a promising anti-dementia drug.

Studies have shown that the release of inflammatory factors, free radical generation and accumulation of excitatory amino acids [46] may occur in cerebral ischemic areas, which can activate the p38 MAPK signaling pathway and participate in the process that damages target cells. Additionally, p38 MAPK activation plays a negative regulatory role in LTP [47], leading to a decline in cognition and memory. Some reports suggest that p38 MAPK activity is increased in AD or VD [48, 49], and the levels of MKK6, an upstream activator, have also been found to be upregulated in a rat AD model [42]. Thus, drugs that inhibit the p38 MAPK signaling pathway can ameliorate learning and memory deficits [39]. According to these studies, p38 mRNA, p-p38, MKK6, and p-MKK6 levels decreased following TLXN administration, indicating that the effect of TLXN on VD rats may be partially due to the inhibition of the p38 MAPK signaling pathway.

Several reports have shown that activated ERK1/2 may play an important role in regulating the induction and maintenance of LTP and synaptic plasticity, which eventually influence learning and memory [50]. Although activated ERK1/2 is involved in the memory process, overactivated ERK1/2 or its nonspecific phosphorylation may be partially responsible for behavioral disorders due to an altered signaling pathway [51]. On the one hand, activated ERK1/2 may result in the release of excitatory amino acids [52] to influence cerebral memory damage. On the other hand, activated ERK1/2 may enhance the expression or synthesis of pro-inflammatory cytokines such as IL-1 during early cerebral ischemia [53], ultimately activating the p38 MAPK signaling pathway. Henriksson et al. demonstrated that the MEK1/2 inhibitor U0126 altered the response of endothelin receptors to alleviate cerebral ischemic injury [54]. Other studies have revealed a protective effect via the inhibition of ERK phosphorylation. Moreover, c-jun, as a downstream transcription factor, reaches peak activation [55] following ischemia, likely indicating overactivation of the ERK signaling pathway. Therefore, targeting the ERK signal pathway may alleviate the damage induced by ischemia. In this study, p-ERK1/2 and ERK1/2 mRNA levels increased in the VD model group compared with those in the sham group. However, the levels of p-ERK1/2 and ERK1/2 mRNA decreased following TLXN administration, indicating that the effect of TLXN on learning and memory may also be mediated via inhibition of the ERK signaling pathway to a certain extent.

As shown in LC analysis, ferulic acid, baicalin, baicalein and wogonin are major constituents of TLXN. Baicalin has been reported to exert a neuroprotective effect on cerebral ischemia [56]. Baicalin inhibits inflammatory factors such as TNF-α, IL-1β and IL-6 via the downregulation of p-ERK, p-p38 and p-MEK1/2 or the NF-κB signaling pathway [57]; baicalin also regulates the level of certain apoptotic proteins via the ERK/p38 MAPK pathway [58]. Baicalein, a free radical scavenging agent [59], ameliorates learning and memory impairment [60], suggesting a relationship between the therapeutic effect of baicalein on behavior performance and the p38 signaling pathway [61, 62]. Although wogonin and ferulic acid, which are additional constituents in TLXN, have not been demonstrated to ameliorate memory impairment, these compounds may balance calcium levels between the intracellular and extracellular environments [63] or inhibit inflammation [63] to ameliorate ischemic injury. Collectively, we suggest that ferulic acid, baicalin, baicalein and wogonin primarily contribute to the effects of TLXN on memory deficits via the regulation of the p38 and ERK signaling pathways, which further inhibit inflammation or apoptosis.

In summary, TLXN ameliorated the memory impairment induced by chronic ischemia, suggesting a mechanism that involves the regulation of the p38 and ERK MAPK signaling pathways. Thus, TLXN demonstrates the potential for development as a drug for vascular dementia.

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Disclosure of conflict of interest

None.

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