Aerobic exercise combined with huwentoxin-I mitigates chronic cerebral ischemia injury

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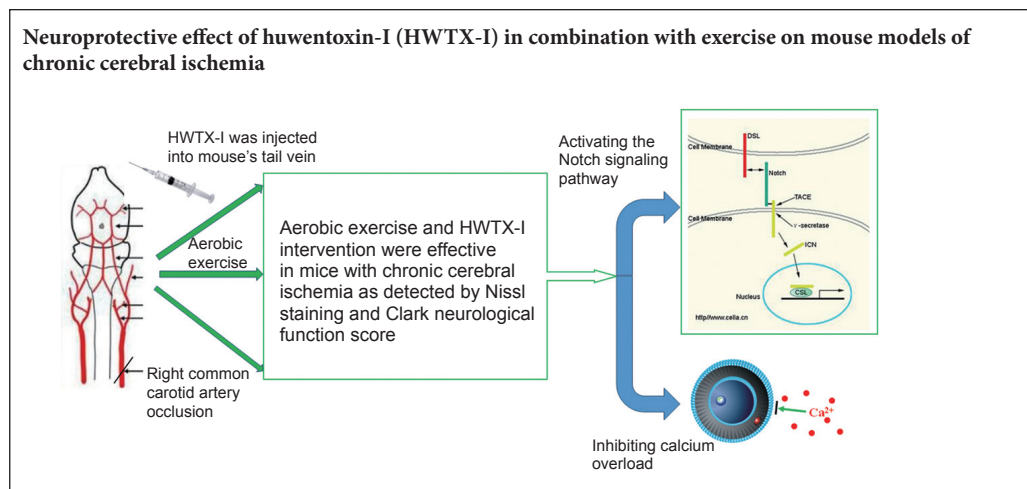
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Graphical Abstract

Neuroprotective effect of huwentoxin-I (HWTX-I) in combination with exercise on mouse models of chronic cerebral ischemia

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Abstract

Ca^{2+} channel blockers have been shown to protect neurons from ischemia, and aerobic exercise has significant protective effects on a variety of chronic diseases. The present study injected huwentoxin-I (HWTX-I), a spider peptide toxin that blocks Ca^{2+} channels, into the caudal vein of a chronic cerebral ischemia mouse model, once every 2 days, for a total of 15 injections. During this time, a subgroup of mice was subjected to treadmill exercise for 5 weeks. Results showed amelioration of cortical injury and improved neurological function in mice with chronic cerebral ischemia in the HWTX-I + aerobic exercise group. The combined effects of HWTX-I and exercise were superior to HWTX-I or aerobic exercise alone. HWTX-I effectively activated the Notch signal transduction pathway in brain tissue. Aerobic exercise up-regulated synaptophysin mRNA expression. These results demonstrated that aerobic exercise, in combination with HWTX-I, effectively relieved neuronal injury induced by chronic cerebral ischemia *via* the Notch signaling pathway and promoting synaptic regeneration.

Key Words: nerve regeneration; chronic cerebral ischemia; aerobic exercise; huwentoxin-I; Notch signaling pathway; calcium overload; neural regeneration

Introduction

Chronic cerebral ischemia, a sustained modest reduction in cerebral blood flow, is associated with neuronal damage and cognitive decline (Cechetti et al., 2012). Previous studies have focused on the mechanisms involved in chronic cerebral ischemia (Antipenko et al., 2016; Edrissi et al., 2016). In recent years, the ischemic mechanism of calcium overload in brain injury has become a hot research topic (Kostic et al., 2015; Zhang et al., 2016; Skovsted et al., 2017; Wang et al., 2017a). The Notch signaling pathway plays an important role in the

regulation of cell differentiation, proliferation, and apoptosis, as well as a series of physiological and pathological processes (Huang et al., 2016; Demitrack et al., 2017; Li et al., 2017; You et al., 2017). Previous studies have shown that the Notch signaling pathway affects neuronal regeneration in cerebral ischemia animal models (Wang et al., 2009; Liu et al., 2011; Tao et al., 2014). Intracellular calcium overload is a main factor in apoptosis with cerebral ischemic injury (Li et al., 2015). Calcium channel blockers also provide protective effects on ischemic brain injury in animal models (Maniskas et al., 2016).

The polypeptide extracted from Conotoxin, ω-CTX-MVI-IA, selectively blocks the N-type voltage-gated calcium channel current, delays ischemic brain damage, and provides neuroprotective effects (Valentino et al., 1993; Yenari et al., 1996; Perez-Pinzon et al., 1997; Colbourne et al., 1999). The three-dimensional structure of HWTX-I (a spider peptide toxin) functions as an inhibitor of the cysteine knot motif, similar to ω-CTX-MVIIA, and HWTX-I is a selective N-type calcium channel blocker (Chen et al., 2005; Wang et al., 2007). Exercise plays a unique role in preventing disease. Our early studies showed that moderate-intensity aerobic exercise as a functional rehabilitation therapy provides significant protection in a variety of animal models of chronic diseases (Wen Tao et al., 2011; Lin et al., 2012; Sheng et al., 2015).

In the present study, the chronic cerebral ischemia model was established in mice to investigate the effects of aerobic exercise and HWTX-I on chronic cerebral ischemia.

Materials and Methods

Animals
Forty healthy, male, Kunming mice, 5–7 weeks of age and weighing 28–32 g, were provided by Hunan Slack Jingda Experimental Animals Co., Ltd., China (license No. SCXK (Xiang) 2011-0003). The study protocol was approved by the Hunan Normal University Medical Ethics Committee (Approval number: 2015(17)). The experimental design followed the national guidelines for the Care and Use of Laboratory Animals, and "Consensus author guidelines on animal ethics and welfare" by the International Association for Veterinary Editors. The article was prepared in accordance with the "Animal Research: Reporting of In Vivo Experiments Guidelines."

Establishment of chronic cerebral ischemia models
The mice were placed in a supine position and a 1.5-cm long skin incision was made in the upper part of the neck. The subcutaneous fat, fascia, and muscle were separated to the trachea. The right common carotid artery was separated and permanently ligated using 3-0 surgical line. The color of the right eye changed from bright-red to grayish-white following ligation of the right common carotid artery. After regaining consciousness, the right eye remained closed and became dark red, although it was determined that a low-flow blood supply was obtained *via* the Willis circle after right brain ischemia (**Figure 1**). These symptoms suggested successful establishment of the chronic cerebral ischemia model (Yoshizaki et al., 2008; Zhao et al., 2014).

Three mice died following model establishment and were immediately replaced. The mice were equally and randomly divided into four groups at 3 days after model establishment: chronic cerebral ischemia group (model group), HWTX-I group, aerobic exercise group, HWTX-I + aerobic exercise group.

Drug administration
HWTX-I (200 μg/ampoule, batch number: 010620, purity: 99.5%; license number: 260085434; Sinobioway Biomedicine Co., Ltd., Xiamen, Fujian Province, China) was provided by Institute of Protein Chemistry and Molecular Biology Laboratory of Hunan Normal University, China. Four days after group assignment, mice in the HWTX-I and HWTX-I + aerobic exercise groups were administered 0.15 mL toxin solution to the tail vein at a dose of 0.05 μg/g. Mice in the model and aerobic exercise groups were administered the same volume of physiological saline, once every 2 days, for a total of 15 times over 30 days.

Exercise training
Following model establishment, mice in the aerobic exercise and HWTX-I + aerobic exercise groups were subjected to treadmill exercise by gradually increasing the running speed and time (**Figure 2**) (Sheng et al., 2015) for 5 weeks as follows: a) on days 1–3 of week 1, the speed was 7 m/min for 30 minutes, with a slope of 0 degrees; b) on days 4–6 of week 1, the training time was increased by 10 minutes, and the running speed increased by 1 m/min for each training; c) on day 6, the training time was 60 minutes, and the running speed was 10 m/min; d) on day 7, the mice rested; 3) weeks 2–5, the speed was 10 m/min for 60 minutes, with a slope of 0 degrees, six times a week until the end of week 5.

Neurological function assessment
A 28-point neurological deficit score first developed by Clark et al. (1997) was used to assess neurological functions in the mice, which included focal and general functional impairment scores. Each mouse group was scored according to the Clark neurological function scale at immediately after group assignment and at the fifth weekend. A higher score represented greater damage.

Tissue extraction
The mice in each group were anesthetized on the day after exercise. Blood was drawn from the eyeballs and placed into anticoagulant tubes. Total RNA was extracted within 2 hours. The mice were placed on ice and the entire brain was removed. Brain tissue (30 mg) from right anterior fontanel was preserved in 1 mL of Trizol at −70°C. The coronal plane, approximately 4-mm thick from the rear of the right brain, was fixed in 4% paraformaldehyde phosphate buffer (0.1 M, pH 7.4) for Nissl staining.

Nissl staining
Tissues were embedded in paraffin wax. Tissue sections approximately 5 μm thick were prepared using a rotary microtome (Jinhua YIDI Medical Appliance Co., Ltd., Jinhua, Zhejiang Province, China). The sections were then subjected to Nissl stained using toluidine blue (ShangHai EKEAR Bio@ Tech Co., Ltd., Shanghai, China). The staining was observed and photographed using a light microscope (Olympus, Tokyo, Japan).

Real-time polymerase chain reaction (RT-PCR)
Brain tissue and blood total RNA was extracted using Trizol reagent (TakaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China) and the Magnetic Total RNA Kit

Figure 1 Establishment of chronic cerebral ischemia models.
(A, B) Isolating the right common carotid artery. (C) Ligating the right common carotid artery.

Table 1 Primers used for real time-polymerase chain reaction

Primer	Sequence (5'–3')	Annealing temperature (°C)
Notch1	Forward: GAT GGC CTC AAT GGG TAC AAG	60.0
	Reverse: TCG TTG TTG TTG ATG TCA CAG T	
Jagged1	Forward: AAT CGC ATC GTA CTG CCT TTC	58.0
	Reverse: GTG TCA TTA CTG GAA TCC CAG G	
NCX1	Forward: ATG CTT CGA TTA AGT CTC CCA CC	60.2
	Reverse: AAT GGG CAA GAT CAC CCC TTT	
SYP	Forward: AGA CAT GGA CGT GGT GAA TCA	58.0
	Reverse: ACT CTC CGT CTT GTT GGC AC	
CaBP-D28k	Forward: GGC TTC ATT TCG ACG CTG AC	59.8
	Reverse: ACG TGA GCC AAC TCT ACA ATT C	
GAPDH	Forward: GTT TCC TCG TCC CGT AGA CA	59.8
	Reverse: AAT CTC CAC TTT GCC ACT GC	

SYP: Synaptophysin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 2 Aerobic exercise.
The mice were subjected to running on a treadmill.

(code No. E31004; Shanghai GenePharma Co., Ltd., Shanghai, China). The absorbance value of the extracted total RNA was determined using ultraviolet spectrophotometry, with an A_{260}/A_{280} ratio > 1.7. PCR primers were designed using mouse cDNA sequences from Gene Bank and Primer 5.0 software, and were synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China (**Table 1**).

Genomic DNA was removed as follows: 2.0 μL 5 × g DNA Eraser Buffer was mixed with 1.0 μL gDNA Eraser and 7.0 μL total RNA. The samples were incubated at 42°C for 2 minutes, and the reaction was terminated at 4°C. Reverse transcription: 10.0-μL reaction solution contained 1.0 μL PrimeScript RT Enzyme Mix I, 1.0 μL RT Primer Mix, and 4.0 μL 5× PrimeScript Buffer 2. Rnase-free dH$_2$O was added to a final volume of 20 μL. The samples were incubated at 37°C for 15 minutes and 85°C for 5 seconds to obtain first-strand cDNA. The reagents used in the reverse-transcription reaction were PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology (Dalian) Co., Ltd).

A SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa Biotechnology (Dalian) Co., Ltd.) was used for RT-PCR. The total reaction volume was 20 μL and contained the following: 2 μL cDNA template, 10 μL SYBR® Premix Ex Taq II (Tli RNaseH Plus) (2×), 0.8 μL forward primer, 0.8 μL reverse primer, 0.4 μL ROX-Reference Dye II (50×),

6 μL nuclease-free water. Reactions were prepared on ice. PCR was performed using an ABI 7500 Fast Real-time PCR System (Foster, CA, USA) at 95°C for 30 seconds, 95°C for 3 seconds, and 60°C for 30 seconds for 40 cycles. Data were processed using an ABI 7500 Fast.

Statistical analysis
All data, expressed as the mean ± SD, were analyzed with SPSS 22.0 software (IBM, Armonk, NY, USA). The sample means were compared using one-way analysis of variance. $P < 0.05$ represented a significant difference.

Results
Effects of aerobic exercise and HWTX-I on neurological function in chronic cerebral ischemia mice
As shown in **Figure 3**, there was no significant difference in neurological deficits between the groups prior to any interventions. At the fifth weekend, compared with the model group, focal and general neurological deficit scores significantly decreased in the intervention groups ($P < 0.01$). Compared with the HWTX-I group, general neurological deficit scores significantly decreased in the HWTX-I + aerobic exercise group ($P < 0.05$).

Effects of aerobic exercise and HWTX-I on morphological changes in the cerebral cortex of chronic cerebral ischemia mice
Ischemia results in cellular morphological changes (Kitabatake et al., 2015; Arteaga et al., 2016; Zhang et al., 2017). To determine the neuroprotective effects of aerobic exercise and HWTX-1 on these morphological changes, we analyzed Nissl staining in the cerebral cortex. In the model group,
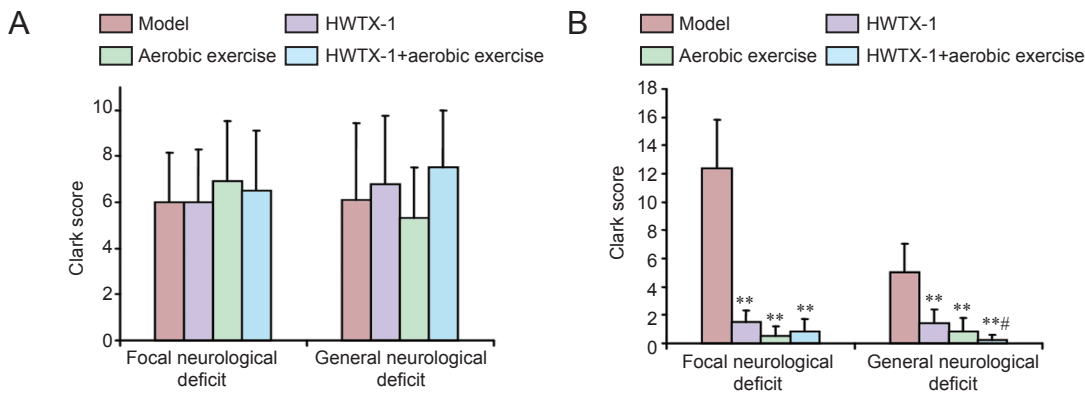
Figure 3 Effects of aerobic exercise and HWTX-I on neurological function in mice with chronic cerebral ischemia.
Neurological function was assessed using a 28-point neurological deficit score (Clark Neurological function score). A higher score represents more severe neurological deficits. (A) Immediately after group assignment; (B) the fifth weekend. Data are expressed as mean ± SD (n = 10 per group). **$P < 0.01$, *vs.* model group; #$P < 0.05$, *vs.* HWTX-I group (one-way analysis of variance). HWTX-I: Huwentoxin-I.

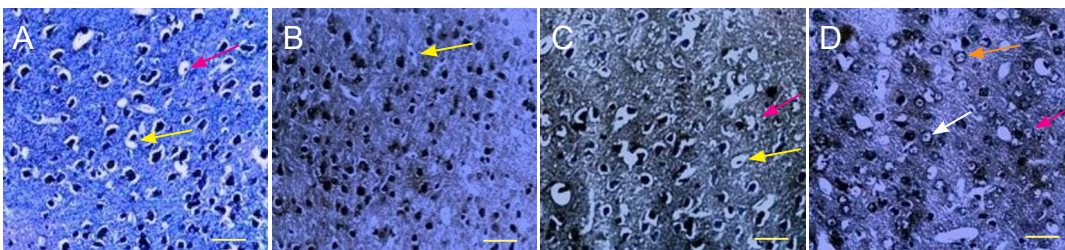**Figure 4 Effects of aerobic exercise and HWTX-I on morphological changes in the cerebral cortex of chronic cerebral ischemia mice (Nissl staining, × 200).**
(A) Model group; (B) HWTX-I group; (C) aerobic exercise group; (D) HWTX-I + aerobic exercise group. Red arrows show vacuoles; yellow arrows show pyknosis; white arrow shows nucleolus; orange arrow shows Nissl body at the periphery. Scale bars: 100 μm. HWTX-I: Huwentoxin-I .
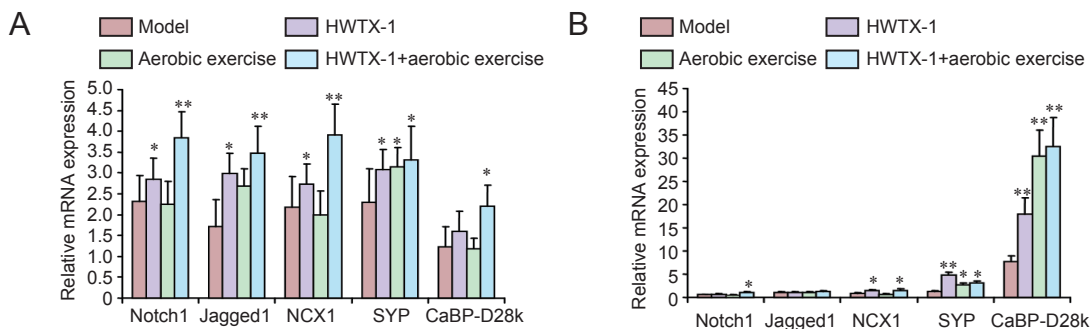

Figure 5 Effects of aerobic exercise and HWTX-I on Notch1, Jagged1, NCX1, SYP, and CaBP-D28k mRNA expressions in the brain (A) and blood (B) of chronic cerebral ischemia mice (real-time polymerase chain reaction).
Data are expressed as the mean ± SD (n = 10 per group). *$P < 0.05$, **$P < 0.01$, *vs.* model group (one-way analysis of variance). HWTX-I: Huwentoxin-I.

neurons in the cerebral cortex were deeply stained, with the presence of pyknosis, obvious vacuoles, nuclear necrosis, and distinct neuronal loss. In the HWTX-I group, vacuoles were not obvious, part of the nuclei and Nissl bodies were visible in the cerebral cortex, but the neurons were darkly stained and exhibited pyknosis. In the aerobic exercise group, the cerebral cortex exhibited a distinct loss of neurons and vacuoles; the neurons were darkly stained, and some neurons exhibited pyknosis, although morphology was better than in the model group. In the HWTX-I + aerobic exercise group, there were less vacuoles in the cortical neurons; the nuclei were lightly stained and exhibited distinct nucleoli; although

pyknosis was still detectable, there was less neuronal loss; the Nissl bodies were clear and gathered to the periphery in the cytoplasm (**Figure 4**).

Effects of aerobic exercise and HWTX-I on Notch1, Jagged1, NCX1, synaptophysin (SYP), and CaBP-D28k mRNA expression in the blood and brains of chronic cerebral ischemia mice
As displayed in **Figure 5A**, mRNA expressions of Notch1, Jagged1, NCX1, SYP, and CaBP-D28k were highest in the HWTX-I + aerobic exercise group compared with the model, HWTX-1, and aerobic exercise groups. Compared with

the model group, mRNA expressions of Notch1, Jagged1, NCX1, and SYP were significantly decreased in brain tissue of mice in the HWTX-I group ($P < 0.05$). Compared with the model group, SYP mRNA expression was significantly increased in brain tissue of mice in the aerobic exercise group ($P < 0.05$). Compared with the model group, mRNA expressions of Notch1, Jagged1, NCX1, SYP, and CaBP-D28k were significantly increased in brain tissues of mice in the HWTX-I + aerobic exercise group ($P < 0.01$ or $P < 0.05$).

As shown in **Figure 5B**, mRNA expressions of Notch1, Jagged1, NCX1, and CaBP-D28k in the HWTX-I + aerobic exercise group, and SYP in the HWTX-I group, were highest. Compared with the model group, mRNA expressions of NCX1, SYP, and CaBP-D28k were significantly increased in the blood of mice in the HWTX-I group ($P < 0.01$ or $P < 0.05$). Compared with the model group, mRNA expressions of CaBP-D28k and SYP were significantly increased in the aerobic exercise group ($P < 0.01$ or $P < 0.05$). Compared with the model group, mRNA expressions of Notch1, NCX1, SYP, and CaBP-D28k were significantly increased in the HWTX-I + aerobic exercise group ($P < 0.01$ or $P < 0.05$).

Discussion

Shamsaei et al. (2015) confirmed a sparse distribution of hippocampal CA1 neurons in rats with cerebral ischemia, with a significantly decreased number of normal cells. Naderi et al. (2017) found that minocycline pretreatment significantly attenuated ischemia-induced pyramidal cell death and microglial activation in the CA1 region, and provided neuroprotective effects on cerebral ischemia-induced memory deficits. Shamsaei et al. (2015) used Nissl staining to show that pre-ischemic exercise significantly reduced necrotic cell death in the hippocampal CA1 region and prevented memory deficits after cerebral ischemia. Wang et al. (2017b) described a series of 11 studies that showed the significant effects of astragaloside IV on ameliorating neurological function scores following ischemic stroke. These studies suggested that different interventions exert neuroprotective effects following cerebral ischemia/reperfusion injury through antioxidant, anti-inflammatory, or anti-apoptotic properties.

The Notch signaling pathway is a conserved and important signal transduction pathway that affects cell fate; it is involved in the proliferation and differentiation of all cells and plays an important role in regulating cell differentiation, proliferation, and apoptosis, as well as a series of physiological and pathological processes (Geng et al., 2015; Ha et al., 2016). The Notch1 receptor is one of four homologous Notch genes; Jagged1 ligand is one of five ligands for the Notch receptor; and the Notch receptor binds to the ligand and transduces signals into the cell *via* the intracellular domain NICD of the Notch receptor. Zhang et al. (2014) showed that increased Notch1, NICD, and Hes-1 (the target gene of Notch signaling pathway) expressions following isoflurane preconditioning in a mouse transient global cerebral ischemia-reperfusion model were due to activation of the Notch signal transduction pathway. Similar results were obtained by Zhao et al. (2015) using electroacupuncture pretreatment

against ischemic injury. Sun et al. (2013) suggested that Notch1 and Jagged1 signaling modulate subventricular zone neurogenesis in the aged brain under normal and ischemic conditions. Together these studies showed that the Notch signaling pathway is involved in recovery following ischemic brain injury. Results from the present study suggested that HWTX-I activated the Notch signaling pathway, delayed neuronal damage, and promoted cell regeneration.

SYP is a 38-kDa integral membrane protein closely related to synaptic structure and function; it has been identified in almost all synaptic terminals of the central and peripheral nervous systems. Pinheiro et al. (2014) showed that SYP protein expression was strongly associated with synaptic formation and function, and SYP protein expression decreased after ischemia. Hou et al. (2011) suggested that willed movement therapy promotes recovery of neurological functions in rats with cerebral ischemia, which could be related to increased SYP expression. He et al. (2017) showed that bone marrow stromal cell treatment significantly improved neurobehavioral performance following ischemic brain injury by SYP and growth-associated protein 43 expression. Fonteles et al. (2016) showed that increased synaptogenic activity and anti-inflammatory action exert protective effects on memory in a permanent middle cerebral artery occlusion mouse model. Results from the present study suggested that the aerobic exercise-induced increase in SYP mRNA expression following cerebral ischemic injury is responsible for recovery of neurological function.

The calcium-binding protein CaBP-D28k is expressed in neurons of most brain regions, and CaBP-D28k-positive neurons have a direct relationship with ischemic brain injury (Yenari et al., 2001). Ca^{2+} overload plays a role in ischemia-reperfusion injury in neurons. Neuronal injury can be delayed or avoided using voltage-gated calcium channel inhibitors to prevent Ca^{2+} influx. CaBP-D28k selectively binds with Ca^{2+} and down-regulates intracellular-free calcium levels. Ouh et al. (2013) determined that ischemic damage reduces expression of calcium-binding proteins, thereby leading to cell death. Results from Lee et al. (2013) showed that longer maintenance of calcium-binding proteins may contribute to less and more delayed neuronal death/damage in young animals. Yenari et al. (2001) concluded that Calbindin D28K overexpression leads to neuroprotection in an animal model of central nervous system injury. Sodium-calcium exchangers (NCX) are ion transporters that are widely expressed in animal cell membranes. Ca^{2+} concentration stability in cells is maintained by pumping Ca^{2+} out from the cytoplasm using reverse transport. It is believed that NCX1 plays a protective role in neuronal injury (Boscia et al., 2012; Vinciguerra et al., 2014; Secondo et al., 2015). NCX1 and the calcium-binding protein Calretinin cooperate within the striatum to confer tolerance against cerebral ischemia (Boscia et al., 2016). Results from the Formisano et al. (2013) study demonstrate that the RE1-silencing transcription factor may represent a potential drug target for treating brain ischemia by regulating NCX1 expression. This resulted in delayed or reduced neuronal damage, and

the effect was increased through aerobic exercise. Although the Ca^{2+} overload took place during the time window prior to intervention, HWTX-I increased NCX1 expression and promoted Ca^{2+} outflow, thereby reducing the Ca^{2+} concentration and delaying neuronal damage. Circulating nucleic acid includes circulating DNA and RNA. Recently, the detection of nucleic acid molecules in peripheral blood circulation has become a hot research topic (Guo et al., 2017; Huang et al., 2017). Although circulating nucleic acid can serve as a sensitive and effective tumor biomarker (Roth et al., 2011; Perkins et al., 2012; Greenberg et al., 2014), very little is known about the application of circulating RNA in cerebral ischemic injury. Our results showed that Notch1 and NCX1 mRNA expression in peripheral blood coincided with a low degree of injury, suggesting that these molecules could serve as peripheral blood markers for cerebral ischemia detection.

Results from this study suggested that HWTX-I and moderate-intensity treadmill exercise for 5 weeks alone and used as a combined intervention in a mouse model of chronic cerebral ischemia effectively reduced brain tissue damage. Aerobic exercise after cerebral ischemia resulted in increased SYP mRNA expression, suggesting a role for SYP in the recovery of neurological function. Although both HWTX-1 and exercise reduced injury, the mechanisms of action were different. HWTX-I combined with aerobic exercise was superior to HWTX-I or aerobic exercise alone.

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