NEURAL REGENERATION RESEARCH
March 2016, Volume 11, Issue 3
www.nrronline.org

RESEARCH ARTICLE

Radix *Ilicis Pubescentis* total flavonoids ameliorates neuronal damage and reduces lesion extent in a mouse model of transient ischemic attack

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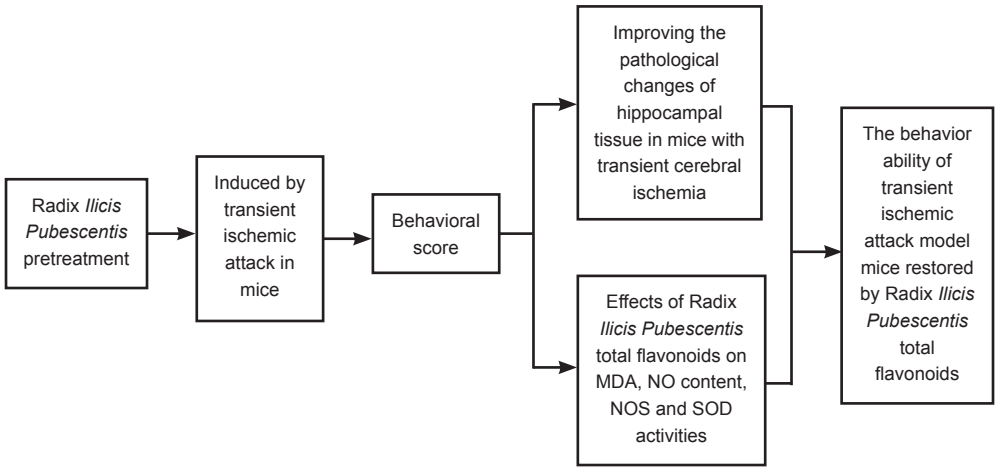
How to cite this article: *Miao MS, Guo L, Li RQ, Zhang XL (2016) Radix Ilicis Pubescentis total flavonoids ameliorates neuronal damage and reduces lesion extent in a mouse model of transient ischemic attack. Neural Regen Res 11(3):441-446.*

Funding: *This study was supported by the State "Major New Drug Creation" Major Science and Technology Project of China, No. 2009ZX09103-324; a grant from the Henan Provincial Science and Technology Innovation Team in University in China, No. 2012IRT-STHN011.*

Graphical Abstract

Radix *Ilicis Pubescentis* total flavonoids alleviate the damage to brain tissue caused by transient ischemic attack



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doi: *10.4103/1673-5374.179056*
http:*//www.nrronline.org/*

Accepted: *2015-10-20*

Abstract

Flavonoids are a major component in the traditional Chinese medicine Radix *Ilicis Pubescentis*. Previous studies have shown that the administration of Radix *Ilicis Pubescentis* total flavonoids is protective in cerebral ischemia. However, to our knowledge, no studies have examined whether the total flavonoids extracted from Radix *Ilicis Pubescentis* prevent or ameliorate neuronal damage following transient ischemic attacks. Therefore, Radix *Ilicis Pubescentis* total flavonoids question and the potential underlying mechanisms. Thus, beginning 3 days before the induction of a mouse model of transient ischemic attack using tert-butyl hydroperoxide injections, mice were intragastrically administered 0.3, 0.15, or 0.075 g/kg of Radix *Ilicis Pubescentis* total flavonoids daily for 10 days. The results of spectrophotometric analyses demonstrated that Radix *Ilicis Pubescentis* total flavonoids enhanced oxygen free radical scavenging and reduced pathological alterations in the brain. Hematoxylin-eosin staining results showed that Radix *Ilicis Pubescentis* total flavonoids reduced hippocampal neuronal damage and cerebral vascular injury in this mouse model of transient ischemic attack. These results suggest that the antioxidant effects of Radix *Ilicis Pubescentis* total flavonoids alleviate the damage to brain tissue caused by transient ischemic attack.

Key Words: *nerve regeneration; transient ischemic attack; Radix Ilicis Pubescentis total flavonoids; tert-butyl hydroperoxide; malondialdehyde; nitric oxide; nitric oxide synthetase; superoxide dismutase; neural regeneration*

Introduction

A transient ischemic attack (TIA) is a transient episode of neurological dysfunction with limited symptoms and signs caused by a temporary local cerebral blood flow block or interruption that may occur because of cerebral vascular lesions (Bai et al., 2012). Focal, transient and recurrent attacks are the most important clinical features (Li, 2012). TIA is not an independent disease, but rather a syndrome involving complex changes that are a special type of acute cerebral vascular disease. TIA may be an important signal, a

warning of impending stroke (Kernan et al., 2014). Lovett et al. (2003) and Shah et al. (2008) reported that within 48 hours after a TIA, the incidence of ischemic stroke was 5%. Sacco (2004) found that 50% of ischemic stroke patients had a history of TIA. Keith et al. (1987) showed that within 5 days after a TIA, the incidence of ischemic stroke was 5%. Du et al. (2011) demonstrated that within 90 days after a TIA, the incidence of ischemic stroke was 17%, and the risk of stroke was highest within 1 week of a TIA. The incidence of ischemic stroke within 5 years of a TIA can be as high as 24–29% (Liu et al., 2014). Therefore, once a TIA appears, risk of ischemic stroke increases. Timely treatment of TIA is important in prevention of ischemic stroke. However, the pathogenesis of TIA is unknown. A previous study showed that TIA is strongly associated with atherosclerosis (Liu et al., 2014). Therefore, treatments following a TIA commonly include anti-platelet aggregation, thrombolysis and anticoagulation drugs (Wang and Zhou, 2015). However, the use of existing drugs for treatment of TIA, such as statins (Han et al., 2015), is limited by their high cost and long-term administration requirements (Ma and Miao, 2011). Therefore, new efficacious drugs with low toxicity are urgently needed for treating TIA, preventing stroke, and advancing research.

Traditional Chinese medicine has a long history of using low-cost medicinals that show few adverse effects for treating cerebral vascular disease. Traditional Chinese medicine suggests that Radix *Ilicis Pubescentis* clears heat-evil, expels superficial evils, and promotes blood circulation (Huang et al., 2014), and the effective components in Radix *Ilicis Pubescentis* are the total flavonoids (RIPTFs) (Zhang et al., 2012a). Modern pharmacological studies indicate that RIPTFs protect against blood stasis combined with cerebral ischemia and may improve blood circulation, enhance energy metabolism, reduce free radicals, and ameliorate neuronal injury (Zhang et al., 2012b). However, to our knowledge, no studies have examined whether RIPTFs prevent or ameliorate neuronal damage following TIAs. The present study explored the answer to this question and potential underlying mechanisms. *Yangxueqingnao* granules, which are used to treat certain cerebral ischemic diseases, were used as a positive control drug (Xu et al., 2009; Xiong et al., 2011; Lou et al., 2014). Thus, we investigated the effects of RIPTFs on the behavior, pathological changes, and redox factors that may be altered in the brains of mouse models of TIA.

Materials and Methods

Experimental animals

A total of 84 (42 males and 42 females) healthy, clean, Kunming mice weighing 30–32 g and aged 5–8 weeks were provided by the Hebei Provincial Experimental Animal Center of China (license No. SCXK (Ji) 2003-1-003). The mice were housed at 25 ± 3°C with 55 ± 10% humidity, and were allowed free access to food and water. Males and females were housed in separate cages. The mice were equally and randomly divided into an untreated control group and five groups that were exposed to conditions inducing a mouse model of TIA following administration of vehicle, *Yangxueqingnao* granules (positive control), or high-, moderate-, or low-doses of RIPTFs. The Animal Ethics Committee of Henan University of Traditional Chinese Medicine in China approved the experiment.

Drug preconditioning

The Radix *Ilicis Pubescentis* extract was provided by the Laboratory of Analytical Chemistry, Henan University of Traditional Chinese Medicine in China. The content of the RIPTFs was 52%. Radix *Ilicis Pubescentis*, the root of the plant IlexpubescensHook.etArn belonging to the family Aquifoliaceae, from Anhui province of China was identified and certified by Professor Cheng-ming Dong from the Henan University of Traditional Chinese Medicine in China. Radix *Ilicis Pubescentis* powder was dissolved in 10 times the amount (w/v) of 70% ethanol for 0.5 hour. The solution was twice extracted using reflux techniques: first for 1.5 hours, and second for 1 hour. After filtering, the filtrates were combined. The decompression and recovery of ethanol were conducted until no alcohol could be detected. The Radix *Ilicis Pubescentis* extract thus obtained was placed in an AB-8 macroporous resin column (Tianjin Haiguang Chemical Co., Ltd., Tianjin, China). The concentration of the sample liquid was 0.4 g/mL, the pH of sample liquid was 4.5, the ratio of crude drug to macroporous resin was 1:8, and the ratio of diameter to height of macroporous resin column was 1:15. The Radix *Ilicis Pubescentis* extract was eluted using 12 times the bed volume of water and 10 times the bed volume of 30% ethanol. The 30% ethanol eluent was collected, followed by decompression and recovery of ethanol. Samples were dried at 50°C, and the RIPTFs were obtained (Feng et al., 2012b). The RIPTFs were dissolved in 0.5% CMC-Na to generate suspensions of 1.5, 0.75, 0.375 mg/mL.

The mice in the *Yangxueqingnao* granule-treated group and the high-, moderate-, and low-dose RIPTFs-treated groups were intragastrically administered *Yangxueqingnao* granules (2 g/kg, dissolved in 0.5% CMC-Na of the 1 g/mL suspension; Tianjin Tasly Pharmaceutical Co., Ltd., batch No. Z10960082) or RIPTF suspensions of 0.3, 0.15, and 0.075 g/kg, respectively, once daily for 10 consecutive days. The mice in the untreated control and TIA model groups were intragastrically administered a volume equal to that given the RIPTF-treated groups (0.2 mL/10 g) of 0.5% CMC-Na (vehicle) once daily for 10 consecutive days.

Induction of TIA in mice

Three days after the first administration of RIPTFs, *Yangxueqingnao* granules, or vehicle, TIA was induced for the first time in all mice except those in the untreated control group. Ten minutes after an intravenous injection of 0.11 M tert-butyl hydroperoxide (7.5 mL/kg; Sinopharm Chemical Reagent Co., Ltd., Beijing, China), mice were placed into a wide-mouth bottle (0.45 L) that was sealed for 15 minutes before the mice were removed. TIA was induced for the second and third times 6 and 9 days after the first pretreatment

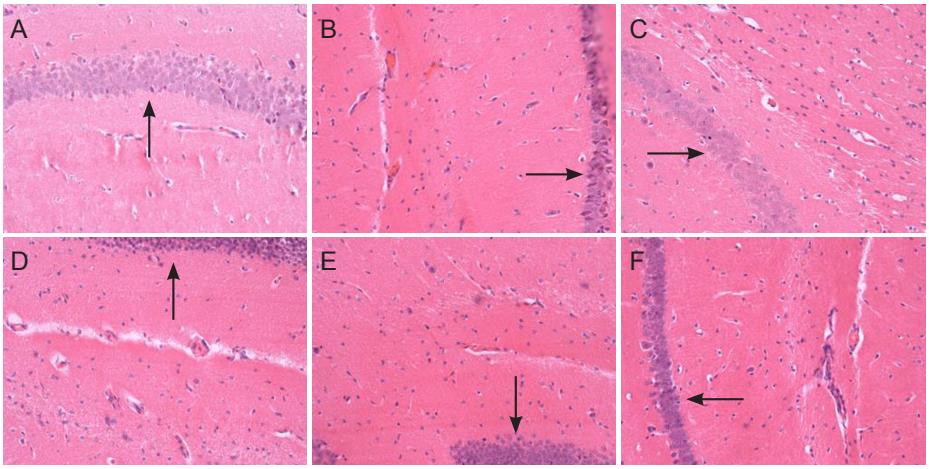

Figure 1 Effect of RIPTFs on pathological changes in the hippocampus in mouse models of transient ischemic attack.
Images are hematoxylin and eosin staining of hippocampal sections captured at × 400. (A) Control group: Large neuronal nuclei, fluffy nuclear chromatin, distinct nucleoli, normal capillaries and endothelial cells. (B) Transient ischemic attack (TIA) model group: Neuronal cell shrinkage, chromatin condensation, indistinct nucleoli, capillary zone expanded, fuzzy endothelial cells. (C) High-dose RIPTF-treated TIA model group: neurons appear normal; nuclear chromatin is fluffy, nucleoli are distinct, capillaries and endothelial cells appear normal. (D) Moderate-dose RIPTF-treated TIA model group: Nuclear volume is obviously reduced, chromatin is condensed, nucleoli are distinct, capillaries and endothelial cells appear normal. (E) Low-dose RIPTF-treated TIA model group: Apparently few neurons, fluffy nuclear chromatin, distinct nucleoli, expanded capillary zone, normal endothelial cells. (F) *Yangxueqingnao* granule-treated TIA model group: Generally normal neuronal cell volume, fluffy nuclear chromatin, distinct nucleoli, normal capillary and endothelial cells. Arrows indicate neuronal cell layer. RIPTFs: Radix *Ilicis Pubescentis* total flavonoids.

Table 2 Effect of RIPTFs on brain MDA and NO levels and NOS and SOD activities in a mouse model of transient ischemic attack

Group	MDA (nmoL/mg)	NO (μmoL/g)	NOS (U/mg)	iNOS (U/mg)	SOD (nmol/mg)
Control	17.843±4.795	1.239±0.597	1.443±0.361	0.576±0.162	58.736±11.346
TIA model	25.700±6.276**	2.372±0.431**	2.558±0.529**	1.283±0.335**	38.646±8.596**
RIPTFs-treated					
High dose	18.344±4.292$^{\#\#}$	1.547±0.352$^{\#\#}$	1.551±0.386$^{\#\#}$	0.811±0.326$^{\#\#}$	53.507±6.184$^{\#\#}$
Moderate dose	20.913±4.008$^{\#\#}$	1.792±0.183$^{\#\#}$	1.990±0.366$^{\#\#}$	0.890±0.325$^{\#\#}$	51.949±10.932$^{\#\#}$
Low dose	18.879±5.979$^{\#\#}$	2.024±0.538	2.051±0.296$^{\#\#}$	0.952±0.337$^{\#}$	49.501±11.308$^{\#}$
Yangxueqingnao granules-treated	19.242±5.414$^{\#\#}$	1.949±0.110$^{\#}$	1.850±0.343$^{\#\#}$	0.851±0.341$^{\#\#}$	51.024±5.130$^{\#\#}$

Data are expressed as the mean ± SD. The number of mice in the control group, transient ischemic attack (TIA) model group, high-, moderate- and low-dose RIPTFs-treated TIA model groups (0.3, 0.15, 0.075 g/kg), and *Yangxueqingnao* granule-treated TIA model group (2 g/kg) was 14, 10, 12, 11, 10 and 11, respectively. Data between groups were compared using one-way analysis of variance. Intergroup comparisons were performed using independent samples *t*-test. $^{**}P < 0.01$, *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs.* TIA model group. RIPTFs: Radix *Ilicis Pubescentis* total flavonoids; MDA: malondialdehyde; NO: nitric oxide; NOS: nitric oxide synthase; iNOS: inducible nitric oxide synthase; SOD: superoxide dismutase.

with RIPTFs, *Yangxueqingnao* granules, or vehicle. Administration of drugs for 3 consecutive days was considered a single cycle. Thus, there was a 48-hour interval between two cycles, with a total of three cycles. Behavioral scoring was performed once per week. The mice that suffered from marked ptosis, a weak droopy eyelid that is characteristic of Horner's syndrome, and limb paralysis that were restored to normal within 24 hours were considered successful mouse models of TIA (Niu et al., 2003).

During the experiment, 16 mice died because of their poor tolerance and more severe symptoms. Data from 68 mice were used in the resulting analyses, including 14 mice in the untreated control group, 10 mice in the untreated TIA model group, 11 mice in the *Yangxueqingnao* granule-treated group, and 12, 11, and 10 mice in the high-, moderate-, and low-dose RIPTFs-treated groups, respectively.

Behavioral score

The following modified standard graded scores were assigned to each mouse based on its appearance, movement, and behavior (Han et al., 1999). Stroke index: Mice with unclean fur, trembling limbs, reduced or dull or strong movements, ear tactile hypoesthesia, or ptosis received a score of 1, and those with hind limb abduction received a score of 3. Nervous system dysfunction score: Mice with spontaneous activity scored 1, walking or crawling in response to a stimulus 1, no movement during stimulus 2. Gait scores: Normal 0, muscle tension high 1, crawling 2, not walking 3. Eating scores: yes 0, no 1. Drinking scores: yes 0, no 1. Responses to painful stimuli: Moving/walking 0, strong head/torso movements or

Table 1 Effect of RIPTFs on pathological changes in hippocampal morphology of mouse models of transient ischemic attack

Group	Pathological grading score			
	–	+	++	+++
Control	14	0	0	0
TIA model	0	2	3	5
RIPTFs-treated				
High dose	8	2	2	0
Moderate dose	5	4	2	0
Low dose	3	2	4	1
Yangxueqingnao granules-treated	5	3	2	1

Data represent the number of mice assigned the score. The number of mice in the control group, transient ischemic attack (TIA) model group, high-, moderate- and low-dose RIPTFs-treated TIA model groups and the *Yangxueqingnao* granule-treated TIA model group were 14, 10, 12, 11, 10 and 11, respectively. Ranked data were analyzed using the Ridit test. The observed pathological changes were scored using the following four levels of criteria: "–" normal neurons, fluffy nuclear chromatin, distinct nucleoli, normal capillaries and endothelial cells; "+" partially retracted neurons, fluffy nuclear chromatin, distinct nucleoli, normal capillary and endothelial cells; "++" atrophied neurons, condensed chromatin, indistinct nucleoli, telangiectasia, fuzzy endothelial cells; "+++" shrunken neuronal cells, condensed chromatin, blurry nuclei, telangiectasia, fuzzy endothelial cells. RIPTFs: Radix *Ilicis Pubescentis* total flavonoids.

reaction 1, limb retraction or no response 2.

Collection of tissue specimens

Ten days after the first drug administration and 1 hour after the last administration, the mice were sacrificed. Their brains were removed, immediately placed on an ice-cold plate, and midsagittally divided into symmetrical hemispheres. The left hemisphere was used to assess pathological changes, whereas the right hemisphere was placed on filter paper, rinsed, and weighed using an analytical balance. Cold physiological saline was added at a ratio of 1:9 brain tissue (g) to physiological saline (mL). The tissue was homogenized at 4°C and centrifuged at 3,000 r/min for 20 minutes. The supernatant was stored at –20°C before being used as a 10% brain homogenate to determine the levels of malondialdehyde (MDA) and nitric oxide (NO) as well as the activities of nitric oxide synthase (NOS) and superoxide dismutase (SOD).

Observation of hippocampal tissue

The left brain hemisphere was rapidly fixed in 10% formaldehyde, embedded in paraffin, sliced into 5 μm-thick serial sections, and stained with hematoxylin and eosin. Hippocampal neurons as well as capillaries and endothelial cells were observed using a light microscope at 400× magnification (Olympus, Japan). The pathological changes were graded on a four-level scale: "–" was assigned to normal neurons with fluffy nuclear chromatin, distinct nucleoli, and normal capillaries and endothelial cells; "+" indicated partially retracted neurons, fluffy nuclear chromatin, distinct nucleoli, normal capillary and endothelial cells; "++" denoted atrophied neurons, condensed chromatin, indistinct nucleoli, telangiectasia, and fuzzy endothelial cells; "+++" indicated neuronal cell shrinkage, chromatin condensation, blurry nuclei, telangiectasia, and fuzzy endothelial cells (Bao et al., 2015).

Table 3 Effect of RIPTFs on behavior in a mouse model of transient ischemic attack

Group	First score	Second score	Third score
Control	$0\pm0^{**}$	$0\pm0^{**}$	$0\pm0^{**}$
TIA model	9.6±1.4	8.8±1.9	8.3±1.8
RIPTFs-treated			
High dose	$7.3\pm1.4^{\#\#}$	$6.3\pm1.4^{\#\#}$	$5.8\pm1.4^{\#\#}$
Moderate dose	$7.6\pm1.6^{\#\#}$	$6.9\pm2.1^{\#}$	$6.2\pm1.5^{\#\#}$
Low dose	$7.8\pm1.5^{\#\#}$	$7.2\pm1.2^{\#}$	6.5±1.6
Yangxueqingnao granules-treated	$7.9\pm1.7^{\#\#}$	$6.9\pm1.6^{\#}$	$6.2\pm1.5^{\#\#}$

Behavior was evaluated 3, 6 and 9 days after the first drug administration. A high score shows poor neurological function. Data are expressed as the mean ± SD. The number of mice in the control group, transient ischemic attack (TIA) model group, high-, moderate- and low-dose RIPTFs-treated TIA model groups (0.3, 0.15, 0.075 g/kg), and *Yangxueqingnao* granule-treated TIA model group (2 g/kg) was 14, 10, 12, 11, 10 and 11, respectively. Data between groups were compared using one-way analysis of variance. Paired intergroup comparisons were performed using independent samples *t*-test. $^{**}P < 0.01$, *vs*. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs*. TIA model group. RIPTFs: Radix *Ilicis Pubescentis* total flavonoids.

MDA and NO levels and NOS and SOD activities detected using spectrophotometry

For spectrophotometric analysis, all reagents were added to the brain homogenates according to the manufacturer's recommendations (Nanjing Jiancheng Biological Engineering Institute, Nanjing, Jiangsu Province, China).

For the determination of MDA levels, the optical density (OD) values were detected at 532 nm. The MDA level was calculated using the following formula: MDA level = $(OD_{sample} - OD_{control})/(OD_{standard} - OD_{blank}) \times$ standard concentration (10 nmoL/mL)/sample protein concentration (mg/L). The MDA content was determined as nmoL/mg (Feng et al., 2012a).

For the determination of NO levels, the OD values were detected at 550 nm. The NO level was calculated using the following formula: NO level = $(OD_{sample} - OD_{blank})/(OD_{standard} - OD_{blank}) \times$ standard concentration (20 μmoL/mL)/sample protein concentrations (g/L). The NO content was determined as μmoL/g (Yang et al., 2013).

To determine the level of NOS activity, colorimetry was conducted at 530 nm, and the OD value of each sample was recorded. Total NOS activity was calculated using the following formula: NOS activity = $(OD_{sample} - OD_{blank})$/coloring matter nanomolar extinction coefficient × total volume of reaction liquid/sample amount × (colorimetric optical path × reaction time)/sample protein concentrations (mg/L). The activity of total NOS was determined in U/mg. The inducible NOS (iNOS) activity was equal to $(OD_{sample} - OD_{blank})$/coloring matter nanomolar extinction coefficient × the total volume of reaction liquid/sample amount × (colorimetric optical path × reaction time)/sample protein concentrations (mg/L). The activity of iNOS was determined in U/mg (Pang

et al., 2014).

To determine the level of SOD activity, colorimetry was conducted at 550 nm, and the OD of each sample was recorded. SOD activity was calculated as follows: ($OD_{control}$ – OD_{sample})/50% × (total volume of reaction liquid/sample amount × sample protein concentrations (mg/L). SOD activity was determined in nmoL/mg (Li and Yan, 2011; Weng and Zhang, 2011).

Statistical analysis

All data were analyzed using the SPSS 13 software (SPSS, Chicago, IL, USA). Data between groups were compared using one-way analysis of variance and are presented as the mean ± SD. Ranked data were analyzed using the Ridit test. Intergroup comparisons were performed using independent samples *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of RIPTF treatment on hippocampal changes in a mouse model of TIA

Hematoxylin-eosin staining showed that neurons in the hippocampus of mice in the TIA model group demonstrated shrinkage, chromatin condensation, and distinct nucleoli. Significant capillary zone expansion and fuzzy endothelial cells were also visible in the hippocampus of these mice. In comparison to these pathological changes, in the high-, moderate-, and low-dose RIPTFs-treated and *Yangxueqingnao* granule-treated mouse models of TIA, the nuclear volume was increased, nucleoli were distinct, and capillary endothelial cells appeared normal (**Figure 1**, **Table 1**).

Effect of RIPTF treatment on brain MDA and NO levels and NOS and SOD activities in a mouse model of TIA

Compared with those in the control group, MDA and NO levels as well as NOS and iNOS activities were significantly higher, whereas SOD activity was significantly lower in the TIA model group ($P < 0.01$). Compared with those in the TIA model group, MDA levels and NOS and iNOS activities were significantly lower but SOD activity was significantly higher in the high-, moderate- and low-dose RIPTFs-treated and *Yangxueqingnao* granule-treated groups ($P < 0.01$ or $P < 0.05$). In addition, NO levels were lower in the high- and moderate-dose RIPTFs-treated, and *Yangxueqingnao* granule-treated groups than those in the TIA model group ($P < 0.01$ or $P < 0.05$). No significant difference in the NO level was detected between the low-dose RIPTFs-treated group and the TIA model group (**Table 2**).

Effect of RIPTF treatment on behavioral activity in a mouse model of TIA

Compared with that in the control group, the behavioral score was significantly higher in the model group ($P < 0.01$). Compared with that in the model group, the behavioral scores were significantly lower in the high- and moderate-dose RIPTFs-treated and *Yangxueqingnao* granule-treated groups ($P < 0.01$). The behavioral score was also significantly reduced in the low-dose RIPTF-treated group ($P < 0.05$; **Table 3**). These results indicate that RIPFT treatment significantly ameliorated behavioral dysfunctions in mouse models of TIA.

Discussion

Alterations in the behaviors examined in the present study reflect abnormal changes in the nervous system of mice and are macroscopic indicators of TIAs in a mouse model, with the score level indicating the degree of damage caused by the TIA. We modified the scale of the degree of damage in the TIA mouse model to exclude the scores of symptoms just prior to death, such as seizure-like movement and exhaustion, and mainly scored the consistently observed changes in animal behaviors. This modified scale offered greater objectivity for the analysis of the results.

The results of our hippocampal morphological observations indicated that the high-dose RIPTFs group showed the best effect. This amelioration of the pathological changes might be associated with the effect of RIPTFs to reduce free radical injury. The results from our assays examining the MDA and NO levels and NOS and SOD activities, demonstrated that RIPTFs inhibited lipid peroxidation, effectively removed free radicals, reduced neuronal toxicity, and protected neurons. These results indicate that RIPTFs prevent TIAs, ameliorate brain tissue injury, and reduce the pathological process of brain injury, supporting our hypothesis.

Most previous studies investigating cerebral ischemia examined the effects of chemical-based drugs, with traditional Chinese medicine practices, accounting for only a small fraction of these studies. Although some studies used traditional Chinese medicine, few examined the active ingredients used in this ancient practice for the prevention or amelioration of damage caused by TIAs. By contrast, the present study examined the preventive effect of active ingredients used in Chinese herbal medicine on TIA. Radix *Ilicis Pubescentis* is commonly used as a Chinese herbal medicine to activate blood circulation and dissipate blood stasis, and treatment with this botanical is effective in the prevention and treatment of cardiovascular and cerebrovascular diseases. However, no studies have explored the effects of Radix *Ilicis Pubescentis* in a mouse model of TIA. Thus, our study examining the preventive effects of Radix *Ilicis Pubescentis* in this model is novel. We investigated the effects of the Radix *Ilicis Pubescentis* total flavonoids on TIA and found that it effectively prevented TIA. Our results not only show that this Chinese herbal medicine, which promotes blood circulation to remove blood stasis, is effective in the prevention of TIA, but also provide evidence for the clinical use of RIPTFs as preventive medication for TIA and offer the novel prospect of developing RIPTFs as new therapeutics for the prevention of TIA.

Acknowledgments: *We are very grateful to Professor Jian-guo Li from Henan University of Traditional Chinese Medicine in China for assistance in the implementation of pathological observation.*

Author contributions: *MSM designed this experiment. LG, RQL and XLZ performed experiments and collected data. MSM and LG estimated the study. LG wrote the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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Copyedited by Smith T, Raye W, Yu J, Qiu Y, Li CH, Song LP, and Zhao M