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Persimmon leaf flavonoid promotes brain ischemic tolerance⋯⋯

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Research Highlights

(1) Persimmon leaf flavonoid mitigates pathological injury of brain tissue following cerebral ischemia/reperfusion in brain ischemic tolerance rats and elevates brain ischemic tolerance.
(2) Persimmon leaf flavonoid reduces endothelial cell injury, inhibits intercellular adhesion molecule-1 protein expression, and elevates brain ischemic tolerance.
(3) The effects of high-dose persimmon leaf flavonoid on strengthening brain ischemic tolerance are identical to that of ginaton.

Abstract

Persimmon leaf flavonoid has been shown to enhance brain ischemic tolerance in mice, but its mechanism of action remains unclear. The bilateral common carotid arteries were occluded using a micro clip to block blood flow for 10 minutes. After 10 minutes of ischemic preconditioning, 200, 100, and 50 mg/kg persimmon leaf flavonoid or 20 mg/kg ginaton was intragastrically administered per day for 5 days. At 1 hour after the final administration, ischemia/reperfusion models were established by blocking the middle cerebral artery for 2 hours. At 24 hours after model establishment, compared with cerebral ischemic rats without ischemic preconditioning or drug intervention, plasma endothelin, thrombomodulin and von Willebrand factor levels significantly decreased and intercellular adhesion molecule-1 expression markedly reduced in brain tissue from rats with ischemic preconditioning. Simultaneously, brain tissue injury reduced. Ischemic preconditioning combined with drug exposure noticeably improved the effects of the above-mentioned indices, and the effects of 200 mg/kg persimmon leaf flavonoid were similar to 20 mg/kg ginaton treatment. These results indicate that ischemic preconditioning produces tolerance to recurrent severe cerebral ischemia. However, persimmon leaf flavonoid can elevate ischemic tolerance by reducing inflammatory reactions and vascular endothelial injury. High-dose persimmon leaf flavonoid showed an identical effect to ginaton.

Key Words

neural regeneration; traditional Chinese medicine; persimmon leaf flavonoid; brain injury; brain ischemic tolerance; ischemic preconditioning; ginaton; ischemia/reperfusion injury; intercellular adhesion molecule-1; endothelin; grants-supported paper; neuroregeneration

INTRODUCTION

Modern pharmacological studies have shown that persimmon leaf flavonoid has extensive pharmacological actions, including dilation of blood vessels, a lipid-reducing effect, a glucose-lowering effect, and antioxidant properties[1-2]. Our previous study showed that persimmon leaf flavonoid enhances brain ischemic tolerance[3], but its mechanism of action remains unclear.

Microcirculation disturbances are an important pathophysiological change during ischemic cerebrovascular disease, such as

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the Experimental Animal Ethics Committee, Henan University of Traditional Chinese Medicine, China.

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peroxide production[4], vascular endothelial cell injury[5], increased cell adhesion[6], plasma albumin leakage[7], and inflammatory factor release[8]. After cerebral ischemia, intracerebral microcirculation is blocked. This study aimed to correlate vascular endothelial cell injury and inflammatory factors with cerebral ischemia. A key event in the inflammatory response is the adhesion and extravasation of leukocytes and vascular endothelial cells. This process is mediated by intercellular adhesion molecules and their ligands, which are located on the surface of leukocytes and vascular endothelial cells[9]. Among the adhesion molecules related to cerebral ischemia/reperfusion injury, intercellular adhesion molecule-1 plays the most important role in ischemia/reperfusion injury[10]. Endothelin, von Willebrand factor and thrombomodulin are associated with endothelial cells, and endothelial injury is associated with the pathological process of thrombosis[11]. This study induced brain ischemic tolerance by ischemic preconditioning, and observed changes in endothelin, thrombomodulin, and von Willebrand factor in rat plasma and intercellular adhesion molecule-1 levels in brain tissue after further ischemia. In addition, the effects of various doses of persimmon leaf flavonoid were explored to identify the mechanism of persimmon leaf flavonoid-neuroprotection during brain ischemic tolerance in comparison with ginaton (main component: ginkgo- flavone glycosides), a commonly used treatment for cerebrovascular disease[12].

RESULTS

Quantitative analysis of experimental animals

Rats (*n* = 98) were equally and randomly divided into seven groups: sham surgery group (sham surgery), ischemia/reperfusion group (reperfusion at 2 hours after cerebral ischemia), preprocessing model group (ischemic preconditioning before ischemia/reperfusion), high-, moderate- and low-dose persimmon leaf flavonoid groups (ischemia/reperfusion after administration of 200, 100, 50 mg/kg persimmon leaf flavonoid on the basis of brain ischemic tolerance), and ginaton group (ischemia/reperfusion after administration of 20 mg/kg ginaton on the basis of brain ischemic tolerance). A total of 22 rats were excluded because of surgical death and failure of successful modeling. Therefore, 76 rats were included in the final analysis.

Effects of persimmon leaf flavonoid on pathological lesions of brain tissue in rats that had acquired brain ischemic tolerance after cerebral ischemia/reperfusion

Hematoxylin-eosin staining results revealed normal nerve cells, cytoplasm and nuclei in the sham surgery group. Atrophic nerve cells, reduced cytoplasm and unclear or disappeared nuclei were observed in the ischemia/reperfusion group. Reduced cell size, decreased cytoplasm were observed in the preprocessing model, low- and moderate-dose persimmon leaf flavonoid groups. Increased cell size, abundant cytoplasm and normal nuclei were observed in the high-dose persimmon leaf flavonoid group. Increased cell size, atrophic cells, decreased cytoplasm, lightly stained or disappeared nuclei were detected in the ginaton group (Figure 1).

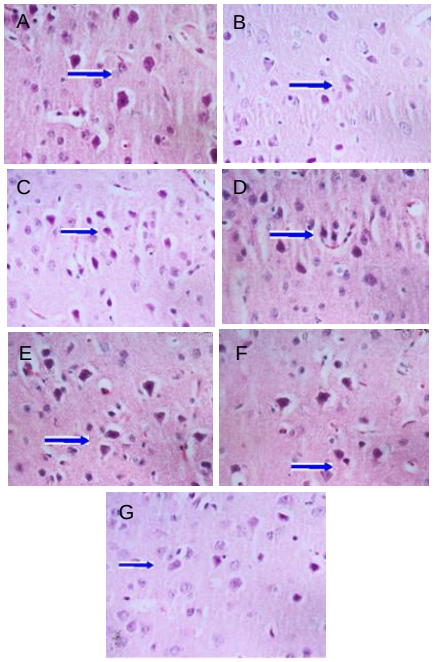

Figure 1 Effects of persimmon leaf flavonoid on pathological lesions in brain tissue of rats that had acquired brain ischemic tolerance at 24 hours after cerebral ischemia/reperfusion (hematoxylin-eosin staining, × 400).

Except for the sham surgery group (A), the rats from the other groups (B–G) were used to establish models of cerebral ischemia/reperfusion injury. The rats of the preprocessing model group (C) were subjected to ischemic preconditioning before ischemia/reperfusion. The rats of the high- (F), moderate- (E) and low-dose (D) persimmon leaf flavonoid groups and ginaton group (G) were administered 200, 100, 50 mg/kg persimmon leaf flavonoid or 20 mg/kg ginaton on the basis of brain ischemic tolerance, followed by modeling of ischemia/reperfusion. Compared with the ischemia/reperfusion group (B), cell size became smaller, the cytoplasmic space was larger, and lightly stained or absent staining of nuclei decreased in rats after intragastric administration of ginaton and persimmon leaf flavonoid. The effect was obvious in the ginaton group and high-dose persimmon leaf flavonoid group. Arrows show nerve cells.

Compared with the sham surgery group, significant pathological lesions were visible in the ischemia/reper-

fusion and preprocessing model groups ($P < 0.01$). Compared with the preprocessing model group, cerebral ischemia-induced pathological lesions were markedly reduced in the high-, moderate- and low-dose persimmon leaf flavonoid groups and ginaton group ($P < 0.05$ or $P < 0.01$), especially in the high-dose persimmon leaf flavonoid and ginaton groups (Table 1).

Table 1 Effects of persimmon leaf flavonoid on degree of pathological lesions (*n*) in brain tissue from rats that had acquired brain ischemic tolerance at 24 hours after cerebral ischemia/reperfusion

Group	*n*	–	+	++	+++
Sham surgery	14	13	1	0	0
Ischemia/reperfusion[a]	11	0	0	4	7
Preprocessing model[a]	9	0	2	5	2
Persimmon leaf flavonoid					
High-dose[c]	9	4	4	0	1
Moderate-dose[c]	11	3	5	2	1
Low-dose[b]	10	1	3	4	2
Ginaton[c]	12	4	5	2	1

The degree of pathological lesions was scored as follows: "–": large cell body, abundant cytoplasm, normal nuclei; "+": reduced cell size, decreased cytoplasm, normal nuclei; "++": some atrophic nerve cells, decreased cytoplasm, lightly stained or absent staining of nuclei; "+++": many atrophic nerve cells, decreased cytoplasm, unclear or absent nuclei. Ridit test was used to compare the difference of intergroup data. [a]$P < 0.01$, *vs*. sham surgery group; [b]$P < 0.05$, [c]$P < 0.01$, *vs*. preprocessing model group.

Effects of persimmon leaf flavonoid on plasma endothelin-1, thrombomodulin, and von Willebrand factor levels in rats that had acquired brain ischemic tolerance after cerebral ischemia/reperfusion

Compared with the sham surgery group, plasma endothelin-1, thrombomodulin, and von Willebrand factor concentrations were significantly higher in the ischemia/reperfusion and preprocessing model groups ($P < 0.01$). Compared with the ischemia/reperfusion group, plasma endothelin-1, thrombomodulin, and von Willebrand factor concentrations were significantly lower in the preprocessing model group ($P < 0.05$), indicating that ischemic preconditioning produced tolerance to recurrent severe cerebral ischemia. Compared with the preprocessing model group, plasma endothelin-1, thrombomodulin, and von Willebrand factor concentrations were significantly lower in the high- and moderate-dose persimmon leaf flavonoid groups, and ginaton group ($P < 0.01$; Table 2).

Effects of persimmon leaf flavonoid on intercellular adhesion molecule-1 expression in brain tissues of rats that had acquired brain ischemic tolerance after cerebral ischemia/reperfusion

Immunohistochemical staining revealed negative expression of intercellular adhesion molecule-1 in the cortex and hippocampus of rats in the sham surgery group. Intense expression of intercellular adhesion molecule-1 (brown color) was observed in the cortex and hippocampus of rats in the ischemia/reperfusion and preprocessing model groups. Intercellular adhesion molecule-1 expression became weak in the cortex and hippocampus of rats treated with various doses of persimmon leaf flavonoid and 20 mg/kg ginaton (Figure 2).

Compared with the sham surgery group, intercellular adhesion molecule-1 expression in brain tissue significantly increased in the ischemia/reperfusion and preprocessing model groups ($P < 0.01$). Compared with the ischemia/reperfusion group, intercellular adhesion molecule-1 expression in brain tissue was significantly lower in the preprocessing model group ($P < 0.01$). Compared with the preprocessing model group, intercellular adhesion molecule-1 expression in brain tissue was lower in the moderate- and low-dose persimmon leaf flavonoid groups ($P < 0.05$), but significantly lower in the high-dose persimmon leaf flavonoid and ginaton groups ($P < 0.01$; Table 3).

Table 2 Effects of persimmon leaf flavonoid on plasma endothelin-1, thrombomodulin, and von Willebrand factor levels (ng/mL) at 24 hours after cerebral ischemia/reperfusion

Group	*n*	Endothelin-1	Thrombomodulin	von Willebrand factor
Sham surgery	14	0.112 0±0.009 6	0.547 4±0.003 8	0.565 9±0.000 8
Ischemia/reperfusion	11	0.239 0±0.012 0[a]	0.644 4±0.000 6[a]	0.604 7±0.001 8[a]
Preprocessing model	9	0.226 1±0.011 4[ac]	0.637 3±0.000 2[ac]	0.603 3±0.000 2[ac]
Persimmon leaf flavonoid				
High-dose	9	0.163 3±0.006 4[b]	0.614 3±0.000 0[b]	0.595 9±0.001 0[b]
Moderate-dose	11	0.198 2±0.006 8[b]	0.619 8±0.000 3[b]	0.598 5±0.000 4[b]
Low-dose	10	0.213 1±0.011 1	0.628 6±0.001 1	0.601 6±0.000 5
Ginaton	12	0.148 7±0.006 7[b]	0.608 0±0.003 3[b]	0.593 5±0.000 2[b]

Results are expressed as mean ± SD. [a]$P < 0.01$, *vs*. sham surgery group; [b]$P < 0.01$, *vs*. preprocessing model group; [c]$P < 0.05$, *vs*. ischemia/reperfusion group (one-way analysis of variance, least significant difference test for pairwise comparison).

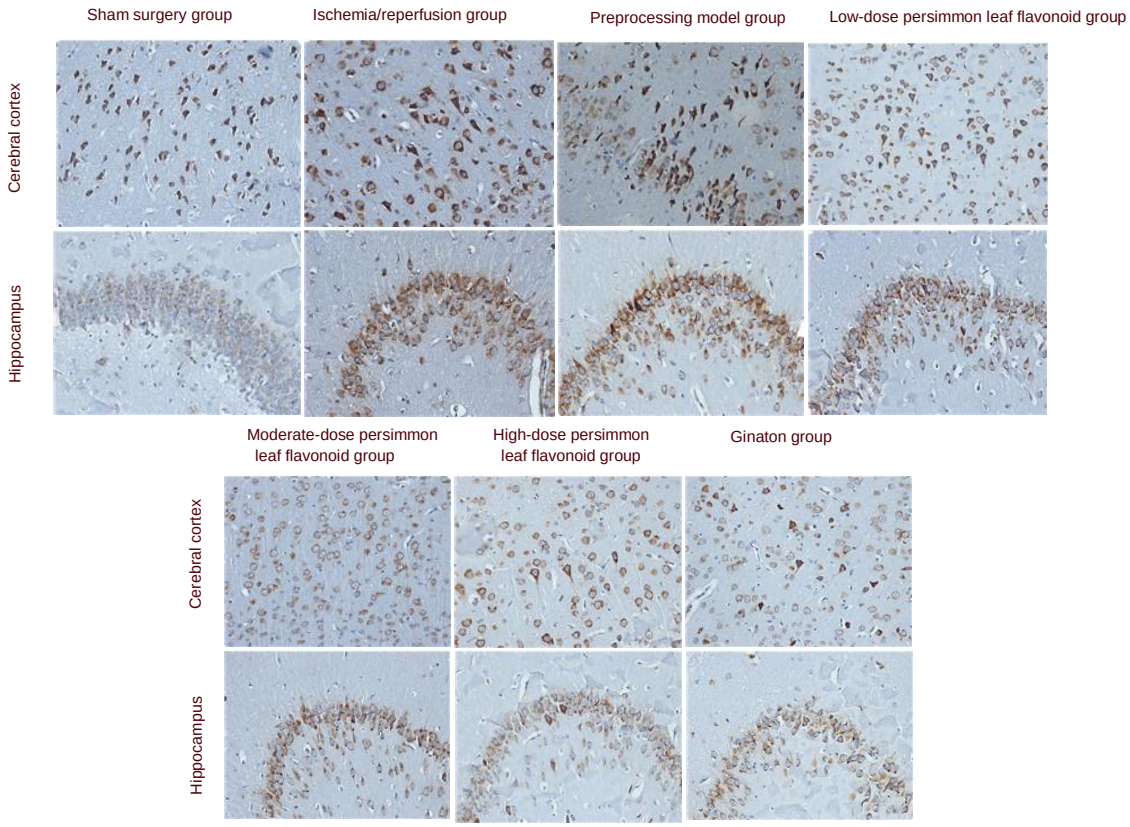

Figure 2 Effects of persimmon leaf flavonoid on intercellular adhesion molecule-1 expression in the cerebral cortex and hippocampus at 24 hours after cerebral ischemia/reperfusion (immunohistochemical staining, × 400).

Except for the sham surgery group, rats from the other groups were used to establish models of cerebral ischemia/reperfusion injury. Rats from the preprocessing model group were subjected to ischemic preconditioning before ischemia/reperfusion. Rats from the high-, moderate-, and low-dose persimmon leaf flavonoid groups and ginaton group were administered 200, 100, 50 mg/kg persimmon leaf flavonoid or 20 mg/kg ginaton on the basis of brain ischemic tolerance, followed by modeling of ischemia/reperfusion. Compared with the ischemia/reperfusion group, intercellular adhesion molecule-1 expression in the cerebral cortex and hippocampus was decreased in rats treated with ginaton and persimmon leaf flavonoid. Moreover, the effect of high-dose persimmon leaf flavonoid and ginaton was obvious.

Table 3 Effects of persimmon leaf flavonoid on intercellular adhesion molecule-1 expression (*n*) in rats that had acquired brain ischemic tolerance at 24 hours after cerebral ischemia/reperfusion

Group	*n*	–	+	++	+++
Sham surgery	14	14	0	0	0
Ischemia/reperfusion[a]	11	0	1	2	8
Preprocessing model[ab]	9	0	1	3	5
Persimmon leaf flavonoid					
High-dose[d]	9	3	4	1	1
Moderate-dose[c]	11	2	5	3	1
Low-dose[c]	10	1	5	3	1
Ginaton[d]	12	4	5	2	1

The degree of intercellular adhesion molecule-1 expression was scored as follows: "–": negative expression in the ischemic region of the cerebral cortex; "+": weakly positive expression in the ischemic region of the cerebral cortex; "++": positive expression in the ischemic region of the cerebral cortex; "+++": intensive expression in the ischemic region of the cerebral cortex. Ridit test was used for intergroup comparisons. [a]$P < 0.01$, *vs*. sham surgery group; [b]$P < 0.01$, *vs*. ischemia/reperfusion group; [c]$P < 0.05$, [d]$P < 0.01$, *vs*. preprocessing model group.

DISCUSSION

The suture method was used to establish a middle cerebral artery occlusion model[13]. Therefore, it was not necessary to open the skull, cause trauma, and potentially induce pathological changes such as cerebral edema or intracranial pressure. In addition, we did not see variability in infarct size due to differences in blood clot size using the autologous blood clot method. The time of ischemia and reperfusion was easy to control. Thus, the suture method has become a popular method to study focal cerebral ischemia. At 10 minutes after pre-ischemia, obvious ischemic tolerance was observed within 3–7 days. The protective effect of 72 hour-induction was strongest[14], and the effect gradually decreased with time.

This study found that 10 minutes of ischemic precondi-

tioning relieved recurrent ischemia-induced brain injury, and that intervention with various doses of persimmon leaf flavonoid and ginaton reduced tissue injury in rats after cerebral ischemia/reperfusion, especially with 200 mg/kg persimmon leaf flavonoid.

Vascular endothelial injury was strongly associated with cerebral ischemia. Endothelin was mainly expressed in vascular endothelial cells, and partially in endothelial cells and nerve cells in the nervous system, showing effects of promoting endothelial cell proliferation. In the acute stage of cerebrovascular disease, the release of von Willebrand factor increased, and plasma von Willebrand factor content increased. Thrombomodulin is a molecular marker of vascular endothelial injury and a sensitive index of vascular endothelial injury. Immunohistochemical staining revealed that intercellular adhesion molecule-1 protein was mainly expressed in the microvascular endothelium of the ischemic cortex[15].

Endothelin, a strong long-acting angiotonic, participates in the pathophysiological process of cerebral hemorrhage, and is associated with the severity of the condition[16]. When vascular endothelial cells are injured, thrombomodulin is released, and plasma thrombomodulin levels increase[17]. Recently, thrombomodulin has been shown to be a new marker of vascular endothelial injury. Plasma von Willebrand factor is known to be an index of vascular endothelial injury[18], and has important significance in thrombosis and determining the severity of a patient's condition[19-21]. Intercellular adhesion molecule-1, a member of the immunoglobulin superfamily, has a similar molecular structure to immunoglobulins, and is an essential adhesion molecule expressed in vascular endothelial cells and leukocytes[22]. Intercellular adhesion molecule-1 mainly mediates neutrophil and activated endothelial cell adhesion. Endothelin-1, thrombomodulin and von Willebrand factor levels were diminished in the medication groups, suggesting that persimmon leaf flavonoid could protect the vascular endothelium and improve cerebral ischemia.

Intercellular adhesion molecule-1 expression induced by pro-inflammatory cytokines was increased after cerebral ischemia. Once blood flow was restored in the ischemic region, intercellular adhesion molecule-1 as a ligand can bind to macrophage-derived chemotactic factor-1 and lymphocyte function-associated antigen-1 in leukocytes[23], as well as mediate neutrophil adhesion. When the microcirculation channel is blocked, blood supply can be affected. However, activated and infiltrated leukocytes releasing inflammatory mediators and cytokines can injure local blood vessels, leading to increased vascular permeability, tissue edema, destruction of surviving neurons, and finally aggravated tissue injury[24]. Therefore, the occurrence of an inflammatory reaction was suppressed, and intercellular adhesion molecule-1 expression was inhibited. Results from this study confirmed that persimmon leaf flavonoid reduced the occurrence of the inflammatory reaction, weakened the expression of intercellular adhesion molecule-1 protein, and elevated the tolerance of the body to cerebral ischemia.

In modern pharmacological studies, persimmon leaf flavonoid has been shown to dilate blood vessels, decrease lipid and glucose levels, and have an antioxidant effect *in vitro* [25]. To reveal the pharmacologic action of persimmon leaf, this study observed the effects of persimmon leaf flavonoid on brain ischemic tolerance in rats. The persimmon leaf flavonoid used in this study was an extract. Ginaton was intragastrically administered. Parallel experiments were conducted in each group. Thus, there was no bias of experimental results. This study demonstrated successful model establishment of brain ischemic tolerance. Plasma endothelin, thrombomodulin, and von Willebrand factor levels were decreased, and intercellular adhesion molecule-1 expression was diminished simultaneously in rats with cerebral ischemia in the ginaton group and various doses of persimmon leaf flavonoid groups. These data indicated that persimmon leaf flavonoid probably has a thrombolytic effect during cerebral ischemia, repairs vascular endothelial cells, enhances the intensity of brain ischemic tolerance, plays a synergistic protective effect, and finally reduces stroke occurrence.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Laboratory of Pharmacology, Henan University of Traditional Chinese Medicine, China from August to September 2009.

Materials

Animals

A total of 98 healthy, clean, male 2-month Sprague-Dawley rats weighing 280–300 g were provided by the Hebei Provincial Experimental Animal Center of China (license No. 907048). The rats were housed at 25 ± 3°C and 55 ± 10%, and were allowed free access to food and

water. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[26].

Drugs

Fresh or dried persimmon leaves of genus *Diospyros kaki L.f*. were purchased from Henan Medical Material Company (Zhengzhou, Henan Province, China). Dried persimmon leaves were decocted three times with water, each for 1.5 hours. After filtering, the filtrates were combined and condensed to a relative density of 1.15–1.20 (60%). Alcohol was added until an alcohol content of 70% (v/v) was achieved, without stirring overnight. The supernatant was leached before use. The sediment was washed twice with 70% (v/v) alcohol. The washing solution was collected, without stirring. The supernatant was collected, and combined with the above-mentioned supernatant. Alcohol was recycled. The residue was dissolved in water, and filtered. The filtrates were extracted five times using ethyl acetate. All ethyl acetate collections were combined and recycled. The condensed residue was dissolved in water, chromatographed on a polyamide column, and washed with distilled water until the eluent was colorless. The specimen was eluted with 70% (v/v) alcohol at a speed of 5–10 mL/min. The eluent was collected, and the alcohol was recycled and condensed at low pressure and temperature. Persimmon leaf extract was obtained by decompression, atomization and drying. The content of persimmon leaf flavonoid was 68%, as determined by ultraviolet spectrophotometry. Persimmon leaf extract (batch No. TY20080116) was provided by the Chemistry Room, Henan College of Traditional Chinese Medicine, China.

Methods

Establishment and intervention of the ischemic tolerance model

Rats were intraperitoneally anesthetized with 10% (v/v) chloral hydrate (0.3 mL/100 g), and fixed on the operation table in a supine position. A median incision was made on the anterior neck. The bilateral common carotid arteries were bluntly dissociated, and occluded using a micro clip to block blood flow for 10 minutes, and then reperfusion was allowed[27]. In the sham surgery group and ischemia/reperfusion group, the bilateral common carotid arteries were exposed, but blood flow was not blocked. In the high-, moderate- and low-dose persimmon leaf flavonoid groups and ginaton group, ischemia/reperfusion rats were subjected to ischemic preconditioning and intragastrically given 200, 100, 50 mg/kg persimmon leaf flavonoid or 20 mg/kg ginaton (batch No. 9900908; Dr Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany). Rats in the sham surgery group, preprocessing model group and ischemia/reperfusion group were intragastrically given saline (1 mL/100 g), once a day for 5 consecutive days.

Preparation of ischemia/reperfusion model

At 5 days, after fasting for 12 hours and 1 hour after administration, the rats were intraperitoneally anesthetized with 10% (v/v) chloral hydrate (0.3 mL/100 g), and fixed on the operation table in the supine position. A median incision was made in the middle of the neck. In accordance with previously published methods[28-29], middle cerebral artery embolism reperfusion models were established. A nylon thread (approximately 0.30 mm diameter) coated with silicone rubber within 1 mm from the tip was inserted 18–20 mm into the proximal end of the middle cerebral artery through the left common carotid artery to block blood supply of the middle cerebral artery for 2 hours. The nylon thread was pulled out and the left middle cerebral artery was embolized. The temperature was maintained at 23–24°C during the surgery. In the sham surgery group, only exposure was performed, but the middle cerebral artery was not embolized. The remaining rats underwent left middle cerebral artery embolism. In accordance with Longa's criteria[30], the rats were graded 24 hours after ischemia/reperfusion. The neurologic findings were scored on a 5-point scale: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) indicated a mild focal neurologic deficit, a score of 2 (circling to the left) indicated a moderate focal neurologic deficit, and a score of 3 (falling to the left) indicated a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. Rats that scored 1 or above indicated successful model establishment.

Sampling of blood

At 24 hours after model induction in each group, blood was collected by removing the eyeball, and treated with the anticoagulant ethylenediaminetetraacetic acid disodium salt for 1 hour, and centrifuging at 1 100 × *g* for 10 minutes. After removal of the supernatant, blood plasma was obtained for the determination of endothelin, thrombomodulin and von Willebrand factor levels.

Endothelin determination

Blood plasma of rats from each group was obtained. Protocols were conducted in accordance with the instructions from the endothelin radioimmunoassay kit (batch No. 20090725; Beijing Puerweiye Biological

Technology Co., Ltd., Technology Development Center Radioimmunity Institute of General Hospital of Chinese PLA, Beijing, China). Relative parameters, calibration curves and sample concentrations were obtained by radioimmunoassay and a Gamma Counter (model SN-695 B; First Rihuan Instrument Factor, Shanghai Atomic Nucleus Institute, Shanghai, China).

Thrombomodulin determination

Blood plasma of rats from each group was obtained. Protocols were conducted in accordance with the instructions from the thrombomodulin immunohistochemistry kit (batch No. 090816; R&D, Minneapolis, MN, USA). Standard curves were drawn on the coordinate system using a thrombomodulin calibrator concentration (ng/mL) at an absorbance of 450 nm. Thrombomodulin content (ng/mL) of detected samples was determined from the standard curve.

Determination of von Willebrand factor

Blood plasma of rats from each group was obtained. Protocols were conducted in accordance with the instructions from the von Willebrand factor immunohistochemistry kit (batch No. 090816; R&D). Standard curves were drawn on the coordinate system using a von Willebrand factor calibrator concentration (ng/mL) at an absorbance of 450 nm. Von Willebrand factor content (ng/mL) of detected samples was determined from the standard curve.

Preparation of brain tissue specimens and pathological observation

After blood collection, the rats were sacrificed by cervical dislocation. Rat brain tissue at 2 mm anterior to the optic root was rapidly obtained, and immersed in formalin for over 24 hours. Brain tissues were embedded in paraffin, sliced into serial 5-μm-thick sections, and stained with hematoxylin and eosin. The cerebral cortex and hippocampus were observed under a 400 × optical microscope (Olympus, Tokyo, Japan). Pathological changes of nerve cells were scored as follows: "–": large nerve cell size, abundant cytoplasm, and normal nuclei; "+": reduced nerve cell size, reduced cytoplasm, and normal nuclei; "++": some cell atrophy, reduced cytoplasm, lightly stained or absent staining of nuclei; "+++": abundant cell atrophy, obviously reduced cytoplasm, unclear or absence of nuclei.

Immunohistochemical method for intercellular adhesion molecule-1 expression

Brain tissues at 3–4 mm posterior to the optic chiasma were obtained from rats, dewaxed, hydrated, incubated in 3% (v/v) H_2O_2 for 5–10 minutes to inactivate endogenous peroxidase, and then washed three times with distilled water, each for 3 minutes. The sections were immersed in 0.01 mol/L citrate buffer (pH 6.0), boiled in a pressure cooker for 5–10 minutes for antigen retrieval, and then naturally cooled at room temperature. After three washes with PBS (each for 2 minutes), 5% (v/v) bovine serum albumin was added at room temperature for 20 minutes. Excess liquid was discarded, without washing. Subsequently, the samples were incubated with rabbit anti-intercellular adhesion molecule-1 polyclonal antibody (1:100; Sigma, St. Louis, MO, USA) at 4°C overnight. Tissue was washed three times with PBS, each for 2 minutes, incubated with biotinylated goat anti-rabbit IgG (1:200; Sigma) at room temperature for 20 minutes, washed three times with PBS, each for 2 minutes, and then incubated in streptavidin-biotin complex (Sigma) at room temperature for 20 minutes, and washed four times with PBS, each for 5 minutes. The sections were visualized with 3,3'-diaminobenzidine (Boster, Wuhan, Hubei Province, China). Reaction time was controlled under the light microscope (Olympus). The reaction was promptly terminated by adding tap water. The sections were lightly counterstained with hematoxylin, washed with running water, differentiated with hydrochloric ethanol, washed with running water, mounted with neutral resin, and then observed under the light microscope (Olympus). The degree of intercellular adhesion molecule-1 expression was scored as follows: "–": negative expression in the ischemic region of the cerebral cortex; "+": weakly positive expression in the ischemic region of the cerebral cortex (light yellow); "++": positive expression in the ischemic region of the cerebral cortex (yellow); "+++": intense expression in the ischemic region of the cerebral cortex (dark yellow).

Statistical analysis

Measurement data were expressed as mean ± SD, and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Intergroup comparisons were made using one- way analysis of variance. Pairwise comparisons were made using least significant difference test. The comparison of ranked data was performed using the Ridit test (α = 0.05).

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