Persimmon leaf flavonoid promotes brain ischemic tolerance

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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\(^1\). Our previous study showed that persimmon leaf flavonoid enhances brain ischemic tolerance\(^2\), but its mechanism of action remains unclear.

Microcirculation disturbances are an important pathophysiological change during ischemic cerebrovascular disease, such as...
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, x 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.
fuson 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).

| Group                  | n  | − | + | ++ | +++ |
|------------------------|----|---|---|----|-----|
| Sham surgery           | 14 | 13| 0 | 0  | 0   |
| Ischemia/reperfusion$^a$ | 11 | 0 | 4 | 0  | 0   |
| Preprocessing model$^a$ | 9  | 0 | 2 | 5  | 2   |
| Persimmon leaf flavonoid |    |   |   |    |     |
| High-dose$^b$          | 9  | 4 | 4 | 0  | 1   |
| Moderate-dose$^b$      | 11 | 3 | 5 | 2  | 1   |
| Low-dose$^b$           | 10 | 1 | 3 | 4  | 2   |
| Ginaton$^a$            | 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 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 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| 0 | 0  | 0   |
| Ischemia/reperfusion$^a$ | 11 | 0 | 4 | 0  | 0   |
| Preprocessing model$^a$ | 9  | 0 | 2 | 5  | 2   |
| Persimmon leaf flavonoid |    |   |   |    |     |
| High-dose$^b$          | 9  | 4 | 4 | 0  | 1   |
| Moderate-dose$^b$      | 11 | 3 | 5 | 2  | 1   |
| Low-dose$^b$           | 10 | 1 | 3 | 4  | 2   |
| Ginaton$^a$            | 12 | 4 | 5 | 2  | 1   |

The 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±0.0096 | 0.547±0.0038    | 0.565±0.0008          |
| Ischemia/reperfusion$^a$ | 11 | 0.239±0.0120 | 0.644±0.0064    | 0.604±0.0018          |
| Preprocessing model$^a$ | 9  | 0.226±0.0114 | 0.637±0.0022    | 0.603±0.0002          |
| Persimmon leaf flavonoid |    |              |                 |                       |
| High-dose$^b$          | 9  | 0.163±0.0064 | 0.614±0.0000    | 0.595±0.0010          |
| Moderate-dose$^b$      | 11 | 0.198±0.0066 | 0.619±0.0033    | 0.598±0.0004          |
| Low-dose$^b$           | 10 | 0.213±0.0111 | 0.628±0.0011    | 0.601±0.0005          |
| Ginaton$^a$            | 12 | 0.148±0.0069 | 0.608±0.0033    | 0.593±0.0002          |

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).
DISCUSSION

The suture method was used to establish a middle cerebral artery occlusion model. 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, 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.

Endothelin, a strong long-acting angiotonic, participates in the pathophysiological process of cerebral hemorrhage, and is associated with the severity of the condition. When vascular endothelial cells are injured, thrombomodulin is released, and plasma thrombomodulin levels increase. 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, and has important significance in thrombosis and determining the severity of a patient’s condition. 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. 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, 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. 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. 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.\textsuperscript{[28]}

**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 the supine position. A median incision was made in the middle of the neck. 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\textsuperscript{[29]}, 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) H₂O₂ 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).

REFERENCES
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