SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 7439-7449, 2017

Neuroprotective effect of total flavonoids from *Ilex pubescens* against focal cerebral ischemia/reperfusion injury in rats

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Received November 2, 2016; Accepted June 26, 2017

DOI: 10.3892/mmr.2017.7540

Abstract. *Ilex pubescens* is commonly used in traditional Chinese medicine to treat cardiovascular and cerebrovascular diseases, such as coronary artery disease and stroke. However, the underlying mechanisms remain to be fully elucidated. The aim of the present study was to investigate the effects of *Ilex pubescens* total flavonoids (IPTF) on neuroprotection and the potential mechanisms in a rat model of focal cerebral ischemia/reperfusion (I/R) injury. Rats were pretreated with intragastric administration of IPTF at doses of 200 and 100 mg/kg for 5 days; middle cerebral artery occlusion surgery was then performed to induce cerebral I/R injury. Neurological deficits were determined using the 5-point neurological function score evaluation system, brain infarct sizes were determined by 2,3,5-triphenyltetrazolium chloride staining and alterations in brain histology were determined by hematoxylin and eosin staining. The neurological deficit score, the infarcted area and the brain tissue pathological injury were significantly reduced when the rats were pretreated with IPTF. In addition, inflammatory mediators and neurotrophic factors in the brain were investigated. IPTF pretreatment decreased the activities of total nitric oxide synthase (TNOS), induced NOS (iNOS) and constitutive NOS (cNOS), and the levels of nitric oxide (NO), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), however, it increased the levels of IL-10 in brain tissues. Furthermore, pretreatment with IPTF also increased the protein expressions of brain-derived neurotrophic factor, glial cell-derived neurotrophic factor and vascular endothelial growth factor, when compared with the model group. In conclusion, the results of the present study demonstrated that IPTF has a neuroprotective effect against focal cerebral I/R injury in rats. The mechanism may be associated with the decreased production of certain proinflammatory cytokines including NO, IL-1β, TNF-α, TNOS, iNOS and cNOS, the increased production of the anti-inflammatory cytokine IL-10 and the increased secretion of neurotrophic factors.

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Abbreviations: I/R, ischemia/reperfusion; IPTF, *Ilex pubescens* total flavonoids; MCAO, middle cerebral artery occlusion; CCA, common carotid artery; ICA, internal carotid artery; TTC, 2,3,5-triphenyltetrazolium chloride; NOS, nitric oxide synthase; TNOS, total NOS; iNOS, induced NOS; cNOS, constitutive NOS; eNOS, endothelial constitutive NOS; nNOS, nervous constitutive NOS; NO, nitric oxide; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; BDNF, brain-derived neurotrophic factor; VEGF, vascular endothelial growth factor; GDNF, glial cell-derived neurotrophic factor; TCM, traditional Chinese medicine

Key words: *Ilex pubescens* total flavonoids, focal cerebral ischemia/reperfusion injury, neuroprotection, inflammatory cytokine, neurotrophic factor

Introduction

Stroke has become a leading cause of mortality and disability worldwide. In the USA and the UK, the morbidity of heart disease and cancer is reportedly 0.2% of the population each year. In China, ~200 million people are affected by stroke every year, and as a result, 70-80% of these patients will suffer with life-changing disabilities (1). Strokes can be ischemic or hemorrhagic in nature; ~80% of strokes are ischemic stroke (2). Currently, therapy for ischemic stroke remains limited, and vascular recanalization and antiplatelet therapy are the only two therapeutic strategies that have been proven to be effective during the acute phase of ischemic stroke (3). For acute ischemic stroke, intravenous thrombolytic therapy within 4.5 h following the acute stroke is the most widely accepted method for vascular recanalization. but the narrow time window, hemorrhage complications and the low recanalization rates in patients with macrovascular occlusion often limit its use (4). Antiplatelet therapy is an important strategy for preventing recurrent stroke by simultaneously blocking different platelet activation pathways. Aspirin is the most widely used antiplatelet agent in current use, however, it has been reported that combination antiplatelet therapy with aspirin and clopidogrel increased the risk of hemorrhage complications (5,6). Therefore, novel, effective and widely applicable pharmacological treatments are urgently required.

The pathophysiology of stroke is very complex and involves a number of factors, including inflammation, oxidative stress, apoptosis and excitotoxicity (7). Ischemic stroke induces the expression of high levels of adhesion molecules, cytokines and inflammatory mediators, including nitric oxide (NO), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These high levels lead to marked local and systemic inflammatory responses (8,9). Inflammation itself may exacerbate the spread of damage to the ischemic penumbra, increase the levels of inflammatory cytokines and thus, further aggravate brain injury (10). Therefore, the identification of novel drugs to regulate the inflammatory response is a promising method for the treatment of cerebral ischemic injury. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, serves an important role in the development and plasticity of the brain. It also protects the brain against injuries induced by stress conditions such as ischemia and trauma, by promoting nerve regeneration, preventing neuronal death, and regulating synaptic plasticity and cell survival (11). Glial cell-derived neurotrophic factor (GDNF) is another important neurotrophin, which serves an important role in reducing apoptosis in neurons and promoting neurological outcomes following hypoxia-ischemia, by inhibiting the production of caspase-3, TNF-α, NO and induced nitric oxide synthase (iNOS) (12). Vascular endothelial growth factor (VEGF) is an angiogenic peptide involved in nerve vascular remodeling, promoting growth in neurons and glial cells, and protecting neuronal tissues from cell death induced by hypoxia or ischemia in order to achieve neuroprotection (13). These observations have indicated that BDNF, GDNF and VEGF may be potential therapeutic agents for the prevention of neuronal cell death following cerebral ischemia.

Traditional Chinese medicine (TCM) is used due to its rich sources, low costs, few side effects and its multi-targeting effects (14,15). Therefore, it is important to investigate the effects and mechanisms associated with anti-cerebral ischemia drugs used in TCM. The TCM Mao Dong Qing, the dry roots of *Ilex pubescens* Hook. et Arn, is an evergreen shrub that is predominantly found in the southern regions of China. It has been used primarily for the treatment of hypercholesterolemia and cardiovascular diseases, such as stroke and coronary artery disease, in addition to peripheral vascular disease (16-18). Preliminary studies by our group identified the beneficial effects of *Ilex pubescens* total flavonoids (IPTF) in rat and mice models of cerebral ischemia (19-21). However, its underlying mechanism and compound activity with regard to its effects against focal cerebral ischemia injury remain to be elucidated. In the present study, the effect of IPTF on a rat model of focal cerebral ischemia/reperfusion (I/R) injury, induced by middle cerebral artery occlusion (MCAO), was investigated. The potential mechanisms underlying the alterations in the levels of inflammation-associated molecules and the increased secretion of neurotrophic factors were also examined.

Materials and methods

Plant collection and identification. Ilex pubescens was purchased from Henan Province Pharmaceutical Co., Ltd. (Zhengzhou, China) and was identified by Professor Chengming Dong and Professor Suiqing Chen from the Department of Medicinal Plants, School of Pharmacy, Henan University of Chinese Medicine (Zhengzhou, China). The voucher specimen (no. XX20140916001) was kept in the group's laboratory.

Preparation of extracts. The total flavonoids were obtained from *Ilex pubescens* using the method described previously (22). Briefly, air-dried *Ilex pubescens* was treated twice with 70% aqueous ethanol (1:10, w/v) for 1.5 h, then a further 1 h under reflux conditions at 80˚C. The filtered extracts were concentrated in a vacuum evaporator at 60˚C to obtain the crude extracts. The crude extracts were dissolved in water (adjusted to pH 4.5), and loaded into glass columns, which were wet-packed with AB-8 macroporous resin. Following elution with water and 10% aqueous ethanol, the elutriant with 40% ethanol was collected, dried using a vacuum at 60˚C and then stored at 4˚C. The total flavonoid content in the extract was >50%, as determined by ultraviolet spectrophotometry, performed as described in a previous study (23).

Animal treatment and administration. A total of 96 adult male Wistar rats [SPF grade; age, 8 weeks; weight, 280-300 g; Laboratory Animal Center of Hebei Province, Shijiazhuang, China (certificate no. 907048)], were employed in the present study. Rats were housed in an environmentally controlled breeding room (22-24˚C; 50-55%; 12-h dark/light cycle), with free access to standard laboratory food and water. The present study was approved by the Ethics Committee of Henan University of Chinese Medicine (Henan, China). Rats were divided into the following 4 groups (n=24/group): Sham operation group [sham; intragastric (ig) administration of saline and surgery without MCAO], ischemic group (model group; MCAO surgery with ig administration of saline), and the third and fourth groups were pretreated with a high (200 mg/kg/day; IPTF-1 group) or low ig dose of IPTF (100 mg/kg/day; IPTF-2) for 5 days, followed by the induction of I/R (MCAO surgery + IPTF pretreatment groups).

Induction of focal cerebral I/R via MCAO surgery. An hour following the last administration of IPTF pretreatment, all rats were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally; Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China) and MCAO surgery was then performed according to Koizumi *et al* (24) and Nagasawa and Kogure (25) with a few modifications. Briefly, the left common carotid artery (CCA) and internal carotid artery (ICA) were isolated via a cervical midline incision. A nylon monofilament pre-coated with silicone rubber (0.26 mm external diameter) was inserted into the CCA and advanced into the ICA to ~19-20 mm from the left CCA bifurcation to occlude the middle cerebral artery. Then, 2 h after the induction of ischemia, the filament was slowly withdrawn and animals were returned to their cages for 22 h of reperfusion. Rats in the sham operation group received the same surgical procedures without filament insertion to induce MCAO. Rat body temperature was maintained at 37.0˚C using an infrared lamp throughout surgical procedures. After the surgery, 22 rats in the sham group, 20 rats in the model group, 20 rats in the IPTF-1 group and 22 rats in the IPTF-2 group survived, respectively.

Neurological deficit scores. Neurological deficit scores were recorded for each rat 22 h after ischemic injury

(n=10-11 rats/group) in a blind-controlled manner, according to Longa's five-point scale method (26). The following grading system was applied: Grade 0, no deficit; grade 1, failure to extend right forepaw; grade 2, circling to right; grade 3, falling to right; grade 4, inability to walk spontaneously and a reduced level of consciousness.

Cerebral infarct volume measurement. Following behavioral testing, rats (n=10-11 rats/group) were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally) and sacrificed, and the brain tissues removed and separated into 6 coronal slices of 2-mm thickness. Brain slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; cat. no. T8877; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution in the dark at 37˚C for 15 min. The cerebral infarct volume was measured using the method described by Belayev *et al* (27). In order to eliminate the contribution of swelling/edema to the ischemic lesion, the cerebral infarct volume was calculated with the following formula: Corrected infarct volume = contralateral hemisphere volume - (ipsilateral hemisphere volume - measured infarct volume) (28).

Tissue preparation. The remaining rats (n=10-11/group) were anesthetized with 10% chloral hydrate (300 mg/kg intraperitoneally), which was followed by rapid removal of the brain. The olfactory bulbs were removed and the brains were cut coronally into two parts at the optic nerve intersection. The first part of the tissue beginning at the rostral end was used to produce a 10% brain homogenate with saline, followed by centrifugation at 2,300 x g for 15 min at 4˚C. The supernatant was collected and stored at -20˚C until assay experiments. The remaining tissue was post-fixed in 4% paraformaldehyde (cat. no. AR1068; Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) in 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4; cat. no. P1010; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 4˚C for 1 week for histological and immunohistochemical examination.

Detection of the levels of IL-1b, TNF-α and IL-10 in brain homogenate. The supernatant of the brain homogenate was used to detect the levels of IL-1β (cat. no. SXM026), TNF-α (cat. no. SX01165) and IL-10 (cat. no. SXB005) using ELISA kits (all from Shanghai Senxiong Biotech Industry Co., Ltd., Shanghai, China) according to the manufacturer's protocols.

Detection of the content of NO and the activities of total NOS (TNOS) and iNOS in brain homogenate. The content of NO in brain tissue homogenate was assayed using the nitrate reductase method and an NO assay kit (cat. no. A012; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The level was expressed as μmol/g protein. The activities of TNOS and iNOS were measured using TNOS and iNOS assay kits (NOS kit, cat. no. A014-1; TP kit, cat. no. A045-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions; results are expressed as U/mg protein.

Hematoxylin and eosin (H&E) staining. A total of 6 fixed brain tissues were randomly selected from each group, dehydrated with graded alcohol series (80, 95 and 100%) and embedded in paraffin. Then, sliced into coronal 5-μm-thick sections, at least 6 from every sample. Then standard H&E staining was performed according to H&E staining kit (cat. no. G1120; Beijing Solarbio Science & Technology Co., Ltd.). Briefly, the sections were dewaxed with dimethylbenzene (Yantai Shuangshuang Chemical Co., Ltd., Yantai, China), and the slices were rehydrated with graded alcohol series (100, 95, 80 and 70%) for 5 min in each concentration and washed with running water twice in turn. Then, the slices were immersed in Harris hematoxylin for 5 min, differentiated for 0.5 min, and immersed in tap water for 15 min to return the nuclei to blue. Next, the slices are immersed in 70-80% alcohol for 1 min and in 0.5% eosin solution for 2 min. dehydrated with graded alcohol series (95 and 100%) for 2 min in each concentration, cleared in dimethylbenzene and sealed with neutral balsam. All of the steps above were carried out at room temperature. Neuronal death in the cortical subfield of the ischemia side was evaluated under a light microscope in 2 high power microscopic fields (magnification, x400; Olympus BX50; Olympus Corporation, Tokyo, Japan). They were then scored with the following histological grading system (29): I, a few neurons damaged; II, numerous neurons damaged; III, majority of neurons damaged; IV, vast majority of neurons damaged.

Immunohistochemical staining. The remaining paraffin-embedded sections were used to detect the expressions of BDNF, GDNF and VEGF. Briefly, 3 sections were deparaffinized, rehydrated with a graded alcohol series and washed with PBS. Then the sections were incubated in 3% H_2O_2 at room temperature for 10 min to quench the endogenous peroxidase activity and rinsed in PBS. Following these procedures, an antigen retrieval procedure was performed by treating the samples in 0.01 M sodium citrate buffer solution (pH 6.0, cat. no. C1120; Beijing Solarbio Science & Technology Co., Ltd.) at 98-100˚C for 20 min. Samples were then subjected to incubation in 5% normal goat serum (Wuhan Boster Biological Technology Co., Ltd.) in 0.01 M PBS for 30 min at room temperature. Sections were incubated with rabbit polyclonal anti-BDNF, anti-GDNF or anti-VEGF antibodies (anti-BDNF; cat. no. BA0565-1; anti-GDNF; cat. no. BA0890 and anti-VEGF, cat. no. BA0407; all 1:50 and from Wuhan Boster Biological Technology Co., Ltd.), respectively, in PBS for 2 h at 37˚C. The negative control sections were incubated with 5% normal goat serum for 2 h at 37˚C. Sections were then incubated with biotin-labeled secondary antibody (polyclonal goat anti-rabbit IgG/streptavidin antibody; 1:50; OriGene Technologies, Inc., Beijing, China) at 37˚C for 30 min, and then incubated with a Strept-Avidin-Biotin-Peroxidase complex (SABC-POD; cat. no. SA1025; Wuhan Boster Biological Technology Co., Ltd.) for 30 min at 37˚C. The results were visualized using a diaminobenzidine kit (DAB, cat. no. AR1022, Wuhan Boster Biological Technology Co., Ltd.). Finally, the sections were mounted onto polylysine-coated slides and all slides were photographed under a light microscope (magnification, x400; Olympus BX50). The intensity of BDNF, GDNF and VEGF in the cortex and hippocampus CA1 in each section was evaluated using Image-Pro Plus, version 5.1 (Media Cybernetics, Inc., Rockville, MD, USA) in two high power microscopic fields, and expressed as the positive signal area and the integrated optical density.

Table I. Effect of IPTF on the neurological deficit scores of rats following MCAO.

Group	n	NDS
Sham	11	0.00±0.00[a]
Model	10	2.50±0.71
IPTF-1	10	1.80±0.63[b]
IPTF-2	11	2.00±0.79

Values are expressed as the mean ± standard deviation. The following scoring system was used: 0, no deficit; 1, failure to extend right forepaw; 2, circling to right; 3, falling to right; 4, inability to walk spontaneously and a reduced level of consciousness. [a]P<0.01 and [b]P<0.05 vs. model group. IPTF, *Ilex pubescens* total flavonoids; NDS, neurological deficit score; model, middle cerebral artery occlusion without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF.

Statistical analysis. All statistical analyses were performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Enumeration data were analyzed using the Kruskal-Wallis test, and the Mann-Whitney U-test was conducted for comparisons between groups. Measurement data were analyzed by a one-way analysis of variance and a least significant difference post hoc test was used for comparisons between groups. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

IPTF treatment reduces brain damage in cerebral ischemic rats induced by MCAO. Neurological deficit scores, cerebral infarct volume and brain histopathology were investigated to determine the neuroprotective effects of IPTF in cerebral ischemic rats induced by MCAO. The results of the neurological deficit scores are displayed in Table I. The neurological deficit score of the model group was 2.50±0.71 and when compared with the sham operation group there was a significant difference (P<0.01). Also, when compared with the model group, pretreatment with 200 mg/kg IPTF significantly reduced the impairment in neurological function induced by I/R (IPTF-1 group; neurological deficit score, 1.80±0.63; P<0.05). The results indicated that IPTF pretreatment may improve behavioral injury in rats following MCAO.

The cerebral infarct volume was determined by TTC staining. The infarcted tissue was stained white and normal tissues were red. When compared with the model group, IPTF pretreatment (200 and 100 mg/kg) significantly reduced the infarct volume induced by MCAO (Fig. 1A and B).

The results of H&E staining revealed that the cell membrane and nucleus of neurons in the cerebral cortex subfield were clearly visible in the sham operation group. When compared with the sham operation group, marked pathological lesions were visible in the I/R model group. Neurons that were swollen, atrophic, lightly stained and undergoing necrosis were observed in the I/R model group. Pathological lesions induced by cerebral ischemia were markedly reduced when compared with the model group in the IPTF pretreatment groups. The results of the corresponding histological grading supported the histological results, the number of neurons with edema, that were atrophic or necrotic, were reduced in the IPTF pretreatment groups, particularly in the high-dose IPTF group (IPTF-1; P<0.05; Figs. 1C and 2). These results illustrated that

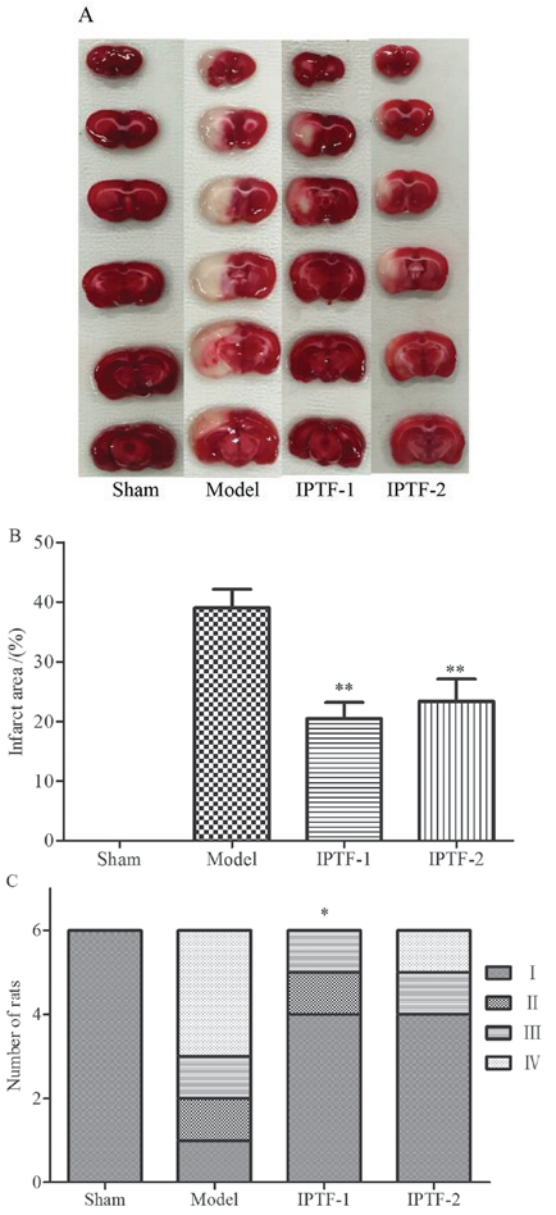

Figure 1. Effect of IPTF on cerebral infarction and the histological grades in the cortex of MCAO model rats. (A) Representative 2,3,5-triphenyltetrazolium chloride-stained brain coronal sections obtained from the sham operated, model (MCAO operated), IPTF-1 (200 mg/kg pretreatment) and IPTF-2 (100 mg/kg pretreatment) groups. The normal tissue was stained red while the infarct area was white. (B) v rate identified in the four groups. Results were quantified using ImageJ software and values are expressed as the mean ± standard deviation. (C) Neuronal death in the cortical subfield of ischemia side was evaluated under a light microscope and scored by histological grade (n=6 random rat brain tissues/group). Data were analyzed using the Kruskal-Wallis test. The number of rat tissues exhibiting each histological grade is presented. The following histological grading system was used: I, a few neurons damaged; II, numerous neurons damaged; III, majority of neurons damaged; IV, vast majority of neurons damaged. *P<0.05 and **P<0.01 vs. model group. IPTF, *Ilex pubescens* total flavonoids; MCAO, middle cerebral artery occlusion; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF.

Table II. Effect of IPTF on the content of IL-1β, TNF-α and IL-10 in the brain tissue of rats with middle cerebral artery occlusion.

Group	n	IL-1β (ng/g prot)	TNF-α (ng/g prot)	IL-10 (ng/g prot)
Sham	10	9.83±2.76[a]	2.00±0.95[b]	9.34±4.06
Model	9	13.01±2.86	4.24±1.61	12.00±3.24
IPTF-1	9	9.28±3.89[a]	2.85±1.19[a]	19.75±10.07[a]
IPTF-2	9	11.85±3.34	2.84±1.54[a]	18.08±10.38

Values are expressed as the mean ± standard deviation. [a]P<0.05 and [b]P<0.01 vs. model group. IPTF, *Ilex pubescens* total flavonoids; IL, interleukin; TNF-α, tumor necrosis factor-α; model, middle cerebral artery occlusion without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF.

Table III. Effect of IPTF on the activities of total-, induced- and constitutive nitric oxide synthase, and the content of nitric oxide in brain tissues of rats with middle cerebral artery occlusion.

Group	n	TNOS (U/mg prot)	iNOS (U/mg prot)	cNOS (U/mg prot)	NO (μmol/g prot)
Sham	11	1.86±0.79[a]	0.49±0.23[a]	1.02±0.32[a]	5.64±6.11[a]
Model	10	2.85±0.53	0.89±0.26	2.04±0.59	14.96±5.26
IPTF-1	10	1.58±0.70[a]	0.50±0.17[a]	0.90±0.48[a]	10.27±4.00[b]
IPTF-2	11	1.48±0.40[a]	0.41±0.19[a]	1.08±0.43[a]	9.57±4.32

Values are expressed as the mean ± standard deviation. [a]P<0.01 and [b]P<0.05 vs. model group. IPTF, *Ilex pubescens* total flavonoids; NOS, nitric oxide synthase; TNOS, total NOS; iNOS, induced NOS, cNOS, constitutive NOS; NO, nitric oxide; model, middle cerebral artery occlusion without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF.

pretreatment with IPTF may reduce brain injury induced by MCAO in rats.

Effects of IPTF on the inflammatory response in cerebral ischemic injury. The levels of IL-1β, IL-10 NO and TNF-α in rat brains were detected in order to determine the influence of IPTF on the inflammatory response in cerebral I/R injury. The results are presented in Table II. The levels of IL-1β and TNF-α in the ischemic hemispheres of the model group were significantly increased when compared with the sham operation group (P<0.05 and P<0.01, respectively). The level of IL-1β in the IPTF-1 treatment group (200 mg/kg IPTF) was significantly decreased when compared with the model group (P<0.05). In addition, the levels of TNF-α were significantly decreased in the two IPTF pretreatment groups (P<0.05) when compared with the model group. By contrast, the expression of IL-10 in the model group exhibited no significant change when compared with the sham operation group. The 100 mg/kg IPTF pretreatment group had a tendency to increased expression of IL-10, but no significant difference (P>0.05) was observed when compared with the model group. However, it was significantly increased in the 200 mg/kg IPTF treatment group (IPTF-1; P<0.05; Table II).

NO, as a pre-inflammatory mediator, is derived from NOS and serves an important role in cerebral ischemia and the effects of an ischemic insult (30). Table III indicates that the levels of TNOS, iNOS, constitutive NOS (cNOS) and NO in the ischemic hemispheres were significantly increased in model group when compared with the sham operation group. In addition, IPTF pretreatment (100 and 200 mg/kg) significantly downregulated the levels of TNOS, iNOS, cNOS and NO in the ischemic hemispheres of rats when compared with the model group (P<0.05 and P<0.01; Table III).

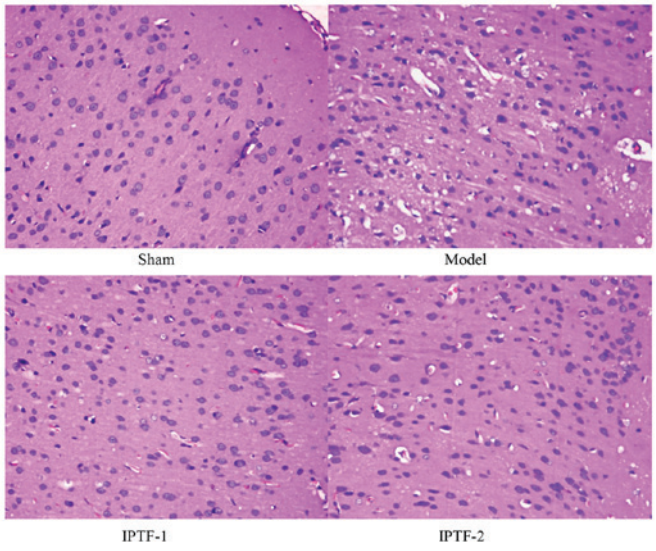

Figure 2. Effect of IPTF on the histopathological changes in the cortex of rats following MCAO. Representative images of hematoxylin and eosin stained tissues are presented (magnification, x400). IPTF, *Ilex pubescens* total flavonoids; MCAO, middle cerebral artery occlusion; model, MCAO operated without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF.

In conclusion, the results demonstrated that IPTF reduced the inflammatory response by regulating the levels of IL-1β,

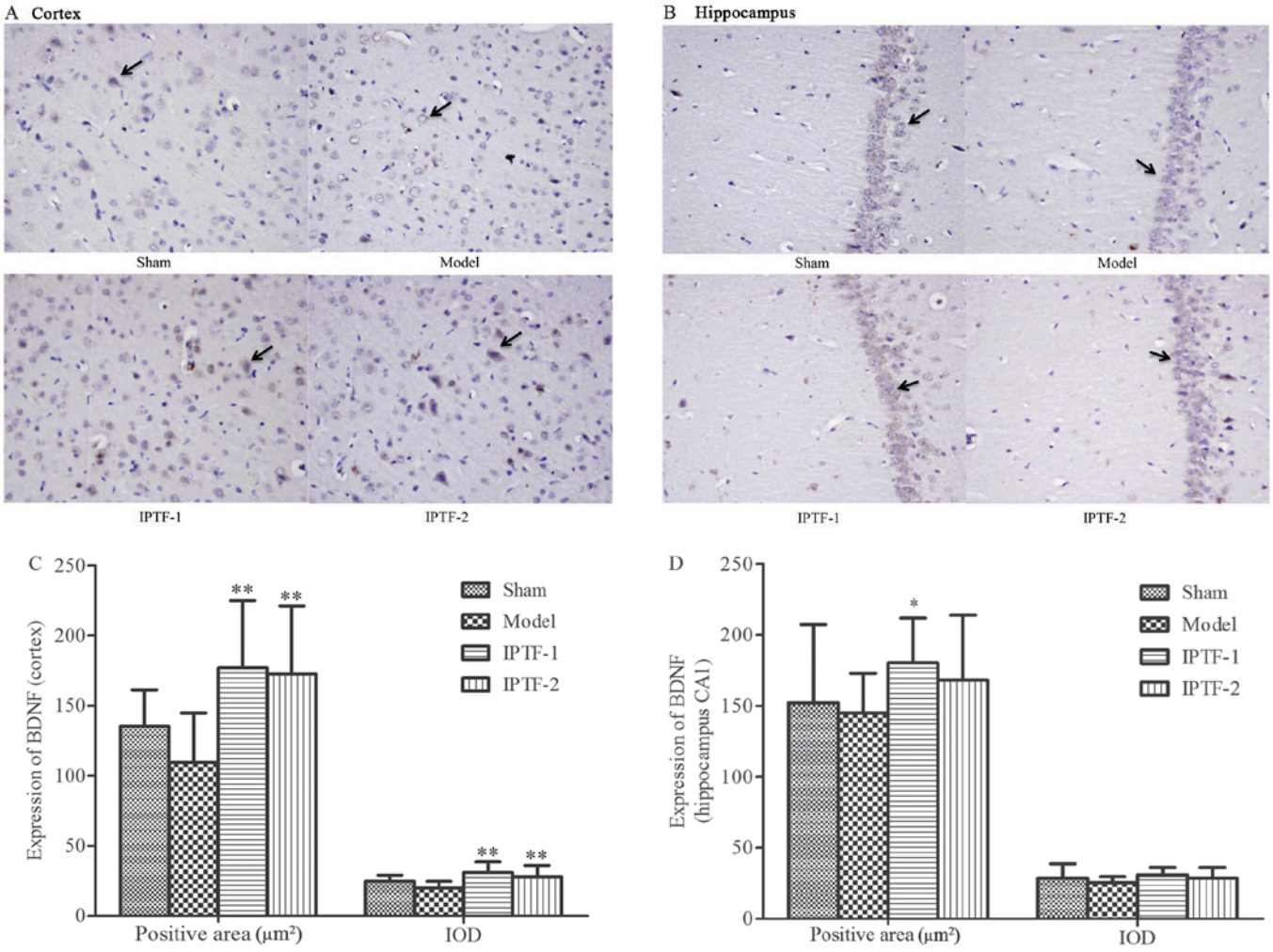

Figure 3. Effect of IPTF on the expression of BDNF in the cortex and hippocampus CA1 subfields of MCAO rats. Protein expression levels of BDNF were detected by immunohistochemical staining in (A) the cortex and (B) in hippocampus CA1 subfields. BDNF is stained brown and indicated by arrows; it was primarily localized in the cytoplasm of neurons. Magnification, x400. BDNF-positive immunohistochemical products in (C) the cortex and (D) hippocampus CA1 subfields were counted using the Image-Pro Plus 5.1 system, and are expressed as the positive signal area (μm^2) and the IOD. Values are expressed as the mean ± standard deviation (n=6 random rat brain tissues/group). *P<0.05 and **P<0.01 vs. model group. IPTF, *Ilex pubescens* total flavonoids; BDNF, brain-derived neurotrophic factor; MCAO, middle cerebral artery occlusion; model, MCAO operated without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF; IOD, integrated optical density.

IL-10, TNF-α and NO and the activity NOS against ischemic injury.

Effects of IPTF on the expression of BDNF, GDNF and VEGF in the cortex and hippocampus CA1 subfields of MCAO rats. The expressions of BDNF, GDNF and VEGF in the cortex and hippocampus CA1 subfields were detected by immunohistochemical staining. Immunohistochemistry demonstrated that IPTF pretreatment significantly increased the expression of BDNF, GDNF and VEGF in cortex and hippocampus CA1 subfields of ischemic hemispheres when compared with the model group (P<0.05 and P<0.01; Figs. 3-5).

Discussion

Mao Dong Qing is derived from the dry roots of *Ilex pubescens* Hook et Arn. The extracts prepared from the *Ilex pubescens* roots have a number of biological activities including analgesic, dilating blood vessels, reducing plasma viscosity, inhibiting platelet aggregation, thrombus and enhancing hypoxia-resistance ability (17,31-34). The authors' previous studies (19-21) demonstrated that flavonoids are the prominent active compound in *Ilex pubescens* and they had neuroprotective effects against cerebral ischemia, which, to the best of our knowledge, has not been reported previously. However, the specific mechanisms underlying the neuroprotective effects of *Ilex pubescens* remain unknown. In the present study, the potential anti-inflammatory and neuroprotective effects of IPTF were examined.

It was observed that IPTF pretreatment effectively improved neurological deficits, reduced cerebral infarct volume and attenuated brain pathological lesions in a dose-dependent manner in rats with cerebral I/R injury (Figs. 1 and 2; Tables I and II). Therefore, these results observations indicate that IPTF has neuroprotective effects on the cerebral ischemic injury.

The inflammatory response is partly attributable to the pathogenic progression of cerebral ischemic injury, and the associated inflammatory cells and mediators (35). It is well known that cytokines are a group of small glycoproteins (~25 kDa) that serve important roles in the immune response.

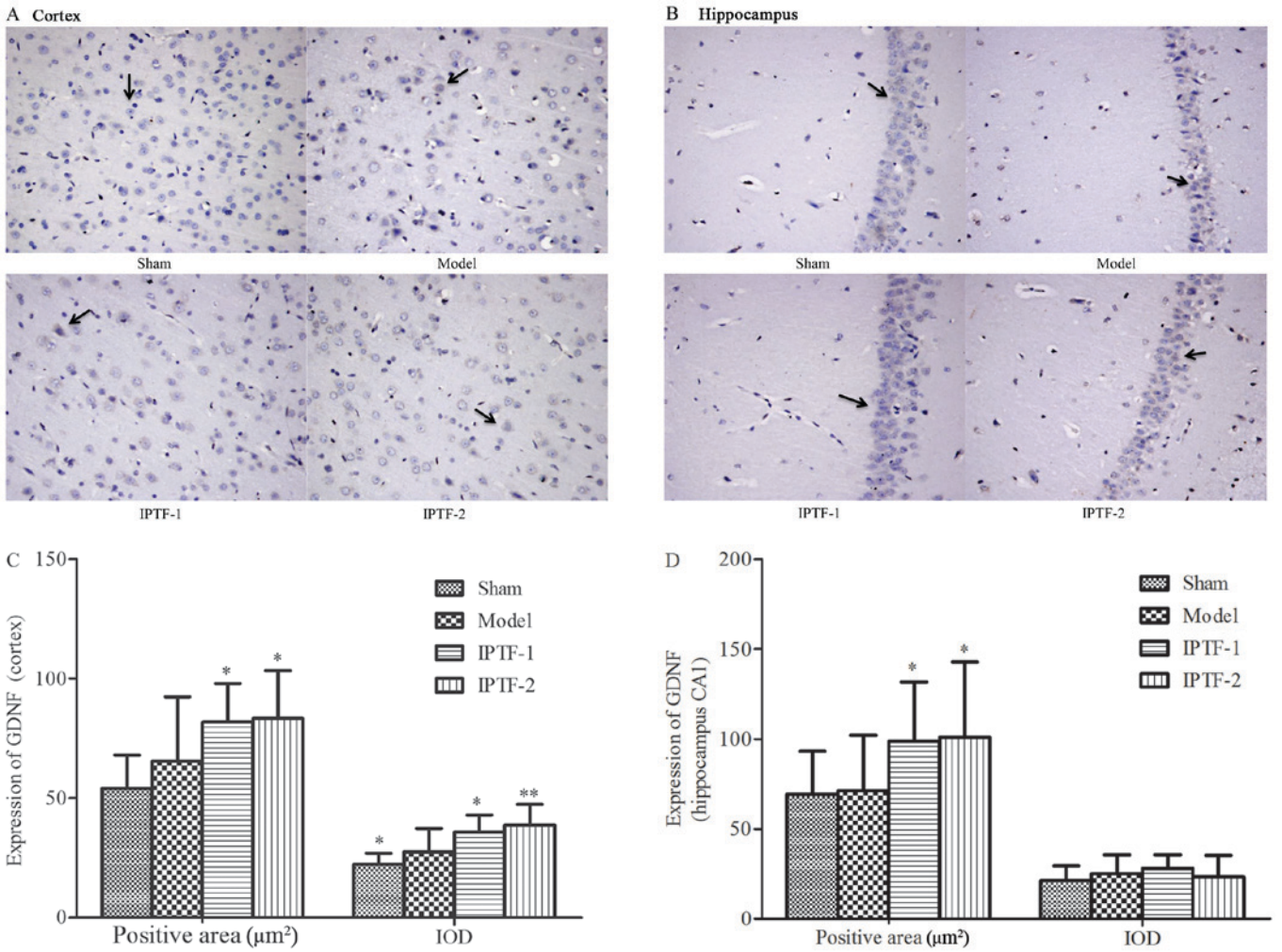

Figure 4. Effect of IPTF on the expression of GDNF in the cortex and hippocampus CA1 subfields of MCAO rats. Protein expression levels of GDNF were detected by immunohistochemical staining in (A) the cortex and (B) the hippocampus CA1 subfields. GDNF is stained in brown and is indicated by arrows; it was primarily localized in the cytoplasm of neurons and gliacytes. Magnification, x400. GDNF positive immunohistochemical products in the (C) cortex and (D) hippocampus CA1 subfields were counted using Image-Pro Plus 5.1 software, and are expressed as the positive signal area (μm^2) and the IOD. Values are expressed as the mean ± standard deviation (n=6 random rat brain tissues/group). $^{*}P<0.05$ and $^{**}P<0.01$ vs. model group. IPTF, *Ilex pubescens* total flavonoids; GDNF, glial cell-derived neurotrophic factor; MCAO, middle cerebral artery occlusion; model, MCAO operated without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF; IOD, integrated optical density.

Cytokines are produced in response to a variety of stimuli including pathogens, inflammation or injury, and exert their effects by binding to their receptors. The major cytokines associated with inflammation in cerebral ischemic injury are IL-1β, TNF-α and IL-10 (36). Under normal conditions, there is a balance between the pro-inflammatory cytokines IL-1β and TNF-α, and the anti-inflammatory cytokine IL-10. Inflammatory cells secrete proinflammatory and anti-inflammatory cytokines during cerebral ischemic injury; their interactions determine the progression of inflammation. When excessive levels of pro-inflammatory cytokines are released as opposed to anti-inflammatory cytokines, inflammation is promoted, thus inducing tissue damage. The levels of IL-1β and TNF-α were upregulated following MCAO or global brain ischemia, however, the level of IL-10 was downregulated (37). A previous study demonstrated that the proinflammatory cytokines IL-1β and TNF-α were important mediators involved in brain injury, and IL-10 was observed to inhibit the production of pre-inflammatory cytokines, thereby exhibiting neuroprotective properties (38). The present study confirmed that IL-1β and TNF-α were induced by ischemia; IL-1β was downregulated in the brain tissues of the IPTF-1 group (200 mg/kg IPTF pretreatment) and TNF-α was downregulated in the brain tissues of both IPTF pretreatment groups (200 and 100 mg/kg; IPTF-1 and -2, respectively). The level of IL-10 in the brain tissue increased in response to cerebral ischemic injury when comparing the model and sham operation groups, however there was no significant difference, while the levels significantly increased when pretreated with 200 mg/kg IPTF (IPTF-1 vs. model group).

NO is a pre-inflammatory mediator derived from NOS that is involved in inflammation during cerebral ischemic injury. There are at least two isoforms of NOS: cNOS and iNOS (39-41). The cNOS class is comprised of endothelial constitutive NOS (eNOS), which is present in vascular endothelial cells, and nervous constitutive NOS (nNOS), which is present in the central and peripheral nervous system (42,43). eNOS and nNOS are predominantly expressed in the early phase of the ischemia, whereas iNOS is primarily expressed in the late phase. In the early phase of cerebral ischemia, NO is

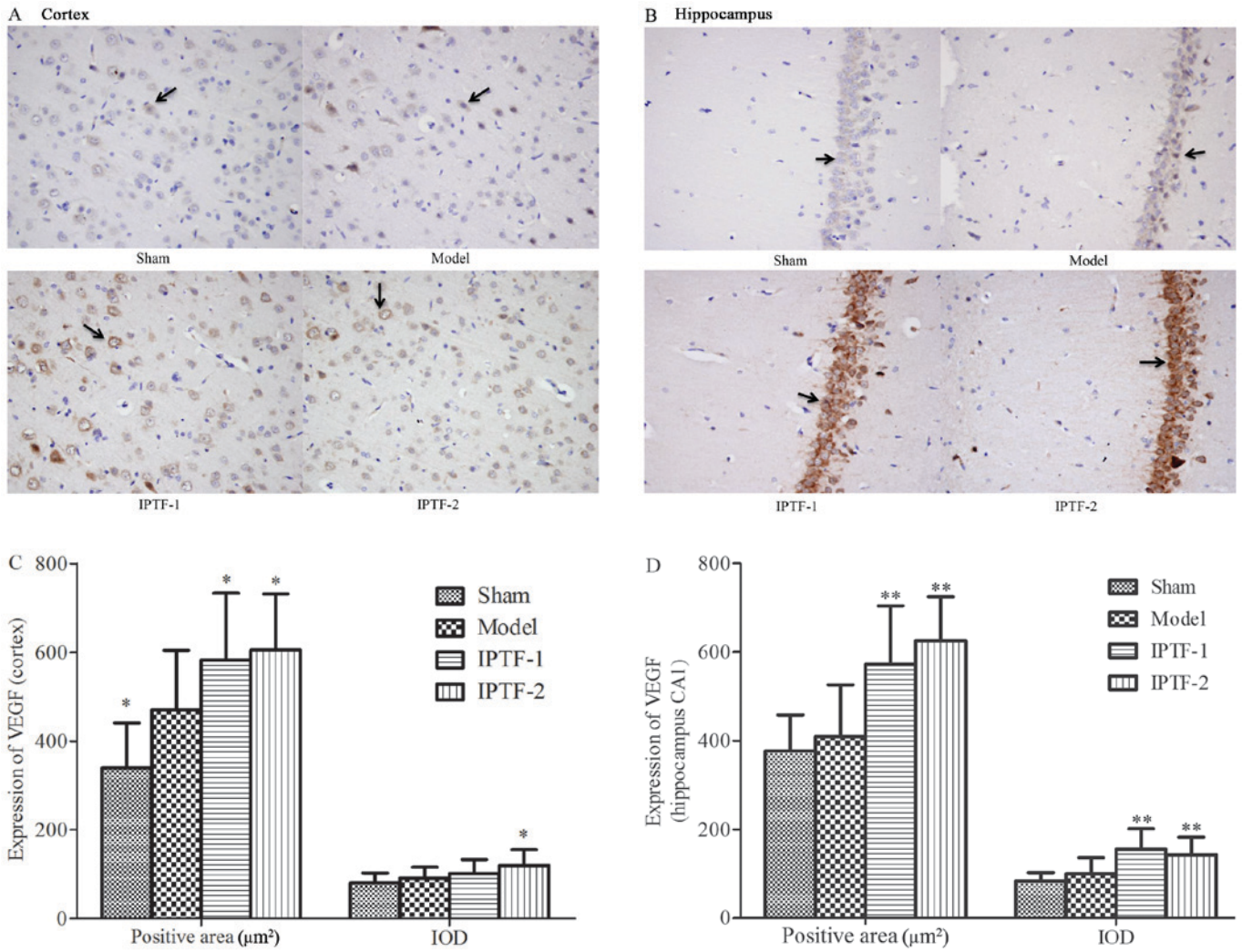

Figure 5. Effect of IPTF on the expression of VEGF in the cortex and hippocampus CA1 subfields of MCAO rats. Protein expression levels of VEGF were detected by immunohistochemical staining in (A) the cortex and (B) the hippocampus CA1 subfields. VEGF is stained in brown and is indicated by arrows; it was primarily localized in the cytoplasm of neurons and vascular endothelial cells. Magnification, x400. VEGF positive immunohistochemical products in the (C) cortex and (D) hippocampus CA1 subfields were counted using Image-Pro Plus 5.1 software, and are expressed as the positive signal area (μm^2) and the IOD. Values are expressed as the mean ± standard deviation (n=6 random rat brain tissues/group). $^{*}P<0.05$ and $^{**}P<0.01$ vs. model group. IPTF, *Ilex pubescens* total flavonoids; VEGF, vascular endothelial growth factor; MCAO, middle cerebral artery occlusion; model, MCAO operated without pretreatment; IPTF-1, rats pretreated with 200 mg/kg IPTF; IPTF-2, rats pretreated with 100 mg/kg IPTF; IOD, integrated optical density.

predominantly produced by nNOS to cause brain damage, and in the late phase, NO is predominantly produced by iNOS (44). In addition, iNOS mediates cerebral ischemic injury, potentially via the action of NO on the mitochondrial respiratory chain, resulting in energy depletion (30). A previous study has indicated that the expression levels of iNOS and nNOS increased, however, eNOS expression decreased in MCAO rats (45). A nNOS blocker, 7-NI, has been observed to attenuate the disruption in the blood-brain barrier following transient focal cerebral ischemia; it also improved neurological deficits and reduced the area of cerebral infarction (46,47). In the present study, the level of NO, and the activities of TNOS, iNOS and cNOS were detected 24 h following the induction of cerebral ischemia. The levels of NO, TNOS, iNOS and cNOS significantly increased in the MCAO model group, which is consistent with the results of previous studies (8-10). The levels of NO, TNOS, iNOS and cNOS significantly decreased in the two IPTF pretreatment groups when compared to the model group. These results indicated that the neuroprotective effects of IPTF might be achieved via a decrease in the levels of inflammation-associated molecules in brain tissues.

Neurotrophins are a family of peptide growth factors that include BDNF, GDNF and VEGF. These growth factors have been demonstrated to protect neurons from damage, and are therefore very important in treatments for neurological disorders including Parkinson's disease, ischemic stroke and Alzheimer's disease (11,13,48-53). Neurotrophins have also been reported to improve angiogenesis, neurogenesis and neurite outgrowth in the brain, and overexpression of these factors has demonstrated their therapeutic actions in cerebral ischemia (54). BDNF was first identified in cerebral ischemia, promoting neurological function recovery and improving neurogenesis (55).

Previous research has demonstrated that the mechanism underlying the neuroprotective effect of GDNF is associated with antiapoptosis and antioxidative effects, and also the induction of progenitor cell proliferation in rats with stroke (56-58). A previous report indicated that BDNF and GDNF may be

potential therapeutic target proteins for ischemic stroke (59). Therefore, the present study investigated the changes in BDNF and GDNF expression in the cortex and hippocampus CA1 of MCAO rats pretreated with IPTF. The results indicated that the expression of BDNF and GDNF in the cortex and hippocampus CA1 subfields were upregulated in the two IPTF groups following cerebral I/R. Taking the results of the aforementioned previous studies into account, it was proposed that the effect of IPTF on reducing neuronal necrosis may be associated with the upregulation of BDNF and GDNF expression.

A previous study indicated that the expression of VEGF increases following cerebral I/R (60). VEGF is a factor associated with angiogenesis and vascular permeability. Hypoxic conditions can induce the transcriptional and post-transcriptional regulation of VEGF expression in the brain tissue, which is associated with an increase in angiogenesis (61-64). The expression of VEGF is also increased follow cerebral ischemia, which accelerates the formation of novel blood vessels in the brain (65,66). It has been reported that the neuroprotective effects of VEGF are primarily focused on activating the phosphoinositide 3-kinase/protein kinase B pathway, inhibiting the activation of caspase-3, the specific potassium currents, the extracellular signal-regulated kinase signaling pathway and the endoplasmic reticulum stress pathway, and increasing the proliferation, migration and differentiation of neuronal progenitors simultaneously (13). In the present study, the expression of VEGF was observed in the hippocampus CA1 and cortex and the findings were consistent with those of previous reports (67,68). The results demonstrated that the expression of VEGF in the hippocampus CA1 and cortex were upregulated in the MCAO model group, particularly in the cortex, which was significantly different to the expression observed in the sham operation group. In addition, pretreatment with IPTF significantly upregulated the expression of VEGF in comparison with the model group. These results indicated that the neuroprotective effects of IPTF may be achieved via the upregulated expression of VEGF in the hippocampus CA1 and cortex.

The recovery of nervous function is a complex process, which is the result of interactions between a number of factors (69-74). The behavioral tests used in the present study were designed to evaluate the level of brain damage in rats, and neurological deficit scores were significantly decreased in rats pretreated with a high dose of IPTF. In addition, IPTF ameliorated the histological injury in brain tissues. In conclusion, the results of the present study suggested that IPTF pretreatment may have neuroprotective effects in cerebral ischemic injury, which may be closely associated with the decreased production of certain proinflammatory cytokines including NO, IL-1β and TNF-α, the increased production of the anti-inflammatory cytokine IL-10, the inhibition of TNOS, iNOS and cNOS activities, and the upregulation of BDNF, GDNF and VEGF expression. With further elucidation of its underlying mechanisms of action and clinical verification, IPTF may be a potential neuroprotective treatment for cerebral ischemic injuries.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. U1204827 and 81503206) and the Fundamental Scientific Research Funds for the Henan Universities (grant no. 2014KYYWF-QN27). The authors would like to thank Professor Chengming Dong and Professor Suiqing Chen (Department of Medicinal Plants, School of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, China) for identifying the plant material.

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