Evidence for a therapeutic effect of Braintone on ischemic brain damage

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Research Highlights

(1) The link between Braintone and pro-apoptotic genes (AT2 receptor, Fas, Bax and Bcl-XS) has been previously described by Kok-Poh Loh, Wan-Hui Wong and Li-Shan Low from the Department of Pharmacology, National University of Singapore in "Mechanisms of cerebral protection of Chinese herbal extract-Braintone on middle cerebral artery occluded rats".

(2) This study combined novel *in vivo* and *in vitro* experiments to show that Braintone dose- dependently increased the expression of hypoxia inducible factor 1α, heme oxygenase-1 and vascular endothelial growth factor in the ischemic cortex of rats with middle cerebral artery occlusion. The Chinese herbal extract Braintone is composed of *Radix Rhodiolase Essence*, *Radix Notoginseng Essence*, *Folium Ginkgo Essence* and *Rhizoma Chuanxiong*. Braintone-containing serum increased levels of hypoxia-inducible factor 1α mRNA and protein, and elevated vascular endothelial growth factor mRNA and heme oxygenase-1 protein expression in a dose-dependent manner in human umbilical vein endothelial cells after glucose-oxygen deprivation.

(3) Braintone has a neuroprotective effect, which is mediated by the up-regulation of hypoxia inducible factor 1α, heme oxygenase-1 and vascular endothelial growth factor expression.

Abstract

This study used a novel combination of *in vivo* and *in vitro* experiments to show that Braintone had neuroprotective effects and clarified the molecular mechanisms underlying its efficacy. The Chinese herbal extract Braintone is composed of *Radix Rhodiolase Essence*, *Radix Notoginseng Essence*, *Folium Ginkgo Essence* and *Rhizoma Chuanxiong*. *In vivo* experiments showed that cerebral infarction volume was reduced, hemispheric water content decreased, and neurological deficits were alleviated in a rat model of permanent middle cerebral artery occlusion after administration of 87.5, 175 or 350 mg/kg Braintone for 7 consecutive days. Western blot analysis showed that Braintone enhanced the expression of hypoxia-inducible factor 1α, heme oxygenase-1 and vascular endothelial growth factor in the ischemic cortex of these rats. The 350 mg/kg dose of Braintone produced the most dramatic effects. For the *in vitro* experiments, prior to oxygen-glucose deprivation, rats were intragastrically injected with 440, 880 or 1 760 mg/kg Braintone to prepare a Braintone-containing serum, which was used to pre-treat human umbilical vein endothelial cells for 24 hours. Human umbilical vein endothelial cell injury was alleviated with this pre-treatment. Western blot and real-time PCR analysis showed that the Braintone-containing serum increased the levels of hypoxia-inducible factor 1α mRNA and protein, heme oxygenase-1 protein and vascular endothelial growth factor mRNA in oxygen-glucose deprived human umbilical vein endothelial cells. The 1 760 mg/kg dose produced the greatest increases in expression. Collectively, these experimental findings suggest that Braintone has neuroprotective effects on ischemia-induced brain damage *via* the up-regulation of hypoxia-inducible factor 1α, heme oxygenase-1 and vascular endothelial growth factor expression in vascular endothelial cells.

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Key Words

neural regeneration; traditional Chinese medicine; Braintone; ischemic brain damage; oxygen-glucose deprivation; endothelial cells; hypoxia inducible factor 1α; heme oxygenase-1; vascular endothelial growth factor; grants-supported paper; neuroregeneration

INTRODUCTION

Stroke is the major cause of human neurological disability, and ischemic stroke accounts for approximately 80–90% of all stroke cases. Although primary brain tissue damage in the central core is irreversible after ischemic stroke, secondary brain damage in the penumbra caused by the ensuing pathophysiological cascade can be prevented or diminished by proper intervention[1]. However, an effective treatment remains challenging due to the complex pathophysiology of acute ischemic stroke.

Historically, neuronal death after stroke has been the focus of stroke research[2]. Recently, the importance of the neurovascular unit, which is comprised of neurons, endothelial cells and astrocytes, has received great attention in the field of stroke, because stroke affects not only neurons, but also astrocytes and microvessels[2]. Within the neurovascular unit, endothelial cells are critical for maintaining normal hemodynamic and metabolic homeostasis[2]. Vascular damage during ischemia often leads to the disruption of the blood-brain barrier and dysregulation of vascular tonus, eventually causing substantial cell death[2].

Hypoxia-inducible factor 1α is a transcription factor that is important for cell survival under hypoxic/ischemic conditions[3-8]. Hypoxia-inducible factor 1α increases the expression of its target genes, including vascular endothelial growth factor[3], heme oxygen-1 and erythropoietin[4]. Hypoxia-inducible factor 1α has been shown to have neuroprotective effects under hypoxic/ischemic conditions by promoting angiogenesis, erythropoiesis, vasodilation and cell proliferation, and by regulating energy metabolism[5]. An accumulating body of evidence indicates that during stress, such as cerebral ischemia, hypoxia or nerve growth factor deprivation, an increase in hypoxia-inducible factor 1α expression confers protection against neuronal cell death and endothelial cell injury[4, 6-9]. Therefore, pharmacological induction of hypoxia-inducible factor 1α expression following ischemia is a highly promising therapeutic approach for stroke.

The Chinese herbs *Rhodiolase*, *Notoginseng*, *Folium Ginkgo* and *Rhizoma Chuanxiong* have been used for stroke ancillary treatment in China for years[10-13]. *In vitro* studies have demonstrated that all of these Chinese herbs have protective effects on endothelial cell injury[10-13]. However, the molecular mechanisms underlying these protective effects are largely unknown. Braintone contains four major active ingredients: *Radix Rhodiolase Essenc*e (a major constituent of *Rhodiola rosea L.*), *Radix Notoginseng Essence*, *Folium Ginkgo Essence* and *Rhizoma Chuanxiong*. Braintone has a neuroprotective effect in a rat model of ischemic stroke[14]. However, the molecular mechanisms of neuroprotection are largely unknown. In this study, we demonstrated that Braintone's neuroprotective action against ischemic stroke might be associated with its ability to protect against ischemia-induced endothelial cell injury *via* the up-regulation of hypoxia-inducible factor 1, heme oxygenase-1 and vascular endothelial growth factor expression in these cells.

RESULTS

Quantitative analysis of experimental animals

One hundred and sixty rats were randomly divided into six groups: control group

(intragastric saline + sham operation; $n = 25$); ischemic control group (intragastric saline + permanent middle cerebral artery occlusion; $n = 40$); Danshen group (intragastric Danshen + permanent middle cerebral artery occlusion; $n = 20$); 87.5, 175 and 350 mg/kg Braintone groups (87.5, 175 or 350 mg/kg Braintone intragastric administration + permanent middle cerebral artery occlusion; $n = 25$). All rats were included in the final analysis.

Braintone reduced infarct volume and hemispheric water content, and alleviated ischemia-induced motor deficits in rats with permanent middle cerebral artery occlusion

To examine the potential of Braintone to prevent ischemic injury, Braintone (87.5, 175 and 350 mg/kg) and Danshen (67.5 mg/kg) were administered intragastrically for 7 days before ischemia. In rats subjected to permanent middle cerebral artery occlusion for 24 hours, extensive infarction was detected in the ipsilateral cerebral cortical and subcortical areas over a series of brain sections. Compared with the ischemic control group, the infarct sizes in the Braintone and Danshen groups were smaller ($P < 0.01$; Figure 1A, B).

Hemispheric water content in permanent middle cerebral artery occluded rats was significantly increased compared with the control group ($P < 0.01$). Braintone at 175 and 350 mg/kg, and Danshen at 67.5 mg/kg significantly reduced hemispheric water content ($P < 0.05$ or $P < 0.01$; Figure 1C).

Following ischemic injury with or without drug treatment, rats were examined and scored for motor deficits using a 12-point scale as previously described[15-17]. Rats in the ischemic control group showed robust motor behavioral deficits. Braintone and Danshen treatments resulted in a remarkable reduction in motor deficits ($P < 0.01$; Figure 1D).

Braintone reduced oxygen-glucose deprivation-induced endothelial cell damage

To assess whether Braintone has a protective effect against ischemia-induced endothelial cell damage, we next examined the effect of Braintone-containing serum on the viability of human umbilical vein endothelial cells subjected to oxygen-glucose deprivation. Braintone-containing serum at a dose range of 440 to 1 760 mg/kg did not affect the viability of human umbilical vein endothelial cells compared with the control group. During oxygen-glucose deprivation, cell viability, determined using the thiazolyl blue tetrazolium bromide (MTT) assay, decreased in a time-dependent manner (Figure 2A). Braintone-containing serum at doses of 880 and 1 760 mg/kg significantly prevented the decrease in viability at both 3 and 12 hours of oxygen-glucose deprivation ($P < 0.05$; Figure 2B, C).
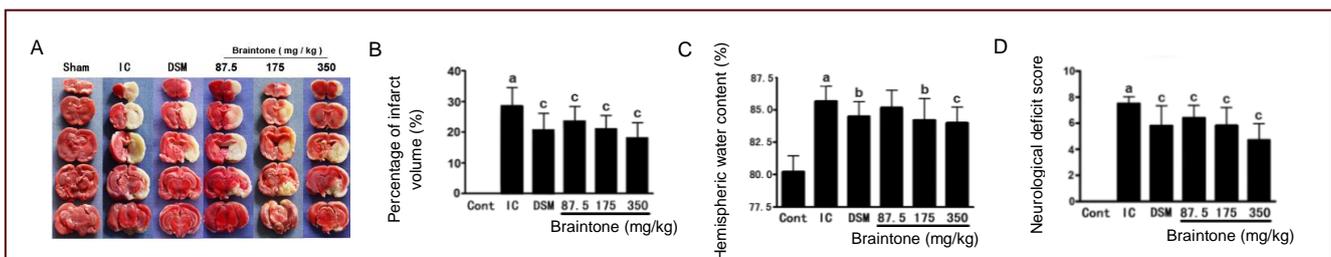
Figure 1 Effects of Braintone on infarct volume, hemispheric water content and ischemia-induced motor deficits in permanent middle cerebral artery occluded rats.

Focal ischemia was induced by permanent middle cerebral artery occlusion. Braintone (87.5, 175 and 350 mg/kg), Danshen (67.5 mg/kg) or vehicle was administered intragastrically for 7 days before ischemia.

(A) Representative photographs of coronal sections, stained with tetrazolium chloride, obtained 24 hours after ischemia in control (Cont), ischemic control (IC), Danshen (DSM)-treated and Braintone-treated rats. White regions are defined as infarcted areas.

(B) Quantitative analysis of total lesion volume in the rat brain. Percentage of infarct volume = [volume of contralateral hemisphere – volume of tetrazolium chloride-stained portion (non-ischemic) in the ipsilateral hemisphere]/volume of contralateral hemisphere × 100%.

(C) Quantitative analysis of hemispheric water content in the rat brain. Hemispheric water content = (hemispheric wet weight – hemispheric dried weight)/hemispheric wet weight × 100%.

(D) Motor deficits were measured 24 hours after ischemic insult. Neurological function was graded on a scale of 0 to 12 (normal score, 0; maximal score (complete loss of functions), 12).

Data are expressed as mean ± SD, $n = 10$. [a]$P < 0.01$, vs. Cont; [b]$P < 0.05$, [c]$P < 0.01$, vs. IC group. Statistical analysis was carried out with one-way analysis of variance followed by a post hoc Tukey test.

Figure 2 Effect of Braintone on oxygen-glucose deprivation (OGD)-induced endothelial cell damage.

Human umbilical vein endothelial cells were treated with Braintone-containing serum or vehicle before OGD insult, and were harvested at the indicated times after OGD. Cell viability was determined using the thiazolyl blue tetrazolium bromide (MTT) assay. The absorbance value (A) was measured at 570 nm using a microplate reader. The percentage of cell death was calculated with the following formula: cell death (%) = $(1 - A_{experiment\ well}/A_{control\ well}) \times 100\%$.

(A) Temporal changes in cell viability after OGD.

(B–C) Braintone-containing serum (440, 880 and 1 760 mg/kg) increased the survival rate of human umbilical vein endothelial cells at 3 hours (B) and 12 hours (C) after OGD.

Data are expressed as mean ± SD, $n = 10$. [a]$P < 0.01$, *vs.* control group (Cont); [b]$P < 0.05$, *vs.* OGD group. Statistical analysis was carried out with one-way analysis of variance followed by a *post hoc* Tukey test.

Braintone/Braintone-containing serum enhanced the expression of hypoxia-inducible factor 1α in the ischemic cortex and in oxygen-glucose deprivation-treated human umbilical vein endothelial cells

Western blot analysis revealed an increase in the levels of hypoxia-inducible factor 1α in the ischemic cortex after permanent middle cerebral artery occlusion, with a maximal induction at 24 hours ($P < 0.01$; Figure 3A, B). Braintone (87.5, 175 and 350 mg/kg) significantly increased hypoxia-inducible factor 1α protein expression compared with the control group ($P < 0.01$; Figure 3C, D).

Real-time PCR analysis showed that during oxygen-glucose deprivation, hypoxia-inducible factor 1α mRNA expression was increased and peaked at 3 hours ($P < 0.01$; Figure 4A). Braintone-containing serum at 880 and 1 760 mg/kg significantly increased hypoxia-inducible factor 1α mRNA expression compared with the oxygen-glucose deprivation group ($P < 0.05$; Figure 4B).

Western blot analysis revealed that the protein levels of hypoxia-inducible factor 1α in oxygen-glucose deprivation-treated human umbilical vein endothelial cells was enhanced, with a maximal induction at 12 hours ($P < 0.01$; Figure 4C, D). Braintone-containing serum at 880 mg/kg and 1 760 mg/kg significantly increased hypoxia-inducible factor 1α protein expression compared with the oxygen-glucose deprivation group ($P < 0.01$; Figure 4E, F).

Immunofluorescence analysis showed that no hypoxia-inducible factor 1α expression was detected in cells under normal culture conditions. Obvious fluorescence was seen in the cytoplasm and nuclei at 3– 12 hours of oxygen-glucose deprivation, and reached a peak at 12 hours ($P < 0.01$; Figure 5A, C). Braintone-containing serum at 880 and 1 760 mg/kg significantly increased hypoxia-inducible factor 1α expression compared with the oxygen-glucose deprivation group ($P < 0.01$; Figure 5B, D).

Braintone/Braintone-containing serum increased the expression of heme oxygenase-1 in the ischemic cortex and in oxygen-glucose deprivation-treated human umbilical vein endothelial cells

Western blot analysis revealed an increase in heme oxygenase-1 protein levels in the ischemic cortex, with a maximal induction occurring 24 hours after permanent middle cerebral artery occlusion ($P < 0.01$; Figure 6A, B). Braintone at 87.5, 175 and 350 mg/kg significantly increased heme oxygenase-1 protein expression compared with the control group ($P < 0.01$; Figure 6C, D).

Immunofluorescence analysis revealed that there was no heme oxygenase-1 expression in cells under normal culture conditions. Obvious fluorescence was detected in the cytoplasm and nuclei 3–12 hours after the start of oxygen-glucose deprivation, peaking at 12 hours (Figure 7A, C). Braintone-containing serum at 880 and 1 760 mg/kg significantly increased heme oxygenase-1

protein expression compared with the oxygen-glucose deprivation group ($P < 0.05$ or $P < 0.01$; Figure 7B, D).

Figure 3 Braintone enhanced the expression of hypoxia-inducible factor 1α (HIF-1α) in the ischemic cortex.

Focal ischemia was induced by permanent middle cerebral artery occlusion (pMCAO). Braintone (87.5, 175 and 350 mg/kg) or vehicle w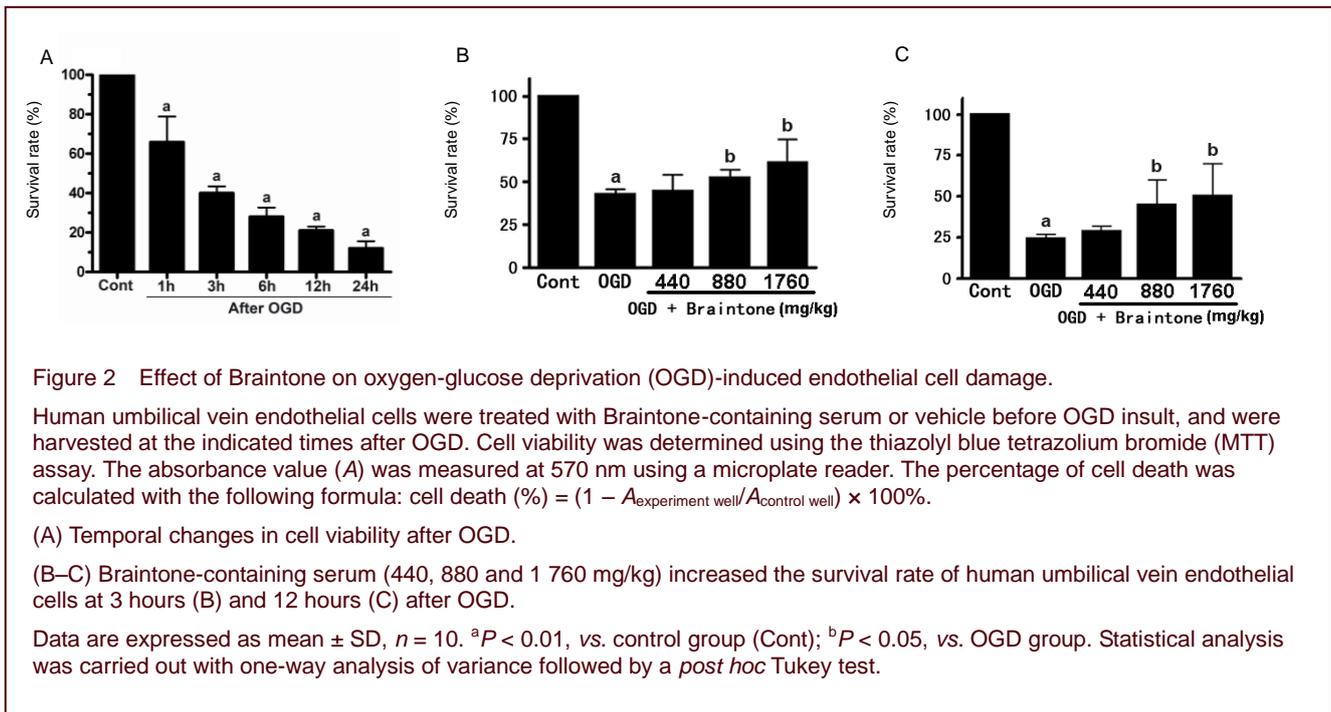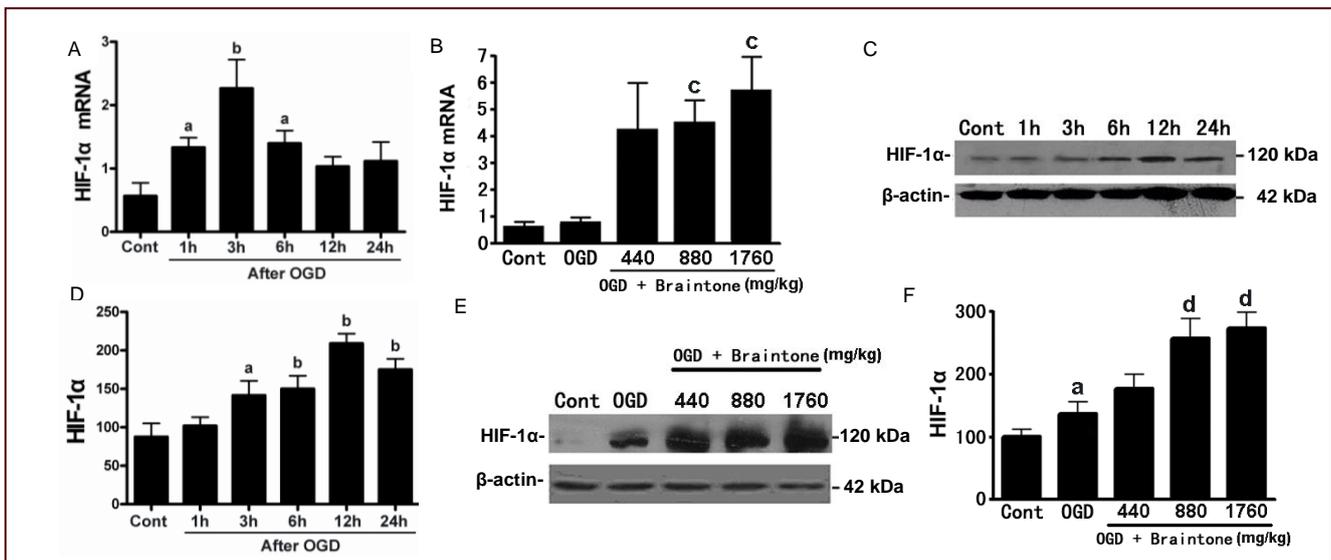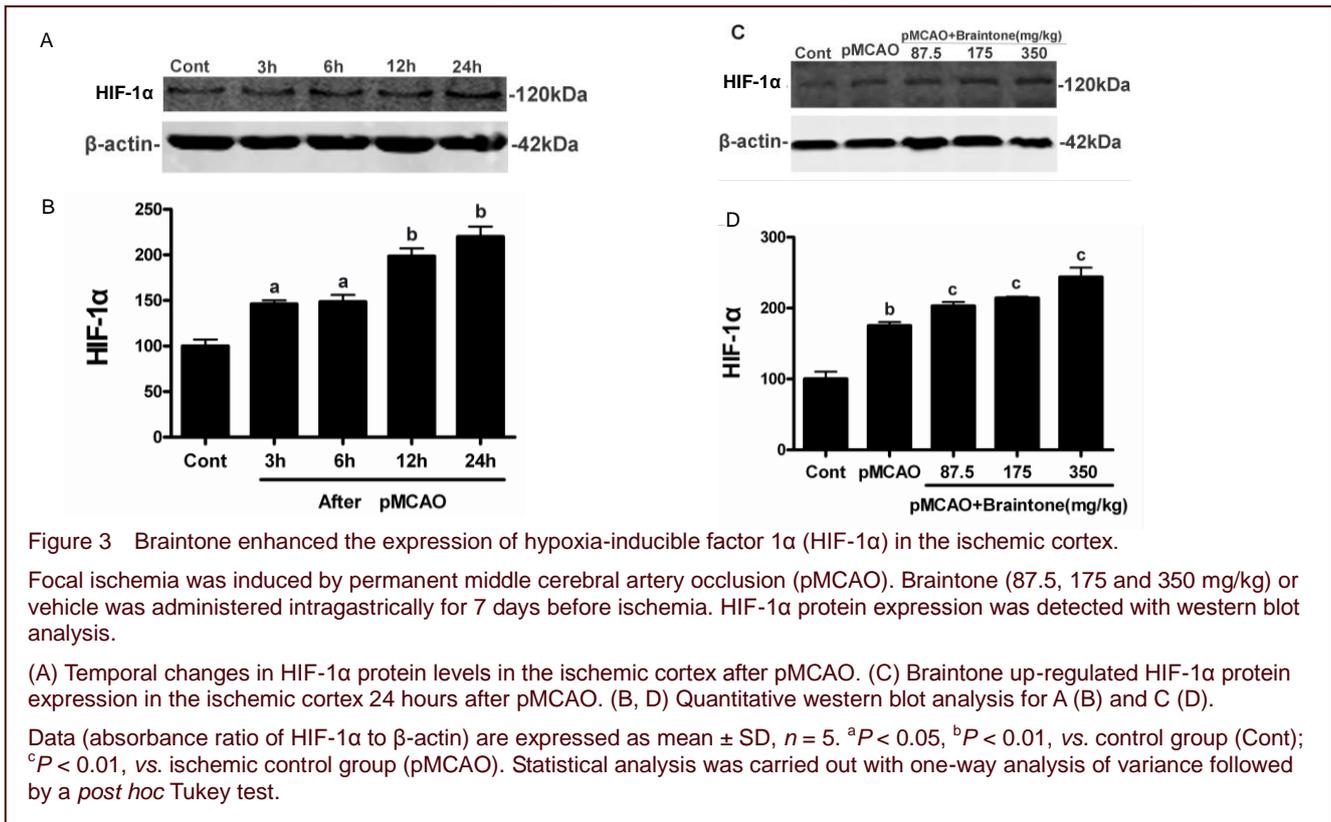as administered intragastrically for 7 days before ischemia. HIF-1α protein expression was detected with western blot analysis.

(A) Temporal changes in HIF-1α protein levels in the ischemic cortex after pMCAO. (C) Braintone up-regulated HIF-1α protein expression in the ischemic cortex 24 hours after pMCAO. (B, D) Quantitative western blot analysis for A (B) and C (D).

Data (absorbance ratio of HIF-1α to β-actin) are expressed as mean ± SD, $n = 5$. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* control group (Cont); [c]$P < 0.01$, *vs.* ischemic control group (pMCAO). Statistical analysis was carried out with one-way analysis of variance followed by a *post hoc* Tukey test.

Figure 4 Braintone-containing serum enhanced the expression of hypoxia-inducible factor 1α (HIF-1α) in human umbilical vein endothelial cells after oxygen-glucose deprivation (OGD).

Human umbilical vein endothelial cells were treated with Braintone-containing serum or vehicle during OGD insult, and were harvested at the indicated times after OGD. HIF-1α mRNA and protein expression were detected with real-time PCR and western blot analysis, respectively.

(A) Temporal changes in HIF-1α mRNA levels in cells after OGD.

(B) Braintone-containing serum up-regulated HIF-1α mRNA expression in cells 12 hours after OGD. Braintone-containing serum (880 and 1 760 mg/kg) significantly increased HIF-1α mRNA expression compared with the OGD group.

(C, E) Representative western blot images. (C) Temporal changes in HIF-1α protein levels in cells after OGD. (E) Braintone-containing serum up-regulated HIF-1α protein expression in cells 12 hours after OGD.

(D, F) Quantitative western blot analysis for C (D) and E (F).

Data (absorbance ratio of HIF-1α protein/gene to β-actin) are expressed as mean ± SD, $n = 10$. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* control group (Cont); [c]$P < 0.05$, [d]$P < 0.01$, *vs.* OGD group. Statistical analysis was carried out with one-way analysis of variance followed by a *post hoc* Tukey test.
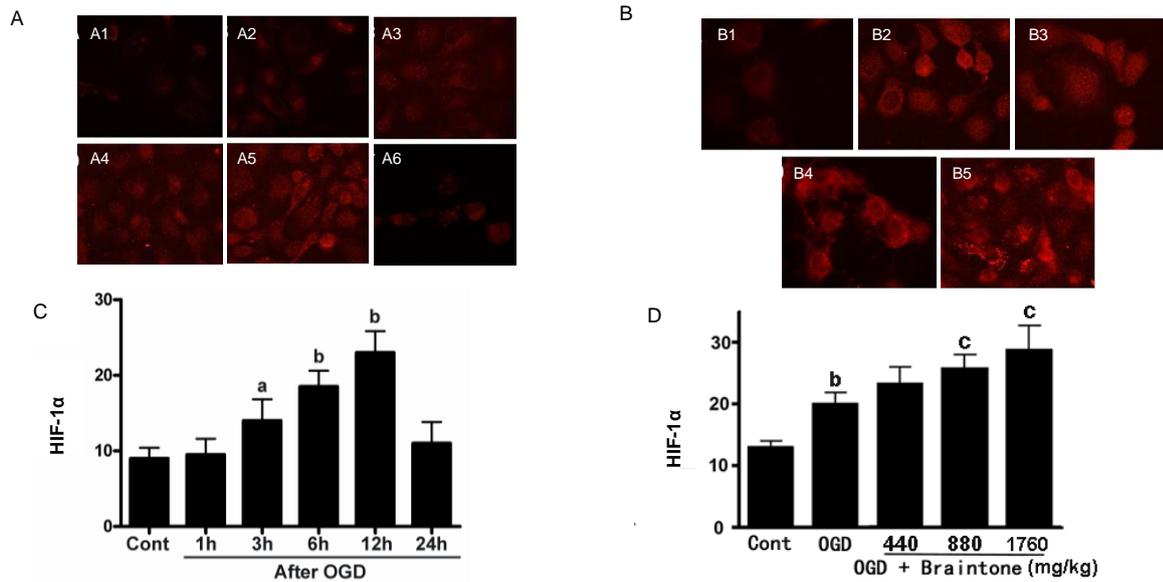
Figure 5   Hypoxia-inducible factor 1α (HIF-1α) expression increased after oxygen-glucose deprivation (OGD), and Braintone enhanced HIF-1α expression in human umbilical vein endothelial cells.

Human umbilical vein endothelial cells were treated with Braintone-containing serum or vehicle during OGD insult, and were harvested at the indicated time after OGD, and then HIF-1α immunofluorescence staining was performed.

(A–B) Representative immunofluorescence photographs (immunofluorescence staining, × 400).

(A) Temporal changes in HIF-1α expression in cells after OGD. A1: Control group (Cont); A2–A6: 1, 3, 6, 12, 24 hour (h) OGD groups.

(B) Braintone-containing serum up-regulated HIF-1α expression in cells 12 hours after OGD. B1: Cont; B2: OGD group; B3–B5: Braintone groups at 440, 880 and 1 760 mg/kg.

(C–D) Quantitative analysis of immunofluorescence for A (C) and B (D).

Data (number of immunoreactive cells in 400-fold visual field) are expressed as mean ± SD, n = 10. [a]P < 0.05, [b]P < 0.01, vs. Cont; [c]P < 0.01, vs. OGD group. Statistical analysis was carried out with one-way analysis of variance followed by a post hoc Tukey test.
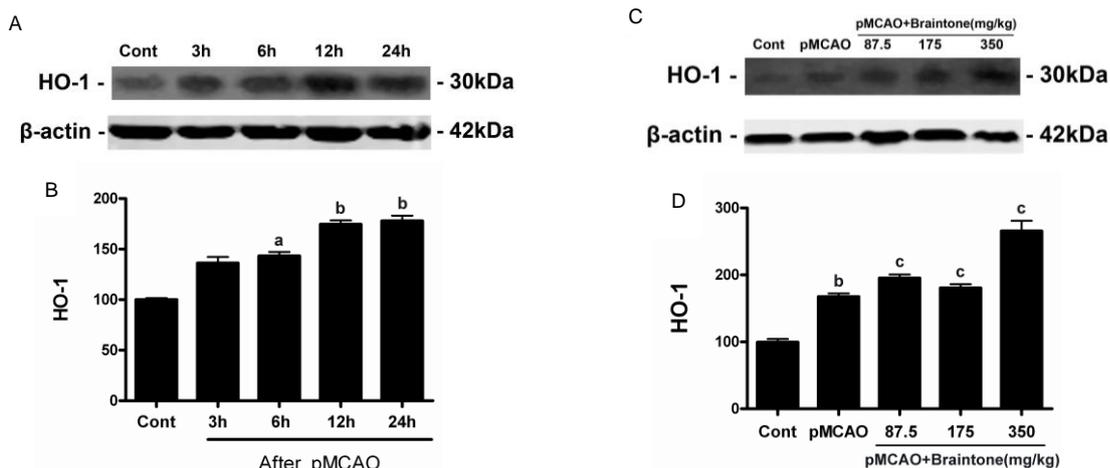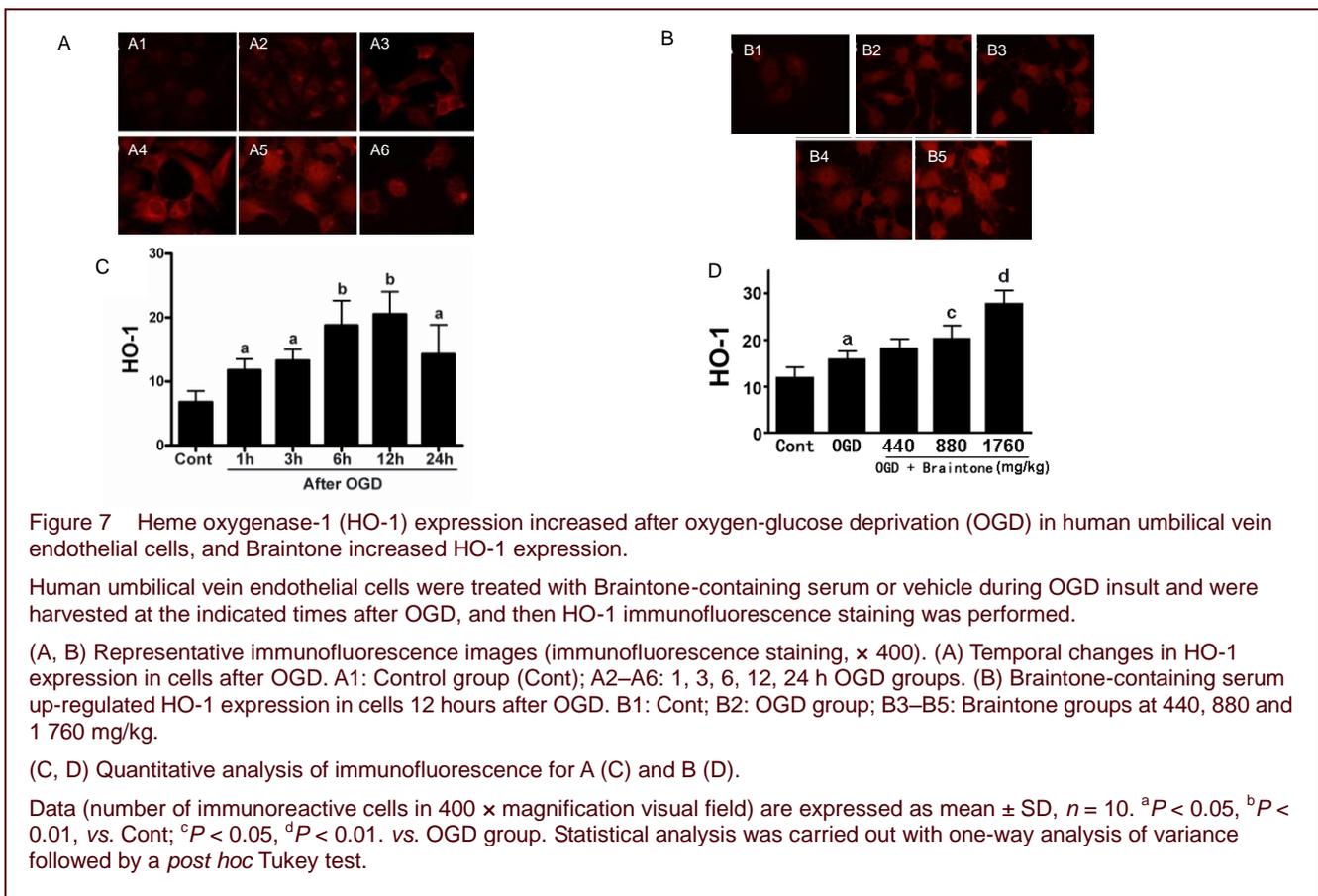

Figure 6   Braintone enhanced the expression of heme oxygenase-1 (HO-1) in the ischemic cortex.

Focal ischemia was induced by permanent middle cerebral artery occlusion (pMCAO). Braintone (87.5, 175 and 350 mg/kg) or vehicle was administered intragastrically for 7 days before ischemia. HO-1 protein was detected with western blot analysis.

(A) Temporal changes in HO-1 protein levels in the ischemic cortex after pMCAO. (C) Braintone up-regulated HO-1 protein expression in the ischemic cortex 24 hours after pMCAO. (B, D) Quantitative western blot analysis for A (C) and B (D).

Data (absorbance ratio of HO-1 to β-actin) are expressed as mean ± SD, n = 5. [a]P < 0.05, [b]P < 0.01, vs. control group (Cont); [c]P <0.01, vs. ischemic control group (pMCAO). Statistical analysis was carried out with one-way analysis of variance followed by a post hoc Tukey test.

Figure 7 Heme oxygenase-1 (HO-1) expression increased after oxygen-glucose deprivation (OGD) in human umbilical vein endothelial cells, and Braintone increased HO-1 expression.

Human umbilical vein endothelial cells were treated with Braintone-containing serum or vehicle during OGD insult and were harvested at the indicated times after OGD, and then HO-1 immunofluorescence staining was performed.

(A, B) Representative immunofluorescence images (immunofluorescence staining, × 400). (A) Temporal changes in HO-1 expression in cells after OGD. A1: Control group (Cont); A2–A6: 1, 3, 6, 12, 24 h OGD groups. (B) Braintone-containing serum up-regulated HO-1 expression in cells 12 hours after OGD. B1: Cont; B2: OGD group; B3–B5: Braintone groups at 440, 880 and 1 760 mg/kg.

(C, D) Quantitative analysis of immunofluorescence for A (C) and B (D).

Data (number of immunoreactive cells in 400 × magnification visual field) are expressed as mean ± SD, $n = 10$. [a]$P < 0.05$, [b]$P < 0.01$, vs. Cont; [c]$P < 0.05$, [d]$P < 0.01$. vs. OGD group. Statistical analysis was carried out with one-way analysis of variance followed by a *post hoc* Tukey test.

Braintone/Braintone-containing serum up-regulated vascular endothelial growth factor protein expression in the ischemic cortex and mRNA expression in oxygen-glucose deprivation-treated human umbilical vein endothelial cells

Western blot analysis revealed an increase in the protein levels of vascular endothelial growth factor in the ischemic cortex, with a maximal induction at 24 hours after permanent middle cerebral artery occlusion ($P < 0.01$; Figure 8A, B). Braintone at 87.5, 175 and 350 mg/kg significantly increased vascular endothelial growth factor protein expression compared with the permanent middle cerebral artery occlusion control ($P < 0.01$; Figure 8C, D).

Vascular endothelial growth factor mRNA levels were up-regulated during oxygen-glucose deprivation, peaking at 6 hours (Figure 9A). Braintone-containing serum at 1 760 mg/kg significantly increased vascular endothelial growth factor mRNA expression compared with the oxygen-glucose deprivation group ($P < 0.05$; Figure 9B).

DISCUSSION

The present study demonstrates the following: (1) Braintone has neuroprotective effects against cerebral ischemia; (2) Braintone directly protects against ischemic endothelial cell injury by up-regulating the expression of hypoxia-inducible factor 1α and its target genes, heme oxygenase-1 and vascular endothelial growth factor.

The pharmacological induction of hypoxia-inducible factor 1α expression in the ischemic region or in affected endothelial cells following oxygen-glucose deprivation is linked to neuroprotection and protection of endothelial cells, respectively[8]. During normoxia, hypoxia-inducible factor 1α is not stable and is easily targeted for degradation by prolyl hydroxylases. Prolyl hydroxylases are activated by oxygen, which is a rate limiting factor for the enzymes. In hypoxic conditions, the prolyl hydroxylase degradation pathway is inhibited and hypoxia-inducible factor 1α is stabilized[18]. Inhibition of hypoxia-inducible factor 1α expression by specific small interfering RNA transfection increases reactive oxygen species generation and cell death[5]. In contrast, an increase in hypoxia-inducible factor 1α expression, achieved by inhibiting prolyl 4-hydroxylase, has been shown to provide neuroprotection in permanent focal ischemia[6]. *In vitro*, iron chelators up-regulate hypoxia-inducible factor 1α, which protects

against oxidative stress-induced death in cultured cortical neurons[3].
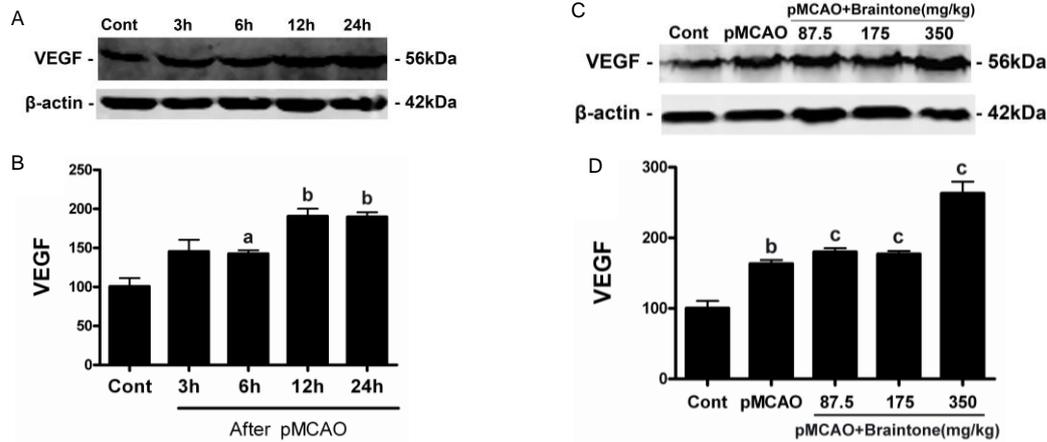

Figure 8   Braintone enhanced the expression of vascular endothelial growth factor (VEGF) in the ischemic cortex.

Focal ischemia was induced by permanent middle cerebral artery occlusion (pMCAO). Braintone (87.5, 175 and 350 mg/kg) or vehicle was administered intragastrically for 7 days before ischemia. VEGF protein was detected with western blot analysis.

(A) Temporal changes in VEGF protein levels in the ischemic cortex after pMCAO. (C) Braintone up-regulated VEGF protein expression in the ischemic cortex 24 hours after pMCAO. (B, D) Quantitative western blot analysis for A (B) and C (D).

Data (absorbance ratio of VEGF to β-actin) are expressed as mean ± SD, $n = 5$. [a]$P < 0.05$, [b]$P < 0.01$, vs. control group (Cont); [c]$P < 0.01$, vs. ischemic control group (pMCAO). Statistical analysis was carried out with one-way analysis of variance followed by a post hoc Tukey test.
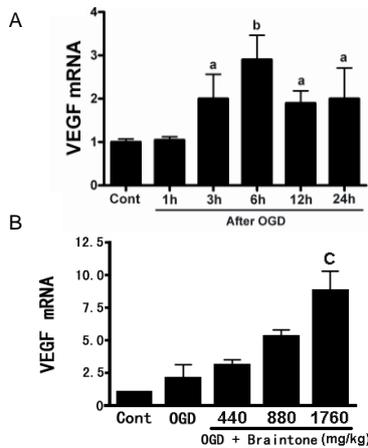

Figure 9   Vascular endothelial growth factor (VEGF) mRNA expression increased after oxygen-glucose deprivation (OGD) in human umbilical vein endothelial cells, and Braintone-containing serum up-regulated VEGF mRNA expression.

Human umbilical vein endothelial cells were treated with Braintone-containing serum or vehicle during OGD insult and were harvested at the indicated times after OGD, and then VEGF mRNA was detected with real-time PCR.

(A) Temporal changes in VEGF mRNA levels in cells after OGD. The expression of VEGF mRNA was increased after OGD and peaked at 6 hours.

(B) Braintone increased VEGF mRNA expression in cells 6 hours after OGD.

Data (absorbance ratio of VEGF gene to β-actin) are represented as mean ± SD, $n = 10$. [a]$P < 0.05$, [b]$P < 0.01$, vs. control group (Cont); [c]$P < 0.05$, vs. OGD group. Statistical analysis was carried out with one-way analysis of variance followed by a post hoc Tukey test.

Furthermore, hypoxia-induced or genetic overexpression of hypoxia-inducible factor 1α protects sympathetic neurons against nerve growth factor-deprivation-induced neuronal death[7]. These findings demonstrate the protective effects of hypoxia-inducible factor 1α against these stressors. More recently, it was shown that the up-regulation of hypoxia-inducible factor 1α by pharmacological treatment protects against endothelial cell injury induced by oxygen-glucose deprivation[8].

An important finding of the current study is that Braintone enhances the ischemia- and oxygen-glucose deprivation-induced increases in expression of hypoxia-inducible factor 1α its target genes, heme oxygenase-1 and vascular endothelial growth factor. Enhancement of the oxygen-glucose deprivation-induced increase in hypoxia-inducible factor 1α and vascular endothelial growth factor expression by Braintone may be mediated by notoginseng, which promotes vascular endothelial growth factor expression in vascular endothelial cells[19], and by Radix Rhodiolase Essence, which increases the expression of hypoxia-inducible factor 1α and vascular endothelial growth factor during hypoxia-induced cardiomyocyte death[20]. Whether Braintone increases hypoxia-inducible factor 1α expression by inhibiting prolyl 4-hydroxylase or through another molecular mechanism remains to be clarified.

Braintone's neuroprotection against endothelial cell injury may, at least in part, be mediated by its induction of heme oxygenase-1, which is a crucial antioxidant. In normal biological conditions, there is a balance between the production and clearance of reactive oxygen species. During oxygen-glucose deprivation, this balance is disrupted, resulting in excessive levels of reactive oxygen species. Heme oxygenase is an endoplasmic reticulum-associated protein that is responsible for the conversion of heme to biliverdin, carbon monoxide and iron. There are two isoforms of heme oxygenase, the 32 kDa inducible heme oxygenase-1, and the 38 kDa constitutive heme oxygenase-2. Whereas heme oxygenase-2 (abundant in brain) regulates normal physiological cell function, heme oxygenase-1 is induced in response to various noxious stimuli. Decreased oxygen availability and oxidative stress are among these noxious stimuli[21]. Carbon monoxide, one of the products generated from heme catabolism, has positive effects as a vasodilator and regulator of vascular tone[22]. Biliverdin is an antioxidant. In the cell, biliverdin generated by heme oxygenase is immediately transformed into bilirubin, a pigment with potent antioxidant properties, by biliverdin reductase. Although free iron is considered cytotoxic because of its participation in the Fenton reaction, current studies show that the protective effects of heme oxygenase-1, mediated by carbon monoxide and bilirubin, exceed the potentially damaging effects of iron[23]. Ischemic preconditioning increases heme oxygenase-1 protein levels and simultaneously protects against brain damage in both transient and permanent middle cerebral artery occlusion models in wild type mice. In contrast, ischemic preconditioning does not protect against brain injury in heme oxygenase-1 knockout mice. These findings indicate that the neuroprotective effects of ischemic preconditioning are partially mediated by heme oxygenase-1[24]. In the present study, we found that Braintone increased the expression of the hypoxia-inducible factor 1α target gene heme oxygenase-1, which might mediate the protective effect of Braintone against ischemic endothelial cell injury.

Another mechanism by which Braintone protects against endothelial cell injury is the up-regulation of vascular endothelial growth factor expression. Vascular endothelial growth factor is involved in many central nervous system disorders, including stroke, and confers neuroprotection in cerebral ischemia. Although the route and timing of delivery might determine the outcome of vascular endothelial growth factor therapy after an ischemic insult, the growth factor has strong therapeutic potential in cerebral ischemia because of its angiogenic and neuroprotective effects[25]. In adult models of middle cerebral artery occlusion, vas-cular endothelial growth factor administration decreases infarct volume, and improves injury scores and neurocognitive outcome[26-27]. Conversely, vascular endothelial growth factor receptor-2 inhibition exacerbates injury and cell death in an adult traumatic brain injury model[28]. Vascular endothelial growth factor has a remarkable neuroprotective effect on vascular endothelial cells by stimulating their growth and survival[29]. Shimotake *et al* [30] found that, in a neonatal model of middle cerebral artery occlusion, vascular endothelial growth factor receptor-2 inhibition reduces endothelial cell proliferation in the injured caudate. Our present study suggests that Braintone might protect endothelial cells against ischemic injury by up-regulating vascular endothelial growth factor expression *via* the induction of hypoxia-inducible factor 1α.

Maintaining mitochondrial stability is critical for protecting cells from ischemic insult. Recently, a direct link between mitochondrial dysfunction and reduced hypoxia-inducible factor 1α levels was established[31]. When mitochondrial respiration is impaired, oxygen in mitochondria is redistributed toward non-respiratory oxygen-dependent targets in the cytosol that activate prolyl hydroxylases, which in turn increase hypoxia-inducible factor 1α degradation. When mitochondrial function is preserved, the redistribution of oxygen will not occur, and the inhibition of prolyl hydroxylases by low oxygen tension will not be released. As a result, more hypoxia-inducible factor 1α will accumulate[31]. Whether Braintone can preserve mitochondrial integrity in endothelial cells and minimize functional loss remains to be investigated.

Recently, the importance of the neurovascular unit, composed of neurons, endothelial cells and astrocytes, has received great attention in the field of stroke, because stroke affects not only neurons, but also astrocytes and microvessels[1]. Within the neurovascular unit, endothelial cells are critical for maintaining normal hemodynamics and metabolic homeostasis. Vascular damage during ischemia often leads to the disruption of the blood-brain barrier and dysregulation of vascular tonus, eventually causing substantial cell death. Therefore, the present findings suggest that Braintone's neuroprotective action against ischemic stroke is associated with its protective effect against ischemia-induced endothelial cell injury *via* the up-regulation of hypoxia-inducible factor 1α, heme oxygenase-1 and vascular endothelial growth factor in endothelial cells.

MATERIALS AND METHODS

Design
A randomized, controlled, animal and cell biological experiment.

Time and setting
The experiment was performed at the Department of Pharmacology and Laboratory of Cerebrovascular Pharmacology, College of Pharmaceutical Science, Soochow University, China from February 2007 to May 2008.

Materials
A total of 172 healthy male Sprague-Dawley rats, aged 3–4 months and weighing 290–200 g, were purchased from the Center for Experimental Animals, Soochow University, China (certificate No. 20020008, grade 2). Four rats were housed per cage in a standard animal room with a 12-hour light/dark cycle and given free access to food and water. In the end, 160 rats were used for animal experiments and 12 rats were used for preparation of Braintone-containing serum. The experimental procedure was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[32].

Methods
Drug treatment of rats
Braintone (87.5, 175 or 350 mg/kg Braintone powder dissolved in saline; production lot 3GB081001; Herbs Products Ltd, Hong Kong, China), *Danshen* (67.5 mg/kg; *Danshen* powder; production lot 20020922; Tianjin TASLY Pharmaceutical Co., Ltd., Tianjin, China) or vehicle (Saline, 3 mL/100 g) was administered intragastrically for 7 days before ischemia. Permanent middle cerebral artery occlusion operations were conducted 2 hours after the last drug or vehicle administration.

Establishment of permanent middle cerebral artery occlusion model
The rat permanent middle cerebral artery occlusion model was generated as previously described[33]. Briefly, rats were anesthetized with intraperitoneal injection of 4% choral hydrate (350 mg/kg). Through a ventral midline incision, the right common carotid artery, external carotid artery and internal carotid artery were isolated, and the external carotid artery and the common carotid artery were ligated. A 30-mm length of monofilament nylon suture (diameter 0.22–0.24 mm), with its tip rounded, was inserted from the right common carotid artery to the internal carotid artery through a small incision in the common carotid artery, and then advanced to the Circle of Willis to occlude the right middle cerebral artery. The suture was retained until the rats were sacri-

ficed. Body temperature was closely monitored with a rectal probe and maintained at 37.0 ± 0.5°C with a heating pad (Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, Tianjin, China) during and after surgery until recovery from anesthesia. Sham-operated rats underwent the same procedure, except that the incision of the carotid and insertion of the monofilament nylon suture into the artery was not performed.

Behavioral testing of rats
Behavioral tests were performed 24 hours after permanent middle cerebral artery occlusion by an investigator who was blinded to the experimental groups. The battery consisted of two tests that have been used previously to evaluate various aspects of neurological function: (1) postural reflex test, developed by Bederson *et al* [15] to examine upper body posture while the animal is suspended by the tail; and (2) forelimb placing test, developed by De Ryck *et al* [16] to examine sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli. Neurological function was graded on a scale of 0 to 12 (normal score, 0; maximal score, 12)[17].

Infarct volume in permanent middle cerebral artery occluded rats detected with tetrazolium chloride
Sixty rats were included in the detection of infarct volume. Brain slices were stained with 4% 2,3,5-triphenyltetrazolium chloride (Fluka, Buchs, Switzerland). Infarct volume for each rat was calculated as the difference between the volume of the contralateral hemisphere and the volume of the stained portion (non-ischemic) of the ipsilateral hemisphere.

Hemispheric water content in permanent middle cerebral artery occluded rats
Sixty rats were included in the detection of hemispheric brain water content. After the brain wet weight was measured, the brains were desiccated at 105°C for 48 hours until the weight was constant, and brain water content was calculated as follows: Hemispheric water content = (hemispheric wet weight – hemispheric dried weight)/ hemispheric wet weight × 100%[34].

Preparation of Braintone-containing serum
Twelve Sprague-Dawley male rats were randomly divided into four groups (*n* = 3 for each group). Rats in the Braintone-containing serum groups were orally administered Braintone solution at 440, 880 or 1 760 mg/kg, twice daily for 3 days[14]. Blood was collected 1 hour after the last administration, centrifuged at 3 000 r/min for 10 minutes, and the serum was collected, termed Brain-

tone-containing serum. In the control group, rats were orally administered normal saline with the same protocol, and their serum was used as control serum. Both Braintone-containing serum and control serum were inactivated by heating at 56°C for 30 minutes, filtered through a 0.22-µm filter, and stored at –80°C.

Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (Geneticell Bioengineering Inc, Shanghai, China) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C with 5% CO_2 and 95% air (v/v) at 90% humidity.

Braintone-containing serum treatment and oxygen-glucose deprivation

Oxygen-glucose deprivation was conducted as previously described[8]. Briefly, human umbilical vein endothelial cells were seeded at a density of 1×10^6/L with Braintone-containing serum at different concentrations or with normal serum in separate culture plates, and 24 hours later, the culture medium was changed to glucose-free DMEM containing either Braintone-containing serum in the Braintone-treated groups or normal serum in the oxygen-glucose deprivation group. The cells were then placed into an anaerobic chamber (Billups-Rothenberg, Del mar, CA, USA) flushed with 5% CO_2 and 95% N_2 (v/v). The cell cultures within the anaerobic chamber were kept in a humidified incubator at 37°C for various time intervals in different experiments. In the control group (non-oxygen-glucose deprivation), cell cultures were subjected to the same experimental procedures with vehicle only and without exposure to the glucose-free DMEM or anoxia.

Cell survival assay

Cellular viability was assessed using the MTT assay kit (Fluka) according to the manufacturer's instructions and published methods[30]. Briefly, 10 µL of MTT labeling reagent (Fluka) at a final concentration of 50 mg/mL was added into each well at the end of oxygen-glucose deprivation, and cultures were incubated in a humidified incubator at 37°C with 5% CO_2 and 95% air (v/v) at 90% humidity for 4 hours to allow formation of purple formazan crystals. Four hours later, 100 µL of solubilization reagent (10% sodium dodecyl sulfate-HCl) was added into each well. Finally, the spectrophotometric absorbance of the solubilized purple formazan crystals was measured using a microplate reader (BioTek Instruments, Winooski, Vermont, USA) at an absorbance wavelength

of 490 nm. All the MTT results for the oxygen-glucose deprivation groups were normalized and expressed as the percent average of the absorbance in the normal control group.

Real-time PCR

Human umbilical vein endothelial cells were quickly homogenized in Trizol (Trizol Reagent, TaKaRa, Dalian, China) and total RNA was extracted. RNA samples (500 ng) were converted to first-strand cDNAs, and then real-time PCR was performed with an iCycler 5 instrument (Bio-Rad, Hercules, CA, USA) using the Fast start DNA Master SYBR Green I kit (TaKaRa). Primer set and annealing temperatures were as follows:

Primer	Sequence	Product length (bp)
Hypoxia-inducible factor 1α	Forward: 5′-AGC CAG ACG ATC ATG CAG CTA CTA-3′ Reverse: 5′-TGT GGT AAT CCA CTT TCA TCC ATT G-3′	213
Vascular endothelial growth factor	Forward: 5′-TGT CCT CAC ACC ATT GAA ACC ACT A-3′ Reverse: 5′-AAT GGG CAC GTG GAT CCT G-3′	323
β-actin	Forward: 5′-ACT AAG CGA GCA CTG CCC AAC-3′ Reverse: 5′-CCT CAT TCA GCT CTC GGA ACA TC-3′	474

Real-time PCR reaction conditions were: 1 cycle of reverse transcription at 37°C for 45 minutes; 50 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds and elongation at 70°C for 1 minute. Expression of each target gene was normalized to the β-actin gene with the iCycler 5 software (Bio-Rad).

Western blot analysis

Brain tissues from the ischemic cortex and the corresponding area of control rats were homogenized in lysis buffer. Each sample was centrifuged at 12 000 r/min at 4°C for 10 minutes and the supernatant was preserved at –80°C. Human umbilical vein endothelial cells were cultured in 60-mm dishes, harvested, and rinsed with ice-cooled PBS twice after oxygen-glucose deprivation. Three volumes of western blot lysing buffer, containing protease inhibitor cocktail, for each volume of cell pellet was added, and the mixture was sonicated on ice (5 sonications, 1 s/mL each, with a 30-second period on ice between each sonication). The sample was centrifuged at 12 000 r/min at 4°C for 10 minutes and the supernatant was preserved at –80°C. The protein concentrations were determined with a BCA detection kit (Pierce, Rockford, IL, USA) and adjusted to equal concentrations

across different samples. The proteins were separated on a 6–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to nitrocellulose membranes, and incubated with rabbit anti-hypoxia-inducible factor 1α polyclonal antibody (1:1 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-heme oxygenase-1 polyclonal antibody (1:500; Boster, Wuhan, China) and rabbit anti-vascular endothelial growth factor polyclonal antibody (1:500; Bioss, Beijing, China) at 4°C overnight. The primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:5 000; Sigma) for 1 hour at room temperature. The blot was developed using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, England) and visualized by autoradiography. β-actin (rabbit anti-rat β-actin monoclonal antibody, 1:5 000; Sigma) was used as the loading control. The absorbance of primary antibody binding was quantitatively analyzed with Sigma Scan Pro 5.0 and was normalized to the loading control, β-actin[14].

Immunofluorescence staining

Human umbilical vein endothelial cells grown on glass coverslips were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes, washed with PBS, permeabilized with 0.1% Triton X-100 for 30 minutes, and rinsed with PBS. Following the blocking of nonspecific binding sites by incubation with blocking buffer (bovine serum albumin, 1%) for 1 hour, cells were incubated for 1 hour at room temperature with a rabbit anti-hypoxia-inducible factor 1α polyclonal antibody (1:400) and a rabbit anti-heme oxygenase-1 polyclonal antibody (1:400; Boster). Antibody binding was detected using MaxVision™ horseradish peroxidase polymer anti-rabbit IHC kit (1:1 000; Maixin Bio, KIT-5010, Fuzhou, China) with diaminobenzidine (Maixin Bio, DAB-0031) as the substrate. After repeated washes with PBS, the slides were mounted and analyzed under high power magnification (200 ×) with a fluorescence microscope (Eclipse TE2000, Nikon, Tokyo, Japan) attached to a Nikon digital camera, and the images were observed on a computer monitor. Fluorescence images were captured with identical exposure settings. For negative controls, sections stained without primary antibodies showed no signals (data not shown). The number of immunoreactive cells in each field was counted by an examiner who was blinded to the experimental conditions.

Statistical analysis

Data were statistically processed using SPSS 10.0 software (SPSS, Chicago, IL, USA) and statistical analyses were performed by one-way analysis of variance followed by a *post hoc* Tukey test. All data were expressed as mean ± SD. A *P* value < 0.05 was considered to indicate a significant difference.

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