Research report

Cornel iridoid glycoside promotes neurogenesis and angiogenesis and improves neurological function after focal cerebral ischemia in rats

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ARTICLE INFO

Article history:
Received 30 October 2008
Received in revised form 16 December 2008
Accepted 16 December 2008
Available online 15 January 2009

Keywords:
Cornel iridoid glycosides
Cerebral infarction
Middle cerebral artery occlusion
Neurogenesis
Angiogenesis
Vascular endothelial growth factor

ABSTRACT

The aim of this study was to investigate the effects of cornel iridoid glycoside (CIG), an ingredient extracted from a traditional Chinese herb Corus officinalis, on neurological function and neurogenesis after ischemic stroke. CIG was intragastrically administered to rats in doses of 20, 60 and 180 mg/kg/day, starting 3 h after the onset of middle cerebral artery occlusion (MCAO). The behavioral test was performed by using the modified neurological severity score (mNSS). Rats were sacrificed 7, 14, or 28 days after ischemia occurred. Neurogenesis and angiogenesis were detected by using immunofluorescence staining. The messenger ribonucleic acid (mRNA) expression of vascular endothelial growth factor (VEGF) and its receptor Flk-1 was measured by RT-PCR, and the protein expression of VEGF was determined by Western blotting analysis. The treatment with CIG at the doses of 60 and 180 mg/kg/day significantly improved neurological function, and increased the number of bromodeoxyuridine (BrdU)-positive cells and nestin-positive cells in the subventricular zone of rats 7, 14 and 28 days after ischemia. The number of newly mature neurons and blood vessels in striatum, as indicated by BrdU/NeuN and vWF immunoreactivity, respectively, was also increased in CIG-treated rats 28 days after stroke. CIG treatment obviously enhanced the mRNA expression of VEGF and its receptor Flk-1 and the protein expression of VEGF 7 and 28 days after ischemia. The results indicated that CIG promoted neurogenesis and angiogenesis and improved neurological function after ischemia in rats, and the mechanism might be related to CIG’s increasing VEGF and Flk-1 in the brain.

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1. Introduction

Neural stem/progenitor cells are localized in the subventricular zone (SVZ) of the lateral ventricle and dentate gyrus of the hippocampus in the mammalian adult brain [13]. Neurogenesis and angiogenesis are coupled in the brain [38,40]. Ischemic stroke caused by middle cerebral artery occlusion (MCAO) triggers angiogenesis and increases cell proliferation in the SVZ [18,38,40,55]. The newly formed neuroblasts migrate into the damaged striatum, where they mature. A substantial portion of the new neurons express markers characteristic of striatal medium spiny neurons that have died [2,19,41]. The newly produced striatal neurons survive for several months after stroke [48].

Vascular endothelial growth factor (VEGF) is generally accepted as the major factor involved in the process of angiogenesis in the central nervous system (CNS). VEGF plays an important role in angiogenesis by stimulating revascularization and by increasing the number of blood vessels. Recent studies have shown that VEGF is also a possible direct neurogenesis and neuroprotective factor [15,47]. Several studies have reported the upregulation of VEGF and its receptors in the brain following focal cerebral ischemia [34,42].

Corus officinalis Sieb. et Zucc is a member of the Cornaceae family. Use of this herb was first recorded in Shen Nong’s Materia Medica about 2000 years ago in China. Cornel iridoid glycoside (CIG) is a main component extracted from C. officinalis. Previous studies in our laboratory found that intragastrical administration of CIG enhanced the expression of growth-associated protein-43 (GAP-43) and nerve growth factor (NGF), inhibited the expression of chondroitin sulfate proteoglycans (CSPG) and reduced the loss of neurons in the medial septum of rats with bilateral fornix/fimbria transaction [31,32]. Our recent study showed that the treatment of CIG improved neurobehavioral deficits, decreased cerebral infarct size, reduced nitric oxide and inhibited nuclear factor kappa B (NF-κB) expression in the brain of rats 24 h after focal cerebral ischemia [27,53]. These results indicate that CIG may improve the microenvironment of the CNS by increasing growth/trophic factors and decreasing inflammation-related factors. Since the microenvironment plays an important role in neurogenesis, we hypothesized that CIG might promote neurogenesis and angiogenesis in the brain after stroke through increasing growth factors, including VEGF. To verify this hypothesis, we observed the effects of CIG on the neurogenesis, angiogenesis, VEGF expression and neurological...
function in rats 7–28 days after focal cerebral ischemia induced by MCAO.

2. Materials and methods

2.1. Extraction and test assay of purity of CIG

The sarcocarp of C. officinalis was purchased from Tong-Ren-Tang Company, Beijing, China. The air-dried sarcocarp material was extracted with water. The solution was concentrated to afford a residue. Ethanol was added to the residue until the concentration of ethanol reached 80%. Ethanol was reclaimed from the solution. After the residue was dissolved in water, the aqueous solution was concentrated and chromatographed on a SP70 resin column using 20% EIOH as eluant. The 20% EIOH eluant was concentrated and then chromatographed on a silica gel column using CHCl3–MeOH mobile phase (15:1–8:1) as eluants to yield iridoid glycosides.

The purity of cornel iridoid glycoside was determined by RP-HPLC assay. The sample was dissolved in methanol. Octadecylsilyl bonded silica gel was used as immobilize phase, and methanol–water (30:70) as mobile phase. Iridoid glycoside was detected at the wavelength of 240 nm. The purity of CIG was 71.19%, in which morroniside accounted for 67% and loganin 33%.

2.2. Animals and middle cerebral artery occlusion

Male Sprague–Dawley rats weighing from 250 to 270 g were purchased from Beijing Vitalriver Experimental Animal Co., Beijing, China (certificate no: SCXK2002-0003), and were housed under a 12/12 h light/dark cycle and specific pathogen-free (SPF) conditions. During the entire experiment, the rats had free access to food and water. They were randomly divided into five groups (n = 27 in each group): (1) a sham-operated group, (2) a vehicle-treated ischemic model group, (3) a 20 mg/kg/day CIG-treated ischemic group (CIG-L), (4) a 60 mg/kg/day CIG-treated ischemic group (CIG-M), and (5) a 180 mg/kg/day CIG-treated ischemic group (CIG-H).

The middle cerebral artery occlusion was induced by the intraluminal filament technique [30]. Rats were anesthetized with 10% chloral hydrate (0.4 mL/kg, i.p.). Then, a piece of nylon monofilament was inserted into the left internal carotid artery. After 90 min of ischemia, the filament was withdrawn. In sham-operated animals, a piece of monofilament was inserted into the left internal carotid artery but the middle cerebral artery (MCA) was not occluded. All experimental procedures were performed in accordance with the Guidelines and Recommendations of Chinese Experimental Animal Administration Legislation, and all efforts were made to minimize both the number of animals used and any suffering by the animals.

CIG was dissolved in normal saline and administered intragastrically once a day at the doses of 20, 60, and 180 mg/kg, respectively, starting 3 h after MCAO. The vehicle control groups of ischemic rats and sham-operated rats received an equal volume of normal saline.

To label proliferating cells, bromodeoxyuridine (BrdU, 50 mg/kg; Sigma, St. Louis, MO, USA) was injected intraperitoneally twice daily for 4 consecutive days beginning at the doses of 20, 60, and 180 mg/kg, respectively, starting 3 h after MCAO. The vehicle control groups of ischemic rats and sham-operated rats received an equal volume of normal saline.

2.3. Neurological functional test

Modified neurological severity score (mNSS) measurement is a composite of motor, sensory, reflex and balance tests, as previously described [7,28]. In the present study, mNSS was used to determine the sensorimotor deficit 7, 14, and 28 days after MCAO. Only animals that showed no or incomplete forelimb placing with rotational asymmetry 24 h after MCAO were included in the subsequent analysis.

2.4. Immunohistochemistry

Immunohistochemistry staining for brain tissue was performed on ice-cold sections (40 µm). Primary antibodies were mouse anti-BrdU (1:1000; Chemicon International, Inc., USA); rabbit anti-BrdU (1:1000; Abcam, Cambridge, UK); mouse anti-nestin (1:1000; BD Pharmingen, Inc., San Diego, CA, USA); goat anti-Double-cortin (anti-Dcx, 1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA); mouse anti-neuronal nuclei (anti-NeuN, 1:200; Chemicon International, Temecula, CA, USA); mouse anti-glial fibrillary acidic protein (anti-GFAP, 1:1500; Sigma), rabbit anti-vein Willebrand factor (anti-vWF, 1:200; Chemicon), mouse anti-VEGF (1:1000; Sigma) and rabbit anti-Fk-1 (1:1000; Abcam).

Secondary antibodies were biotinylated horse anti-mouse and goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA); CY2-conjugated goat anti-mouse and goat anti-rabbit IgG; CY3-conjugated goat anti-mouse and goat anti-rabbit IgG (all 1:200; all from Amersham Biosciences Ltd.–GE Healthcare, USA).

Fluorescence double staining was used for visualization of BrdU with Dcx, NeuN or GFAP. In brief, free-floating sections were incubated in 3% H2O2 in 0.01 M PBS. After incubation with a blocking solution, all sections were incubated with primary antibodies, such as anti-BrdU with anti-Dcx, anti-NeuN, anti-GFAP, anti-vWF or Fk-1 antibodies, respectively, for 4 h at 4 °C. After incubation with the CY2- or CY3-conjugated secondary antibodies for 2 h, fluorescence signals were visualized by the confocal laser scanning microscope system (MRC1024, Bio-Rad, Hercules, CA, USA). For Dcx staining, biotinylated horse anti-goat secondary antibody and Alexa Fluor® 488-conjugated streptavidin (1:200, Molecular Probes®, Invitrogen) were used. For BrdU staining, sections were pretreated with 50% formamide/280 mM NaCl/30 mM sodium citrate at 65 °C for 2 h and incubated in 2% H2O2 in 0.1 M boric acid (pH 8.5) at room temperature for 10 min.

2.5. Image analysis and quantification

All analyses were conducted by observers blinded to treatment conditions. To determine the number of BrdU-labeled cells in the SVZ, every 10th section (each 40 µm) between bregma levels +1.60 and −0.2 mm were selected (total = 3 sections per brain). In each section, a square of 50 µm per side was placed within the dorsal lateral corner of the SVZ, and the total number of BrdU-labeled cells per square was counted. The number of cells in each square was then multiplied by slice thickness, and an average for the three slices per hemisphere was taken [49].

For semi-quantitative measurement of nestin density, Image Pro® Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) was used. Three sections per brain were selected between bregma levels +1.60 and −0.2 mm every 9th section. The digitalized images were then contrast-enhanced to clearly differentiate positivity from background, and a thresholding procedure was established to determine the proportion of immunoreactive area within each fixed field of view. The quantification was not analyzed stereologically. Data are presented as a percentage of area, in which the nestin-immunopositive areas in each field were divided by the total areas in the field (180 µm × 180 µm) [8,9].

The counting of BrdU+/Dcx+, BrdU+/-GFAP+ and BrdU+/NeuN+ cells in the stria
tum was performed using a confocal laser microscope in three sections per animal from the same levels as above. Areas of interest were scanned with a ×40 objective lens in 260.6 µm × 260.6 µm format in the x–y direction.

To count microvessels, the images were converted into gray scale for quantification. Only vWF-positive vessels with a clear lumen and those that formed ring-like or tubular structures were counted [6]. The number of vessels in each field was then converted into the number of vessels/mm² (AOI × 560 µm × 560 µm). The vessel number was obtained by estimating the number of branch points and vessels and dividing the sum by 2. No correction was done for blood vessels that extended spatially over a sufficiently long distance since these vessels comprise only a very low percentage of the total number of vessels.

2.6. RT-PCR assay

The cortex of rats was rapidly dissected 7 days after MCAO, and total RNA was purified from it by using Trizol® Reagent (Invitrogen), according to the manufacturer’s instructions. After deoxyribonuclease (DNase) treatment, 1 µg of total RNA was converted into complementary DNA (cDNA) with a M-MuLV First Strand cDNA Synthesis Kit (SuperScript™, Invitrogen). The following primers were used, respectively, for VEGF: Sense 5′-TCAGGACCCAGAGGCAG-3′, Antisense 5′-TCACCGTGCCTGTCTTA-3′ [17]; the primers for Flk-1: Sense 5′-CCATGAAAGGGAACTGG-3′, Antisense 5′-TCCTGTGGTCTGATC-3′; the primers for GAPDH: Sense 5′-CAAT-3′, Antisense 5′-CCAGCTGCTGATGGTC-3′ [10]. PCR was performed using a Ready-to-use PCR Kit (Bio Basic Inc., Ontario, Canada). For VEGF, the initial melting temperature was 96 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 55 s and 72 °C for 55 s, with a final extension at 72 °C for 10 min. For Flk-1, the initial melting temperature was 96 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 55 s and 72 °C for 55 s, with a final extension at 72 °C for 10 min. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. All samples were normalized against a GAPDH control band. Images were analyzed by image J® software and the results were expressed as relative fold of VEGF/GAPDH and Flk-1/GAPDH.

2.7. Western blotting

Western blotting was performed on the cortex tissue ipsilateral to the ischemic brain. Mouse anti-VEGF antibody (1:1000; Sigma) was used. Using the same mem-

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brane, mouse anti-β-actin immunoblotting (1:2000; Sigma) was performed to standardize the expression of VEGF.

2.8. Statistical analysis

All data were expressed as mean ± standard error (mean ± SE). Behavioral data were analyzed by SPSS 10.0™ (SPSS, Inc., Chicago, IL, USA) using Windows software to conduct two-way analysis of variance (ANOVA, equal variances assumed by S-N-K). Other data were analyzed by SPSS 10.0 using Windows software to conduct one-way ANOVA (equal variances assumed by S-N-K). A P-value < 0.05 was considered significant.

3. Results

3.1. Effects of CIG on neurological function in cerebral ischemic rats

A modified neurological severity score test was performed after MCAO. The higher the score, the poorer the function. The results showed that treatment starting at 3 h after MCAO with CIG-M (60 mg/kg/d) and CIG-H (180 mg/kg/d) significantly decreased mNSS scores 7, 14 and 28 days after MCAO, compared with the vehicle control group of ischemic rats (P < 0.05), indicating that CIG was effective to improve neurological function at both early stage and late stage after cerebral ischemia (Table 1).

3.2. Effects of CIG on cell proliferation in SVZ of cerebral ischemic rats

BrdU is an analog of thymidine, which is incorporated into DNA of cells during the S phase and has been used to investigate cell proliferation [23]. Nestin is a class VI intermediate filament protein, which has been used as the predominant marker for stem and progenitor cells in the mammalian CNS [36]. The results of the present study showed that in the vehicle-treated ischemic rats, the num-

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
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<td></td>
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<td>Day 7</td>
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<tr>
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<td>10</td>
<td>7.17 ± 0.44*</td>
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<tr>
<td>CIG-H</td>
<td>180</td>
<td>8</td>
<td>7.50 ± 0.27*</td>
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CIG was intragastrically administered to the rats starting from 3 h after the onset of middle cerebral artery occlusion (MCAO). The vehicle-treated ischemic model group received an equal volume of normal saline. Values are expressed as mean ± SE.

* P < 0.05 vs. vehicle-treated group.

** P < 0.01 vs. vehicle-treated group.

Fig. 1. Effects of cornel iridoid glycoside (CIG) on cell proliferation in SVZ of rats 7, 14 and 28 days after cerebral ischemia. A through C: BrdU immunoreactive cells in the ischemic ipsilateral SVZ in sham-operated rats (A), a vehicle-treated ischemic group (B) and a group of rats treated with 60 mg/kg/day of CIG (C) 14 days after MCAO. D through F: Nestin immunoreactive cells in the ischemic ipsilateral SVZ in sham (D), vehicle (E), and 60 mg/kg/day CIG groups (F) 14 days after MCAO. (G) Quantitative analysis of the number of BrdU-positive cells. (H) Quantitative analysis of the density of nestin-positive cells. n = 3 for each group. Values are mean ± SE. * P < 0.05, ** P < 0.01, compared with vehicle-treated ischemic group. Bar in F = 100 μm for panels A–F.
Fig. 2. Effects of CIG on neuron maturation in striatum of rats 28 days after cerebral ischemia. Double immunostaining and confocal microscopy were used. Panels A through C: BrdU+/DCX+ cells; Panels D through F: BrdU+/NeuN+ cells; Panels G through I: BrdU+/GFAP+ cells. Panel J: quantitative analysis of the number of neuroblasts (BrdU+/DCX+), mature neurons (BrdU+/NeuN+) and astrocytes (BrdU+/GFAP+). n = 4 for each group. Values are mean ± SE. *P < 0.05, **P < 0.01, compared with vehicle-treated ischemic group. Bar in I = 10 μm for panels A through I.

3.3. Effects of CIG on neuron maturation in striatum of cerebral ischemic rats

In this experiment, double immunostaining and confocal microscopy were used to identify the phenotype of BrdU-labeled cells 28 days after MCAO. DCX was used as a marker for immature neurons and NeuN as a marker for mature neurons, and GFAP was used to identify astroglia. The results of double immunostaining showed that only a few BrdU+/DCX+ cells were found in the ischemic ipsilateral striatum, and there was no significant difference between CIG-treated groups and vehicle-treated ischemic rats (Fig. 2A–C, J). The treatment with CIG at the doses of 60 and 180 mg/kg markedly increased the number of BrdU+/NeuN+ cells (Fig. 2D–F, J); P < 0.05, P < 0.01), and 60 mg/kg CIG significantly decreased BrdU+/GFAP+ cells (Fig. 2G–I, J); P < 0.05).

3.4. Effects of CIG on vessel density in striatum of cerebral ischemic rats

Von Willebrand factor is a specific marker for endothelial cells. In this study, vWF-immunoreactive cells on the coronal slices of striatum were analyzed 28 days after MCAO. The number of vWF-positive vessels in the 60 mg/kg CIG-treated group (Fig. 3C) was more than that in vehicle-treated ischemic rats. In the quantitative analysis, vWF-positive vessels were counted and expressed as the number of vessels/mm². The treatment of CIG at the doses of 60 and 180 mg/kg significantly increased the number of vWF+ vessels in striatum compared with vehicle-treated ischemic rats (Fig. 3D; P < 0.05, P < 0.01).

3.5. Effects of CIG on mRNA expression of VEGF and Flk-1 in cortex of rats with cerebral ischemia

The results of RT-PCR showed that mRNA coding for VEGF₁₆₅ and VEGF₁₂₁ in cortex of rats 7 days after MCAO was detected at the predicted molecular sizes in each group, and VEGF₁₆₅ was more prominent (Fig. 4A). The expression of VEGF mRNA was decreased in ischemic model rats; 60 mg/kg CIG significantly increased VEGF₁₆₅ mRNA expression in cortex as compared with vehicle-treated model rats (Fig. 4A, P < 0.05).

The RT-PCR analysis for VEGF receptor Flk-1 mRNA was performed in cortex of rats 7 days after MCAO. The results indicated that the treatment with CIG at the doses of 60 and 180 mg/kg significantly increased the expression of Flk-1 mRNA in cortex, compared with vehicle-treated ischemic rats (Fig. 4B; P < 0.05, P < 0.01).

3.6. Effects of CIG on protein expression of VEGF in cortex of cerebral ischemic rats

Fig. 5 shows the results of Western blotting with a monoclonal anti-VEGF antibody in cortex of rats 7, 14 and 28 days after MCAO. Compared with the vehicle-treated ischemic rats, the treatment with 60 mg/kg CIG significantly increased the protein expression of VEGF 7 days after MCAO (P < 0.05), and CIG at the doses of 60 and 180 mg/kg obviously enhanced VEGF expression 14 and 28 days after ischemia (P < 0.05, P < 0.01).
Fig. 3. Effects of CIG on vWF+ vessels in striatum of rats 28 days after cerebral ischemia. Panel A, sham-operated group; Panel B, vehicle-treated ischemic rats; Panel C, 60 mg/kg/day CIG-treated group. Panel D, quantitative analysis of the number of vWF+ vessels/mm². n = 4 for each group. Values are mean ± SE. *P < 0.05, **P < 0.01, compared with vehicle-treated ischemic group. Bar in C = 100 μm for panels A–C.

4. Discussion

The previous study in our lab revealed that oral administration of CIG obviously improved the neurological function in rats 24 h after MCAO, and this effectiveness was largely attributed to the inhibition of inflammation and neuronal apoptosis [26,27,53]. The purpose of the present study was to investigate whether CIG might have the protective effect in the later stages after cerebral ischemia. The results demonstrated that treatment with CIG starting from 3 h after MCAO significantly improved neurological function in rats 7, 14 and

Fig. 4. Effects of CIG on mRNA expression of VEGF and its receptor Flk-1 in cortex of rats 7 days after cerebral ischemia by using RT-PCR analysis. Panel A, VEGF₁₆₅ mRNA expression; Panel B, Flk-1 mRNA expression. The bands were quantified by densitometry, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and expressed as relative fold activation. n = 3 (the data of each group were obtained from three rats in three experiments.) Values are mean ± SE. *P < 0.05, **P < 0.01, compared with vehicle-treated ischemic rats.
found that CIG treatment significantly increased the number of newly generated neuronal cells in the SVZ of rats 7, 14 and 28 days after MCAO compared with vehicle-treated ischemic rats, suggesting that CIG may promote neurogenesis in the adult brain. In addition, angiogenesis was observed in the penumbra areas after focal cerebral ischemia in the adult brain [33,50]. In the present study, we also investigated the effect of CIG on angiogenesis by quantitating the number of vessels immunoreactive to vWF, an accepted marker for endothelial cells and angiogenesis [54]. It was found that the number of vWF-positive vessels was increased in CIG-treated rats 28 days after MCAO compared with the vehicle-treated ischemic rats, suggesting that CIG may also promote angiogenesis.

In order to investigate the mechanism by which CIG promote angiogenesis, the expression of VEGF was detected in the present study. There are at least four possible splice variants of VEGF, consisting of VEGF$_{121}$, VEGF$_{165}$, VEGF$_{189}$, and VEGF$_{206}$ [12]. VEGF$_{165}$ and VEGF$_{121}$ are likely to be the most abundant isoforms, and mediate most biological activity of VEGF. Our data showed that CIG increased the mRNA expression of VEGF and its receptor Flk-1 7 days after MCAO, and also elevated the level of VEGF protein in the cortex 7, 14 and 28 days after ischemia, as compared with vehicle-treated ischemic rats.

In addition to its angiogenic role, VEGF also stimulates neurogenesis [20,25,40]. Administration of exogenous VEGF accelerates neurogenesis in vitro and in vivo [20]. In addition, neural stem cells in the SVZ [44] and Dcx+ neuroblasts in the dentate subgranular zone [20] express VEGF receptor Flk-1. VEGF appears to modulate adult neurogenesis by signaling through this receptor, since administration of a dominant-negative mutant Flk-1 reverses the VEGF-induced increase in neurogenesis [4]. Thus we suppose that CIG-induced increase of VEGF and Flk-1 may also promote neurogenesis in the brain after cerebral ischemia.

The local microenvironment in regions of ongoing neurogenesis in the adult mammalian brain regulates the self-renewal, activation and differentiation of stem cells [46,52]. Besides the positive regulation by VEGF and other growth factors, inflammation and apoptosis play a negative role in microenvironment. Nitric oxide (NO), an important factor of inflammatory reaction, is a negative regulator of cell proliferation and a positive promoter of neuronal differentiation toward astrocytes derived from the newly generated cells after cerebral ischemia.

Recently, it has been shown that neural stem cells exist close to blood vessels in the rat dentate gyrus [40] and SVZ [5]. These findings suggest that the microenvironment surrounding blood vessels, which is termed vascular niche, may play an important role in neurogenesis in the adult brain. It is known that BrdU is an analog of thymidine and can be incorporated into DNA of cells during the S phase and thus has been used to investigate cell proliferation [23]. Nestin is a class VI intermediate filament protein that is expressed in stem cells of the adult neuroepithelium [24]. The expression of nestin has been used to label neurogenic activity in cells either within or derived from the SVZ [1,21,43]. Nestin protein is also expressed in reactive astrocytes following injury to the brain [11,14,45]. Several research groups have provided the evidence that focal ischemic injury increases cell proliferation and neurogenesis in the SVZ. The number of BrdU-positive cells peaks from 7 to 14 days after ischemia and returns to control level 3–5 weeks after ischemia [18,29,55]. The expression of nestin after ischemia may be related with cell proliferation [4]. The cell proliferation in the SVZ of rats induced by cerebral ischemia was also observed in our present study, manifested by an increase in the number of BrdU-labeled and nestin-labeled cells with the peak 14 days after MCAO. However, the number of these cells was dropped to control level 28 days after ischemia. The treatment of CIG significantly increased the number of BrdU-labeled and nestin-labeled cells in the SVZ of rats 7, 14 and 28 days after MCAO compared with the vehicle-treated ischemic rats, suggesting that CIG may promote cell proliferation.

Multiple labeling studies, by using BrdU immunohistochemistry and confocal microscopy, reveal that newly generated neuronal cells in the adult brain express a sequence of markers [22,40]. Some were double-labeled with mature neuron marker NeuN or mature astrocyte marker GFAP [41]. In the present study, we found that CIG treatment significantly increased the number of BrdU+/NeuN+ neurons and decreased the number of BrdU+/GFAP+ astrocytes in striatum of rats 28 days after MCAO, compared with the vehicle-treated ischemic rats. However, only a few BrdU+/Dcx+ cells appeared in ischemic striatum, and CIG treatment did not influence the number of BrdU+/Dcx+ cells. Dcx is a marker of neuroblasts, which are transiently expressed (during about 2–3 weeks) in neuronal progenitors [3]. We presume that immature neurons (neuroblasts) had differentiated into mature neurons 28 days after MCAO. The results indicate that CIG has the potential to promote the differentiation toward mature neurons and to inhibit the differentiation toward astrocytes derived from the newly generated cells after cerebral ischemia.

VEGF-induced increase in neurogenesis [4]. Thus we suppose that CIG-induced increase of VEGF and Flk-1 may also promote neurogenesis in the brain after cerebral ischemia.

The local microenvironment in regions of ongoing neurogenesis in the adult mammalian brain regulates the self-renewal, activation and differentiation of stem cells [46,52]. Besides the positive regulation by VEGF and other growth factors, inflammation and apoptosis play a negative role in microenvironment. Nitric oxide (NO), an important factor of inflammatory reaction, is a negative regulator of cell proliferation and a positive promoter of neuronal fate determination in neurogenic regions in adult brain. Nitric oxide synthase (NOS) inhibitors increase SVZ proliferation [37,39]. The number of newly generated cells in the olfactory bulb and dentate gyrus are enhanced significantly in null mutant mice lacking neuronal NOS activity [39]. An increase in Bcl-2 expression leads to improvement of neuron survival in the cerebral ischemia [35,56]. Our previous studies indicated that CIG inhibited inducible nitric oxide synthase (iNOS) activity, decreased NF-κB expression, and regulated apoptosis-related factors by increasing Bcl-2 and decreasing Bax in the brain of cerebral ischemic rats [26]. Therefore, we presume that CIG may provide a beneficial microenvironment for neurogenesis by increasing VEGF and inhibiting inflammation and apoptosis.
Alterations in neurogenesis, and the extent to which the newly generated neurons are integrated into the brain network play an essential role in the modulation of functional activity [16]. Many endogenous molecular mechanisms and exogenous agents promote the post-ischemic neurogenesis. So major challenges seem to be inducing sufficient proliferation to repair the devastating neuronal damage following ischemia, promoting the survival of the newly formed cells and more importantly to induce proper connectivity of the newly formed cells with the existing circuitry [51]. This study demonstrated that the treatment with CIG significantly promoted neurogenesis and angiogenesis in the brain and improved neurological function in rats 7, 14, and 28 days after focal cerebral ischemia, and the mechanism might be related to CIG's increasing the expression of VEGF and Flk-1 in the ischemic brain. The results suggest that CIG may have potential benefits for the treatment of stroke patients.

Conflict of interest
We ensure that all authors have no competing financial interests.

Acknowledgements
This study was supported by the National Key Basic Research 973 Program of China (No. 2003CB517104 to L. Li); National Natural Science Foundation of China (No. 30801526 to R.Q. Yao, No. 90709011 to L. Li, No. 30427184 to L. Li); Beijing Natural Science Foundation (No. 7050001 to L. Li); Beijing Science and Technology Program (No. 77020015 to L. Li). (No. 7050001 to L. Li); Beijing Science and Technology Program (No. 77020015 to L. Li).

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