The Protective Effect of Cerebralcare Granule® on Brain Edema, Cerebral Microcirculatory Disturbance, and Neuron Injury in a Focal Cerebral Ischemia Rat Model

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ABSTRACT

Objective: The present study was to explore the protective effects of CG on rat cerebral injury after focal cerebral I/R.

Methods: Male Sprague–Dawley rats were subjected to right middle cerebral artery occlusion for 60 minutes followed by reperfusion for 60 minutes or 24 hours. CG (0.4 or 0.8 g/kg) was administrated 90 minutes before ischemia. Brain edema was evaluated by Evans’s blue dye extravasations and brain water content, leukocyte adhesion, and albumin leakage were determined with an upright fluorescence microscope, and neuron damage was assessed by p53 upregulated modulator of apoptosis.

Results: Focal cerebral I/R elicited a prominent brain edema, an increase in leukocyte adhesion, and albumin leakage, as well as neuron damage. All the insults after focal cerebral I/R were significantly attenuated by pretreatment with CG.

Conclusions: Pretreatment with CG significantly reduced focal cerebral I/R-induced brain edema, cerebral microcirculatory disturbance, and neuron damage, suggesting the potential of CG as a prophylactic strategy for patients in danger of stroke.

Key words: ischemia and reperfusion, middle cerebral artery occlusion, neuron injury, cerebral microcirculatory disturbance, brain edema

Abbreviations used: BBB, blood–brain barrier; CG, Cerebralcare Granule; dUTP, deoxyuridine-5’-triphosphate; EB, Evans’s blue; FITC, fluorescein isothiocyanate; I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; PUMA, p53 upregulated modulator of apoptosis; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UPLC-TOF-MS, ultra performance liquid chromatography–time-of-flight-mass spectrometer

INTRODUCTION

Stroke, as a serious neurological disease, inflicts a major impact on the public health in almost every country. Ischemic stroke frequently results in brain edema causing severe or even fatal outcome. However, several available strategies, such as decompression craniotomy, hypothermia, and osmotic diuresis therapy, are applied only as a rescue therapy [6,19,24], while options for preventing brain edema are limited.

CG was approved in 1996 by the China State Food and Drug Administration for treatment of headache and dizziness associated with cerebrovascular diseases. CG is composed of 11 herbs, including Radix angelicae sinensis (Dang Gui), Rhizoma chuan xiong (Chuan Xiong), Radix paoniae alba (Bai Shao), Ramulus uncariae cum uncis (Gou Teng), Caulis spatholobi (Ji Xue Teng), Spica prunellae (Xia Ku Cao), Concha margaritifera usta (Zhen Zhu Mu), Radix rehmanniae preparata (Di Huang), Semen cassiae (Jue Ming Zi), Rhizoma corydis yamhusuo (Yan Hu...
Suo), and Herba asari (Xi Xin). Each of these herbs has previously been characterized and commonly used in traditional Chinese medicine. A number of studies have been conducted to identify the active constituent contained in these herbs and the mechanism behind their actions. For example, ligustrazine (the major constituent of Rhizoma chuan xiong) was reported to inhibit neutrophil adhesion to endothelial cells [15,17], and peoniflorin (the major constituent of Radix paeonie alba), ligustrazine, and ferulic acid (the major constituent of Radix angelicae sinensis) were found to decrease the infarct size and brain tissue damage [12,15,17,18,29].

Our previous studies undertaken in Mongolian gerbils demonstrated that CG attenuates the bilateral common carotid artery occlusion-elicited cerebral microcirculatory disturbance, hippocampal neuron injury, as well as BBB disruption, indicative of the potential of CG in protecting brain from edema after ischemia and reperfusion (I/R) [28,30]. The purpose of the present study was to verify the ability of CG as a prophylactic management in the development of brain edema following I/R, in addition to attenuation of microcirculation disturbance and neuron injury, using a focal cerebral I/R model in which rats were subjected to MCAO.

**MATERIALS AND METHODS**

**Animals**

Male Sprague–Dawley rats weighing 270–300 g were purchased from the Animal Center of Peking University Health Science Center. The animals were fasted for 12 hours before each experiment and allowed free access to water. All animals were handled according to the guidelines of the Peking University Animal Research Committee. The surgeries and protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University.

**Cerebralcare Granule**

CG was produced by Tianjin Tasly Pharmaceutical Co. Ltd (Tianjin, China). The batch number of the granules used in this study was Z10960082. No steroid is included in the content of CG. The processing of the product followed a strict quality control, and the ingredients were subjected to standardization. The herbs were manufactured as granules after dynamic cycle extraction and concentration by evaporating and spray drying. Then the CG was packed with aluminum foil composite, 4 g per bag. The compound was dissolved in water to a concentration of 80 or 160 mg/mL before use [30].

**UPLC–TOF-MS Analysis of CG Components in Rat Plasma**

Rats were fasted overnight before experiment, and administered by gavage with 0.8 g/kg CG dissolved in saline. Animals that received equivalent volume of saline were used as control. Blood was harvested from orbit veins one, two, three, and four hours after drug administration, and anticoagulated with heparin. Plasma was separated by centrifugation for 10 minutes at 3000 rpm (956 rcf), and processed for analysis by UPLC–TOF-MS (UPLC Waters ACQUITY and LCT Premier XE TOF-MS) [31]. MassLynx 4.1 Software (Waters Corporation, Milford, MA, USA) was used to acquire data. Data were collected over the mass range of 50–1500 m/z.

**Animal Model and Drug Administration**

Focal cerebral ischemia was induced by MCAO, as described elsewhere [3]. Briefly, animals were anesthetized using 20% urethane. The right common carotid artery, including its bifurcation, was exposed. The external carotid artery was divided, leaving a stump of 3–4 mm. The internal carotid artery was then isolated, and the pterygoplatine artery was ligated close to its origin. The internal carotid artery was then clamped with a small vascular clip. The common carotid artery was also clamped. The stump of the external carotid artery was reopened, and a 4-0 monofilament nylon suture with a slightly enlarged and round tip was inserted up through the internal carotid artery. When a small resistance was felt, insertion was stopped. The distance from bifurcation of the common carotid artery to the tip of the suture was 18–22 mm. After occlusion for one hour, the suture was withdrawn through the internal carotid artery into the external artery, allowing reperfusion. The animals in the sham group underwent the same surgical procedures, but without arterial occlusion. For evaluating the CG effect, animals in the CG + I/R groups received the drug orally 90 minutes before MCAO at either 0.4 g/kg (CG 0.4 + I/R group) or 0.8 g/kg (CG 0.8 + I/R group).

**Evan’s Blue Dye Extravasation and Brain Water Content**

Disruption of the BBB was analyzed 24 hours after the MCAO using EB dye. Briefly, rats were anesthetized with pentobarbital sodium (0.1 g/kg body weight, i.p.), and EB dye (Sigma-Aldrich, St. Louis, MO, USA; 4%, 3 mL/kg) in saline was injected within two minutes into the left femoral vein and allowed to circulate for 120 minutes. Rats were transcardially perfused with PBS until colorless perfusion fluid drained from the right atrium. The amount of extravasated EB in the brain was determined by spectrophotometry at an excitation wavelength of 620 nm. The brain was removed and weighed immediately for determination of wet weight, and weighed after drying in an oven at 105°C for 24 hours for determination of dry weight. The brain water content was presented as [(wet weight − dry weight)/wet weight] × 100% [13].
Observation of Microcirculation
After being anesthetized with 20% urethane (2.0 g/kg body weight, i.m.), the rats were tracheotomized and mechanically ventilated with a breathing machine designed for small animals (ALC-V8). With a hand-held drill, a skull window of 4 × 6 mm was created 1 mm behind the coronal suture, and 1 mm on the right side of the sagittal suture. The dura was superfused contiguously with 37°C warm physiological saline. A single unbranched venule was selected for determination, which was without obvious bend with diameter ranging between 30 and 50 μm and length of about 200 μm. The dynamic of cerebral microcirculation was continuously observed for 60 minutes using a biological microscope (DM-LFS, Leica, Germany) equipped with a color monitor (J2118A, TCL, Huizhou, China), a biological microscope (DM-LFS, Leica, Germany) in combination with a CCD camera (USS-301, APX, Photron, Tokyo, Japan), and a DVD recorder (DVR-R25, Malata, China). The microvessel images were recorded through a high speed video camera system at a rate of 1000 frames/sec (FASTCAM-ultima APX, Photron, Tokyo, Japan), and the recordings were replayed at a rate of 25 frames/sec from the stored images. The RBC velocity in venule was measured with Image Pro Plus software (IPP, Media Cybernetic, Bethesda, MD, USA) before ischemia and 1, 20, 40, and 60 minutes after reperfusion, respectively. Using the same software, the inner diameter of cerebral venules was measured and presented as the mean of three measurements at one location [30]. To assess leukocyte adhesion in venules, the fluorescence tracer Rhodamine 6G (Sigma, St Louis, MO, USA) was administrated (5 mg/kg body weight) to the animal via the femoral vein 10 minutes before ischemia. After craniotomy, the cerebral microvessels were observed under an upright fluorescence microscope (DM-LFS, Leica, Germany) in combination with a CCD camera (USS-301, Uniq, Santa Clara, CA, USA) using a helium-neon laser beam for illumination. The adherent leukocytes were identified as those that attached to the venular walls for more than 30 seconds. The number of adherent leukocytes was scored under basal condition and 1, 20, 40, and 60 minutes after reperfusion. For evaluation of albumin leakage from venular wall, FITC-albumin (Sigma-Aldrich) was infused (50 mg/kg body weight) slowly through femoral vein 10 minutes before ischemia, and the upright fluorescence microscopy was employed. Using IPP software, the fluorescence intensities of FITC-albumin inside the lumen of selected venules (Iv) and in the surrounding interstitial area (Ii) were measured. The ratio I/Iv was calculated and compared with the baseline as an indication of albumin leakage.

Infarct Volume
Twenty-four hours after reperfusion, the rat was anesthetized with 20% urethane (2.0 g/kg body weight, i.m.) and the brain was rapidly excised and sliced coronally into five sections (2 mm thick) beginning from optic chiasma. The slices of the brain were incubated for 30 minutes at 37°C in a 2% solution of TTC in PBS and then photographed as digital images (Digital Sight DS-5M-U1; Nikon, Tokyo, Japan). The infarct volume was calculated based on the ratio of the infarct area of the ipsilateral hemisphere to total non-infarct area from both the ipsilateral and contralateral hemispheres to avoid the influence of tissue edema [3].

Neurological Scores
Twenty-four hours after reperfusion, the neurological scores were performed in a blinded fashion, as previously reported with some modifications [13]. Each rat was subjected to 6 tests (spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch) with the minimum neurological score being 3 and the maximum 18. The score given to each rat at the completion of the evaluation was the summation of the 6 individual test scores [13].

Nissl and Immunohistochemistry Staining
For this propose, the brain samples were collected 24 hours after reperfusion. After transcardiac perfusion with 250 mL of 4% paraformaldehyde under anesthesia, rat brain was removed and postfixed in the same fixative for 48 hours. The brain tissue located in the middle between the optic chiasma and the cerebral caudal end was cut into blocks, embedded in paraffin, and sectioned at 10 μm. The sections were deparaffinized and rehydrated, sequentially, and processed for either Nissl staining or immunohistochemistry. For immunohistochemistry, the sections were incubated with the following primary antibodies: mouse anti-caspase-3, goat anti-p53 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-PUMA (1:1000, Cell Signaling, Boston, MA, USA). After being washed, the samples were incubated with a biotinylated secondary antibody followed by avidin–biotin–peroxidase complex, and visualized with diaminobenzidine. As control, a consecutive section was treated similarly except that the primary antibody was omitted. IPP software was used to assess the number of positive cells of caspase-3, p53, and PUMA in the cortex penumbral region of the ischemic side of brain, as described previously [3,13].

TUNEL Assay
The animals under anesthesia were infused via the left ventricle with 4% paraformaldehyde in 0.01 M PBS (pH 7.4) 24 hours after reperfusion. Brain was removed and postfixed with the same fixative for six hours, and cryoprotected in 30% sucrose in PBS for at least 48 hours at 4°C. The coronal brain sections (10 μm thick) were cut from the
middle between the optic chiasma and the cerebral caudal end on a cryostat (Leica CM1800, Bensheim, Germany). To evaluate the apoptotic neuron cells in the cortex penumbral region, TUNEL was conducted using an *in situ* cell death detection kit (Fluorescein dUTP Kit; Roche Inc., Indianapolis, IN, USA), according to the instruction of manufacturer. The nuclei were counterstained with hoechst33342 (2 μg/mL) for five minutes. The slides were rinsed with PBS, coverslipped with mounting medium and observed under a laser confocal microscope (Axiovert 2000, Zeiss, Germany) with a 63× objective at an excitation wavelength of 480 nm and emission wavelength of 530 nm. Five fields were randomly selected for each rat. The number of TUNEL-positive nuclei and the total number of nuclei in each field were scored, and the ratio of the two values was automatically calculated with IPP 5.0 software. A similarly treated consecutive section without addition of TdT was used as control [31].

**Ultrastructure of Cerebral Cortex**

Another set of rats were anesthetized 24 hours after reperfusion, and perfused with a fixative composed of 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at a speed of 3 mL/min through the left ventricle. The animals were decapitated and a cerebral slice of 1 mm thick was taken from the middle between the optic chiasma and the cerebral caudal end, and tissue samples smaller than 1 mm³ in the cortex penumbral region were prepared as routine for transmission electron microscopy. Ultrathin sections of cortex were examined by an electron microscope (JEM 1230, Jeol, Japan).

**Statistical Analysis**

All parameters were expressed as mean ± SE. Statistical analysis was performed using ANOVA followed by Tukey test for multiple comparisons. A *p*-value <0.05 was considered statistically significant.

**RESULTS**

**Identification and Kinetics of CG Components in Rat Plasma**

UPLC–TOF-MS system is characterized by high sensitivity and specificity. By this approach, two components, corydaline and casialactone, were detected in the plasma of CG treated rats, which are obviously distinct from that in plasma of control animals (Figures 1 and 2). These two components appeared one hour after CG administration, and maintained detectable over the next three hours.

**EB Dye Extravasation and Brain Water Content**

After 24 hours of reperfusion, EB was clearly observed in the ipsilateral hemisphere of the coronal sections (Figure 3A). The EB content of the brain tissue increased significantly in the I/R group compared with the sham group, while CG treatment mitigated the I/R-caused increase in EB content of the brain tissue (Figure 3B). Likewise, a significant increase in brain water content was revealed in the ipsilateral hemisphere 24 hours after the MCAO, when compared with the sham group, which was alleviated significantly by CG treatment at 0.8 g/kg (Figure 3C). No significant differences in brain water content were observed among the groups in the contralateral hemisphere.

**Albumin Leakage**

CG pretreatment prevented FITC-labeled albumin leakage from cerebral venules evoked by I/R, and the representative images are presented in Figure 4A. Transvascular efflux of FITC-labeled albumin was quantified for different groups (Figure 4B). There was no detectable leakage under the basal condition in all groups. In the sham group, only a small amount of albumin was found to have leaked from the venular wall during reperfusion. By contrast, albumin leakage in the I/R group increased markedly after one minute of reperfusion and kept increasing with time. The I/R-induced albumin leakage was diminished significantly in the animals receiving CG at both doses tested.

**Leukocyte Adhesion**

Leukocytes in the microcirculation are easily visualized and quantified after rhodamine injection (Figure 5A). The changes in the number of leukocytes adherent to the venular wall in the four groups are shown in Figure 5B. In the sham group, the number of adherent leukocytes increased slightly after reperfusion but showed no significance when compared with baseline. The number of adherent leukocytes in the I/R group significantly increased immediately after reperfusion and remained increasing until the end of observation. Pretreatment with CG attenuated I/R-elicited enhancement of leukocyte adhesion with the higher dose being more significant than the lower dose.

**RBC Velocity and Venular Diameter**

With the use of the microscope equipped with a high-speed video camera, cerebral microvessels and RBC moving inside the venules were clearly visible in the cerebral cortex (Figure 6A). Impressively, the number of open capillaries after I/R reduced considerably (a2) in comparison with the sham group (a1), indicating a decreased blood supply in this area. CG pretreatment, especially at 0.8 g/kg, obviously attenuated this alteration by I/R (a4).

No significant difference in venular diameter was observed among the sham, I/R, and CG-treated groups at baseline, nor over the 60 minutes reperfusion (Figure 6B). The time courses of RBC velocity in cerebral venules in different groups are depicted in Figure 6C. The mean RBC
velocity in cerebral venules in the sham group did not alter significantly during the period of observation. RBC velocity declined temporarily after I/R compared with baseline with the minimum at one minute of reperfusion and then gradually returned to the basal level. The CG treatment had no significant effect on RBC velocity in comparison with the I/R group.

Cerebral Infarct and Neurological Score
Representative TTC-stained brain sections in different groups are shown in Figure 7A, wherein the areas of gray color represent the infarct regions. The ratios of cerebral infarct volume in four groups are shown in Figure 7B. No infarct was found in the sham group. The ratio of infarct volume in the I/R group reached to 30%, which was diminished significantly by pretreatment with CG with a reduction of 10% and 15% at 0.4 and 0.8 g/kg, respectively.

The neurological scores of the animals in each group were 17.6 ± 0.2 (sham group), 9.3 ± 0.6 (I/R group), 10.8 ± 0.6 (CG0.4 + I/R group), and 12.0 ± 0.5 (CG0.8 + I/R group). Pretreatment with CG at 0.8 g/kg was found to increase the neurological score significantly in comparison with the I/R group (p < 0.05) (Figure 7C).

Nissl Staining
Results of Nissl staining at 24 hours after reperfusion showed that neurons of the sham group exhibited normal morphological features, while diverse neuron damages occurred in the I/R group, such as cell loss, cellular swelling, nuclear pyknosis, and karyorrhexis. CG treatment partially prevented cell loss and protected neuron cells from injury after MCAO (Figure 8).

Immunohistochemistry and TUNEL Staining of Cerebral Cortex
TUNEL assay was conducted to evaluate neuron apoptosis 24 hours after MCAO, and the result revealed the presence of a large number of TUNEL-positive cells in the cortex region of rats subjected to I/R challenge, although TUNEL-positive cells were hardly observed in the sham group. The
number of TUNEL-positive cells was significantly reduced in the CG treatment groups compared with the I/R group, with 10% and 30% reduction at 0.4 and 0.8 g/kg, respectively (Figure 9A and C). Moreover, immunohistochemistry was carried out to address the possible contribution of caspase-3, p53, and PUMA in neuron apoptosis, and the representative pictures and quantification of the immunolabeling positive cells in different conditions are presented in Figure 9B and D, respectively. The result showed that the expressions of caspase-3, p53, and PUMA increased noticeably in response to I/R challenge, all of which were attenuated significantly by CG pretreatment in a dose-dependent manner.

**Ultrastructure of Microvessels and Neurons in Cerebral Cortex**

Figure 10 shows the representative transmission electron micrographs of capillaries and neurons in cerebral cortex 24 hours after reperfusion in the sham, I/R, and CG 0.8 ± I/R groups. Compared with the sham group (a1, a2), I/R elicited a remarkable alteration in the cortical capillary, manifested as narrowing lumen, rough inner surface, and swelling endothelial cells. In addition, swelling perivascular astrocyte processes containing dilated organelles were observed frequently (b1, b2). These ultrastructural alterations were alleviated by pretreatment with 0.8 g/kg CG (c1, c2). The neurons in the sham group displayed normal ultrastructural features (a3). In contrast, in the I/R group, the neurons became obviously irregular in shape and packed loosely, and some neurons were even missing. Swelling processes of glial cells and death of neurons were often observed as well (b3). CG treatment apparently attenuated I/R-induced changes (c3).

**DISCUSSION**

Brain edema is a major determinant in the progression of cerebral dysfunction following I/R, and the development
of strategy for blunting brain edema is considered to be of crucial importance in improving outcome of the patients after stroke. With the focal cerebral I/R model, the present study demonstrated that pretreatment with CG significantly attenuated I/R-induced brain edema, as indicated by the decline in EB dye extravasation and brain water content. Furthermore, the I/R-imposed insults on cerebral microcirculation and neuron were alleviated by pretreatment with
CG as well, which is in accordance with our previous findings using gerbil as a global I/R model \[28,30\]. Finally, the result of neurological score proved the prospective beneficial action of CG on the outcome. The results by UPLC–TOF-MS analysis showed that the protocol used can ensure the appearance of the CG components in the plasma when

Figure 5. The effect of pretreatment with CG on the number of leukocytes adhering to venular wall. (A) Representative images of the sham group (a and b), I/R group (c and d), and CG 0.8 g/kg + I/R group (e and f) acquired at baseline (a, c, e) and 60 minutes after I/R injury (b, d, f). Dotted line arrows: adherent leukocytes; Actual line arrow: rolling leukocyte. Bar = 50 μm. (B) The time course of the number of adherent leucocytes in different conditions. Compared with the I/R group, the number of adherent leukocytes in the CG administration groups decreased significantly starting from 20 minutes after reperfusion. Data are mean ± SE from six animals. I/R, ischemia and reperfusion; V, venule. *p < 0.05 vs. baseline, †p < 0.05 vs. sham group, ††p < 0.05 vs. I/R group.

Figure 6. The effect of pretreatment with CG on rat cerebral cortical venular diameter and RBC velocity in venules. (A) The representative images of venules observed by the high-speed video camera system. Arrows show the venules in the sham group (a1), I/R group (a2), CG 0.4 + I/R group (a3), and CG 0.8 + I/R (a4). (B) The time course of the cerebral venular diameter. (C) The time course of the velocity of RBC in cerebral venule. Data are mean ± SE from six animals. *p < 0.05 vs. baseline, †p < 0.05 vs. sham group.
Figure 7. The influence of CG pretreatment on neuronal injury of rats subjected to focal cerebral I/R. (A) Representatives of TTC-stained brain sections 24 hours after cerebral I/R. The white areas represent the infarct regions. (B) A quantitative evaluation of the influence of CG on infarct volume. (C) Neurological scores 24 hours after MCAO. Grades of 3–18 were used. Administration of CG at 0.8 g/kg significantly improved neurological function compared with the I/R group. Data are mean ± SE from six animals. *p < 0.05 vs. sham group, †p < 0.05 vs. I/R group.

Figure 8. Nissl staining of cerebral cortex in penumbral region of rats. The arrows indicate Nissl-positive neurons. The double arrows indicate nuclear pyknosis with karyorrhexis. a1, a2, a3: sham group; b1, b2, b3: I/R group; c1, c2, c3: CG 0.4 + I/R group; d1, d2, d3: CG 0.8 + I/R group. Data are mean ± SE from six animals.
I/R is initiated, and the CG components do exist in the rat plasma over the observation of microcirculation when given 90 minutes before ischemia. Brain edema following I/R is the consequence of BBB disruption. Various mechanisms are implicated in maintaining the integrity of BBB, among which endothelium, basal lamina, and glial cell processes are thought to be the prominent contributors [1,11,25,27]. We did not undertake an experiment in the present study to discriminate at which target CG exerted action to attenuate brain edema. However, increasing evidence demonstrates the close link between leukocyte recruitment and breakdown of BBB [8,14]. The accumulated leukocytes in postcapillary venules not only increase the hydrostatic pressure within the vessels but also release active oxygen species, which, in turn, impair the vessel wall [2,9,10,20,26]. Thus, the preventing effect of CG on I/R-induced leukocyte adhesion in venules found in the present study, as well as in previous work [30], may account, at least in part, for its favorite role in mitigating brain edema.

The CG is originally developed for treatment of cerebrovascular disturbance, such as headache and dizziness, which is mostly associated with global cerebral ischemia. In an animal model of global cerebral ischemia, we recently reported the potential of CG pretreatment on I/R-induced cerebral cortex microcirculatory disturbance [30]. Microcirculatory disturbance after I/R triggers the disruption of BBB, and is exaggerated by the subsequent brain edema. In the present study, we showed the protective effect of CG on the cerebral vasculature disorder in a focal cerebral I/R model that is more relevant to ischemic stroke in clinic, suggesting a potential use of CG for improving the outcome of the patients suffering from stroke.

The intravital microscopy in the present study revealed that I/R caused only a transient decrease in blood velocity while it had no effect on the venular diameter. On the other hand, however, the number of nonperfused capillaries in the area under investigation increased considerably in animals subjected to I/R, in comparison with control, implying a reduced blood perfusion in the affected territory. The rationale for the occurrence of nonperfused capillaries in the reperfusion phase remains unclear. One possible mechanism is the activated leukocytes built up in
the capillaries, obstructing the blood flux. Nonetheless, these nonperfused capillaries will exaggerate ischemia, and, along with the adherent leukocytes in venules and BBB disruption, lead to neuron damage. Interestingly, CG pretreatment attenuated I/R-evoked capillary nonperfusion as well, particularly at the dose of 0.8 g/kg.

We have previously reported that administration of CG to gerbils ameliorated hippocampal CA neuron damage caused by global I/R [28]. Likewise, CG was found to protect cortex neurons from focal cerebral I/R injury in the current study, as documented by the results of TTC staining, Nissl staining, and electron microscopy. In the acute phase of ischemia, cell death in the ischemic core is commonly considered necrotic, whereas after a short period of cerebral ischemia followed by reperfusion, the neurons in the penumbral regions undergo another wave of delayed cell death, which is dominated by apoptosis [4,32]. Consistent with others, we observed a drastic increase in apoptosis 24 hours after reperfusion in the penumbral regions of cortex in response to I/R challenge, as evidenced by the increase in the number of both TUNEL-positive and caspase-3-positive cells. Noticeably, the enhanced apoptosis in the cerebral cortex evoked by I/R was ameliorated significantly by pretreatment with CG. Neuron death is the consequence of a cascade of reactions in response to I/R challenge, which take place sequentially and interplay with each other. The complexity of neuron death process is superimposed by the multiple components of CG, which renders it extremely difficult to elucidate the mechanism underlying the effect of CG on I/R-induced neuron injury. However, some clues emerge that may plausibly shed light on this issue. In view of the critical importance of oxidative stress in the initiation of neuron death process, the antioxidant potential of CG may contribute most to the observed attenuation effect on neuron death. To this end, we have previously reported that CG is able to ameliorate I/R-induced hydrogen peroxide production [30]. Consistent with this finding, available evidence from in vitro studies revealed that at least six chemicals derived from the composed herbs exhibit antioxidant potential; they are: paeoniflorin from *Radix paeoniae alba* [22], ligustazine from *Rhizoma chuan xiong* [7], ferulic acid from *Radix angelicae sinensis* [23], rehmannioside from *Radix rehmanniae prepara-

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**Figure 10.** The effect of CG pretreatment on the ultrastructure of cerebral cortex in penumbral region of rats subjected to focal cerebral I/R. The brains were processed for electron microscopy 24 hours after reperfusion. a1, a2, a3: sham group; b1, b2, b3: I/R group; c1, c2, c3: CG 0.8 + I/R group. The electron micrographs of low magnification for different conditions are shown in Panel 1, where the capillaries and neurons enclosed in rectangles are further enlarged in Panels 2 and 3. N, nuclei of neurons; S, swelling perivascular astroglials; E, swelling endothelial cells.
ta [21], genistein from Caulis spatholobi [16], and alaternin from Semen cassiae [5]. In the present circumstance, these antioxidants most likely work coordinately, yielding an additive effect. Secondary, the CG-caused attenuations in cerebral microcirculation disturbance, BBB disruption and the resultant brain edema, as observed in present study, may lend themselves to blunt the development of neuron death, as obstruction of these insults improves the cerebral oxygen supply, on the one hand, and alleviates the inflammatory reaction, on the other hand. Finally, the fact that CG attenuated neuron apoptosis after I/R and, meanwhile, depressed the I/R-enhanced expression of p53 and PUMA suggests that the pathway of p53 through PUMA to caspases is at least one of the targets at which CG acts to protect neuron from apoptosis after I/R. We admit that much more works are required to elucidate the mechanism. The speculations above are to point to the likelihood for future exploration of the rationale behind the CG action observed in the present study.

In conclusion, CG is able to alleviate the brain edema induced by focal cerebral I/R in addition to attenuation of microcirculatory disturbance and neuron damage, providing a potentially alternative prophylactic management for the patients in imminent danger of stoke.

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