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THE ANTIOXIDANT CEREBRALCARE GRANULE ATTENUATES CEREBRAL MICROCIRCULATORY DISTURBANCE DURING ISCHEMIA-REPERFUSION INJURY

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ABSTRACT—Cerebralcare Granule (CG) is a compound Chinese medicine used for treatment of headache and dizziness associated with cerebrovascular diseases. To clarify the mechanism underlying the clinical outcome of CG, this study investigated the effects of CG on the structure and function of cerebral microvasculature during I/R injury. A total of 138 Mongolian gerbils were included and divided into four groups, each composed of 36 or 30 animals, for evaluating various parameters of concern. A skull window was prepared for microcirculatory observation in animals, which were subjected to I/R with or without pretreatment with CG (0.4 or 0.8 g/kg). The velocity of red blood cells in the venules was observed by a high-speed video camera system, along with intravital confocal microscopic measurements of microvascular diameters, adherent leukocytes, and albumin leakage in the brain cortex. Changes in the fluorescence intensity of dihydrorhodamine 123 in cerebral microvessels and malondialdehyde level in the cortex were measured. The ultrastructure of the microvessels in the cerebral cortex was analyzed using both transmission and scanning electron microscopy. In addition, cerebral blood flow was monitored using the laser Doppler imaging technique. Pretreatment with CG (0.4 or 0.8 g/kg) significantly alleviated I/R injury–induced disorders in cerebral microvasculature, as evidenced by the data observed at 60 min of reperfusion wherein the values in CG (0.4 g/kg) pretreatment group, CG (0.8 g/kg) pretreatment group, and I/R group were 2.43 ± 0.24, 2.28 ± 0.18, and 6.00 ± 0.35 for leukocyte adhesion, 2.51 ± 0.40, 2.33 ± 0.29, and 4.77 ± 0.24 for albumin leakage, 7.06 ± 0.81, 5.93 ± 0.42, and 28.38 ± 2.70 for dihydrorhodamine 123 fluorescence intensity in cerebral microvessels, 16.35 ± 0.52, 14.34 ± 0.68, and 21.46 ± 0.71 for malondialdehyde level in the cortex, and 0.43 ± 0.07, 0.46 ± 0.02, and 0.17 ± 0.08 for cerebral blood flow, respectively. I/R injury–elicited ultrastructural alterations in microvessels in cerebral cortex were also mitigated impressively by CG administration, manifested as attenuation of the reduced number of opening capillaries and the altered fine structures in endothelium, which were characterized by rough inner surface, increased intracellular vesicles, hypertrophy of digitations of intercellular contact, and swollen perivascular astroglial processes. Cerebralcare Granule is able to attenuate I/R injury–induced functional and structural changes in microvessels in the cerebral cortex of gerbils, an ability that is most likely correlated with its antioxidant potential.

KEYWORDS—I/R, microvascular permeability, leukocyte adhesion, oxidative stress, microcirculation

ABBREVIATIONS—DHR—dihydrorhodamine 123; ROS—reactive oxygen species; RBC—red blood cell; H_2O_2—hydrogen peroxide; FITC—fluorescein isothiocyanate; MDA—malondialdehyde; CBF—cerebral blood flow; O_2^-—superoxide anion; I/R—ischemia and reperfusion

INTRODUCTION

Cerebrovascular I/R injury is a leading cause of death and morbidity associated with stroke, hypertension, atherosclerosis, and trauma. The regimen targeting at cerebral blood vessel has proven beneficial in treating and preventing these devastating diseases. However, the complex interactions underlying microcirculatory disturbance in I/R impose a great challenge to the development of effective therapies. It is well recognized that I/R evokes an array of injurious responses characteristic of oxidative stress and inflammation (1, 2). In the microcirculation, the pathophysiological process typically involves leukocyte and endothelial activation and production of reactive oxygen species (ROS) and cytokines. These inflammatory mediators can interact with blood cells and endothelium, leading to impaired perfusion and plasma leakage. Because multiple factors and cellular pathways participate in the injurious process, therapies that directed at an individual target are insufficient to prevent the global circulatory damage. This is evidenced by the result of the studies with single antioxidant (3, 4) and single antiplatelet aggregation (5, 6) or antileukocyte adhesion molecules (7, 8).

Cerebralcare Granule (CG; Tianjin Tasly Pharmaceutical Co Ltd, Tianjin, China) is a newly developed compound medicine that was approved in 1996 by the China State Food and Drug Administration for the treatment of headache and dizziness associated with cerebrovascular diseases. Cerebralcare Granule is composed of 11 herbs (Table 1). Available evidence from *in vitro* studies reveals that at least six chemicals derived from the composed herbs exhibit antioxidant potential, that is, paeoniflorin that is from *Radix paeoniae alba* (bai shao) (9), ligustrazine from *Rhizoma chuan xiong* (Chuan Xiong) (10), ferulic acid from *Radix angelicae sinensis* (Dang

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TABLE 1. **Characterization of the Herbs Included in CG**

Herbs	Percentage content (%)	Identified compounds	Effects	References
Radix angelicae sinensis (Dang Gui)	6.76	Ferulic acid	Antioxidant	11
			Decreasing the brain infarct size	19
Rhizoma chuanxiong (Chuan Xiong)	6.76	Ligustrazine	Antioxidant	10
			Inhibiting neutrophil adhesion to endothelial cells	15,16
			Decreasing the brain infarct size	15–17
Radix paeoniae alba (bai shao)	5.41	Peoniflorin	Antioxidant	9
			Decreasing the brain infarct size	18
Ramulus uncariae cum *uncis* (Gou Teng)	13.51	Rhynchophylline	Preventing *N*-methyl D-aspartic acid, muscarinic M1, and 5-HT_2 receptors–mediated neurotoxicity during ischemia	42
			Blocking of calcium channel	43
Caulis spatholobi (Ji Xue Teng)	13.51	Genistein	Attenuating oxidative stress and neuronal damage	13
Spica prunellae (Xia Ku Cao)	13.51	Ursolic acid and 2-alpha-hydroxyursolic acid	Inhibiting the production of nitric oxide	44
Concha margaritifera usta (Zhen Zhu Mu)	13.51	Water-soluble extract	Upregulation of Bcl-2	45
Radix rehmanniae preparata (Di Huang)	5.41	Rehmannioside	Antioxidant	12
Semen cassiae (Jue Ming Zi)	13.51	Naphthopyrones	Inhibiting the histamine release	46
		Alaternin	Radical scavenging effect	14
Rhizoma corydalis yanhusuo (Yan Hu Suo)	6.76	L-Tetrahydropalmatine	Inhibiting myocardial infarction	47
			Dopaminergic antagonist	48
Herba asari (Xi Xin)	1.35	Methyleugenol	Inhibiting histamine release	49

Gui) (11), rehmannioside from *Radix rehmanniae preparata* (Di Huang) (12), genistein from *Caulis spatholobi* (Ji Xue Teng) (13), and alaternin from *Semen cassiae* (Jue Ming Zi) (14). In addition, ligustrazine was reported to inhibit neutrophil adhesion to endothelial cells (15, 16), and peoniflorin, ligustrazine, and ferulic acid were found to decrease the infarct size and brain tissue damage (15–19). However, it is not clear whether antioxidants containing compound preparation, CG, confer neuroprotection by improving cerebral microcirculation.

The purpose of the present study was to explore the effect of CG on I/R induced cerebral microcirculatory disturbance in a Mongolian gerbil model of carotid ligation and reperfusion. The results showed that pretreatment with CG attenuated significantly the I/R–induced dysfunction and structural abnormalities in microcirculation, which might be associated with its antioxidant potential.

MATERIALS AND METHODS

Animals

Male Mongolian gerbils weighing 65 to 90 g were purchased from the Animal Center of Capital Medical University (Beijing, certificate no. SCXK 2002-0001). The gerbils were maintained in the Animal Center of Peking University Health Science Center. They were housed individually in cages at 22°C ± 2°C and humidity of 40% ± 5% under a 12-h light/dark cycle with free access to tap water and pellet food. The animals were fasted for 12 h before experiment but allowed free access to water. All animals were handled according to the guidelines of the Peking University Animal Research Committee.

TABLE 2. **Number of animals for different experimental groups and various parameters**

Groups	RBC velocity and oxidative stress	Venular diameter and albumin leakage	Leukocyte adhesion	Ultrastructure examination (60 min after I/R)	Ultrastructure examination (24 h after I/R)	MDA level	CBF	Total
Sham	6	6	6	3	3	6	6	36
I/R	6	6	6	3	3	6	6	36
CG 0.4 + I/R	6	6	6	0	0	6	6	30
CG 0.8 + I/R	6	6	6	3	3	6	6	36
Total	24	24	24	9	9	24	24	138

The same animals were used for determination of both RBC velocity and oxidative stress, and the same is true of determination of venular diameter and albumin leakage. For ultrastructure examination, irrespective of 60 min or 24 h after reperfusion, only three animals were involved in each group, whereas six animals were evaluated in each group for each of the remaining parameters.

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TABLE 3. **Time course of changes in ratio of venular diameter**

Groups	Baseline	Time after reperfusion (min)			
		0	10	30	60
Sham	27.59 ± 2.44	27.81 ± 2.57	28.61 ± 2.76	29.01 ± 2.72	28.76 ± 2.76
I/R	22.00 ± 2.26	32.61 ± 2.16	32.25 ± 2.10	31.64 ± 1.95	31.69 ± 1.93
CG 0.4 + I/R	32.08 ± 3.71	37.56 ± 4.68	37.11 ± 4.41	37.64 ± 4.53	37.29 ± 4.33
CG 0.8 + I/R	26.64 ± 3.57	29.97 ± 3.64	29.44 ± 4.04	29.07 ± 3.60	29.53 ± 3.48

Data are mean ± SE from six gerbils.

Cerebralcare Granule

Cerebralcare Granule was produced by Tianjin Tasly Pharmaceutical Co Ltd. The batch number of the granules used in this experiment was Z10960082. No any steroid is included in the content of CG. The processing of the product followed strict quality control, and the ingredients were subjected to standardization. The herbs were manufactured as granules after dynamic cycle extraction and concentrated 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.

Chemicals

Dihydrorhodamine 123 (DHR) and fluorescein isothiocyanate (FITC) conjugate–albumin were purchased from Sigma Chemical Co (St Louis, Mo). Rhodamine 6G was from Fluka Chemie AG (Buchs, Switzerland), and urethane from Tianlian Fine Chemical Co (Shanghai, China). Assay kit for malondialdehyde (MDA) and total protein quantitation were purchased from Nanjing Jiancheng Bio-Tek Co (Nanjing, China).

I/R injury and drug administration

After being anesthetized with urethane (2.0 g/kg body weight [bw], i.p.), the gerbils were tracheotomized and mechanically ventilated with room air. A midline incision was made in the neck, and a custom-designed occluder (a small noose consisting of a fine polyethylene catheter in a plastic tube) was implanted in bilateral common carotid arteries without compromising blood flow (20). The femoral vein was cannulated for i.v. administration of FITC-albumin or rhodamine 6G. The animal head was secured in a stereotactic frame, and the skull was exposed through a midline incision. A 3 × 3-mm craniotomy was performed before the bregma with a handheld drill. The dura was superfused contiguously with 37°C warm physiological saline. For the animals in I/R group, cerebral ischemia was induced by occluding bilateral common carotid arteries for 30 min, and the occlusion was then released for reperfusion (20). In sham group, the animals underwent a similar treatment but without occlusion. Animals in CG-treated group received the drug orally 90 min before cerebral ischemia at a dose of either 0.4 or 0.8 g/kg, 6- or 12-fold the dose used in clinic (4 g/60 kg). The microcirculation was continuously observed for 60 min. A total of 138 animals were included and randomly distributed into sham group (36 gerbils), I/R group (36 gerbils), CG 0.4 + I/R group (30 gerbils) and CG 0.8 + I/R group (36 gerbils). Of the animals in each group, only six were used for determination of one or two parameters with the exception of ultrastructure examinations, which were undertaken in mere three groups (group sham, I/R injury, and CG 0.8 + I/R injury), and three animals were evaluated in each condition. See Table 2 for further details.

Red blood cell velocity in venules

The cerebral microcirculation was observed using a fluorescence microscope (Leica DM-LFS; Leica Microsystems, Wetzlar, Germany) equipped with a color monitor (TCL J2118A; TCL, Huizhou, China), a video timer (VTG-55B; FOR-A, Tokyo, Japan), and a DVD recorder (DVR-R25; Malata, Xiamen, China). Venules ranging from 30 to 50 μm in diameter and 200 μm in length were selected for study. The microvessel images were recorded through a high-speed video camera system at a rate of 1,000 frames per second (FASTCAM-ultima APX; Photron, San Diego, Calif), and the recordings were replayed at a rate of 25 frames per second from the stored images (21, 22). The red blood cell (RBC) velocity was measured with Image-Pro Plus 5.0 software (Media Cybernetics, Ga) before ischemia (baseline) and 0, 10, 30, and 60 min after reperfusion, respectively.

Oxidative stress in venules

To monitor oxidative stress in venules, the hydrogen peroxide (H_2O_2)–sensitive fluorescent probe dihydrorhodamine 123 (DHR; Molecular Probes, Saint Louis, Mo) was administrated to the superfusate (10 μmol/L) 10 min before ischemia (23). In the presence of oxidants, nonfluorescent DHR is oxidized to fluorescent rhodamine 123 (RH123), providing a measurable signal for the oxidative reaction. Fluorescence intensity in the lumen and surrounding area (Ia) and in the wall (Iw) of selected microvessel segments was acquired and estimated via a SIT camera (EB-CCD Camera C7190; Hamamatsu, Sizouka, Japan) attached to the microscope (excitation, 510 nm; emission, 534 nm) using Image-Pro Plus 5.0 (24). The values were averaged, and the difference between Ia and Iw (ΔI) was used to estimate the relative levels of oxidized DHR. The ΔI values were determined before ischemia (Ib) and at 0, 10, 30, and 60 min after reperfusion (Ix). Because Ib varied depending on DHR loading, the ratio of Ix to Ib for a given experimental condition was used as an index of oxidative stress and expressed as DHR fluorescence ratio.

Leukocyte adhesion

The fluorescence tracer rhodamine 6G was administrated (5 mg/kg bw) to the animal via the femoral vein 10 min before ischemia. After craniotomy, the cerebral cortex venules were observed under a laser confocal microscope (Axiovert 200; Zeiss, Sena, Germany) illuminated with an argon laser beam (wavelength, 543 nm), and fluorescence emission was collected through an objective (Zeiss Plan-neofluar 20×; numerical aperture, 0.70). After being digitized through an 8-bit A/D converter, X-t scan fluorescence images (512 × 512 × 8-bit) were stored and processed. The adherent leukocytes were identified as cells that attached to the venular walls for more than 30 s (25). The number of adherent leukocytes was counted under the basal condition and 0, 10, 30, and 60 min after reperfusion.

Venular diameter and albumin leakage

Fluorescein isothiocyanate–albumin was infused (50 mg/kg bw) through the femoral vein 10 min before ischemia (21). Laser confocal microscopy was used to acquire venular images under irradiation at a wavelength of 488 nm. Using Image-Pro Plus 5.0 software, the inner diameter of cerebral venules was measured before ischemia and at 0, 10, 30, and 60 min after reperfusion. The diameter was presented as the mean of three measurements at one location (21). The fluorescence intensities of FITC-albumin inside the lumen of selected venules (Iv) and in the surrounding interstitial area (Ii) were

TABLE 4. **Time course of changes in ratio of velocity of RBCs in venules**

Groups	Baseline	Time after reperfusion (min)			
		0	10	30	60
Sham	1.64 ± 0.15	1.78 ± 0.19	1.75 ± 0.18	1.37 ± 0.09	1.47 ± 0.14
I/R	1.59 ± 0.16	0.86 ± 0.07*	0.91 ± 0.12*	1.16 ± 0.17	1.42 ± 0.10
CG 0.4 + I/R	1.61 ± 0.15	0.69 ± 0.16*	1.32 ± 0.13	1.55 ± 0.14	1.59 ± 0.17
CG 0.8 + I/R	1.81 ± 0.06	1.04 ± 0.08*	1.47 ± 0.12	1.68 ± 0.08	1.90 ± 0.06

Data are mean ± SE from six gerbils.
*$P < 0.05$ vs. sham group.

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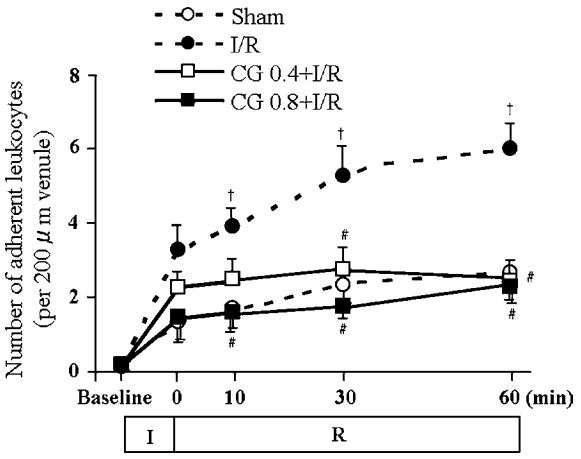

FIG. 1. **The effect of pretreatment with CG on the number of leukocytes adherent to venular wall in the cerebral cortex of gerbils**. Sham: sham group; I/R: I/R group; CG 0.4 + I/R: pretreatment with CG 0.4 g/kg + I/R group; CG 0.8 + I/R: pretreatment with CG 0.8 g/kg + I/R group. Data are mean ± SE from six gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

estimated. The ratio Ii/Iv was calculated and compared with the baseline as an indicator of albumin leakage.

Ultrastructure examination

Sixty minutes or 24 h after reperfusion, the brain was perfused for 40 min with a fixative composed of 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at a speed of 3 mL/min. For transmission electron microscopy, a coronal slice approximately 1 mm thick was taken. The slice was placed in fresh prepared 3% glutaraldehyde overnight at 4°C. After rinsing with 0.1 M phosphate buffer for three times, the tissue block was postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4°C. The samples were dehydrated and then embedded in Epon 812. Ultrathin sections of cortex were stained with uranium acetate and lead citrate and examined in a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan). For scanning electron microscopy, the samples were cut coronally into blocks and placed in fresh prepared glutaraldehyde for 2 h, rinsed with 0.1 M phosphate buffer, and then postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. The specimens were processed as routing and examined under a scanning electron microscope (JSM-5600LV; JEOL) (26). The number of open capillaries in the cortex was counted on a fractured face of 2,000 × 2,500 μm for different experimental groups.

MDA level in the cortex

Malondialdehyde levels in the brain tissue were determined as an indicator of lipid peroxidation. Animals were killed at 60 min of reperfusion. The cortex was dissected and homogenized in ice-cold buffer (0.1 mM $C_{12}H_{22}O_{11}$, 1.5 mM $MgCl_2 \cdot 6H_2O$, Tris 50 mM) with a weight-to-volume ratio of 1:9. The homogenate was centrifuged at 8,000 rpm for 15 min at 4°C. The MDA level was determined according to the manufacturer's instruction. Protein concentration was measured using the Coomassie blue protein-binding assay using bovine serum albumin as a standard.

Cerebral blood flow

Cerebral blood flow (CBF) was measured using laser Doppler perfusion imager (PeriScan PIM3; PERIMED, Stockholm, Sweden). An incision was made through the scalp, and the skin was retracted to expose the skull. The periosteal connective tissue adherent to the skull was removed with a sterile cotton swab. A computer-controlled optical scanner directed a low-powered He-Ne laser beam over the exposed cortex. The scanner head was positioned in parallel to the cerebral cortex at a distance of 18.5 cm. The scanning procedure took 4 s for a measurement covering an area of 0.8 × 1.0 cm. At each measuring site, the beam illuminated the tissue to a depth of 0.5 mm. A color-coded image to denote specific relative perfusion levels was displayed on a video monitor (27). The images were acquired before ischemia (baseline) and at 0, 10, 20, 30, 40, 50, and 60 min after reperfusion. Relative perfusion values for each area were normalized and expressed as percentage of the baseline.

Statistical analysis

All parameters were averaged from six animals (n = 6) and expressed as mean ± SE. Statistical analysis was performed using ANOVA followed by the Bonferroni test for multiple comparisons. $P < 0.05$ is considered to be statistically significant.

RESULTS

Venular diameter and RBC velocity

No significant alteration was observed in the diameter of postcapillary venules over the entire course of observation among the groups (Table 3). The time course of changes in RBC velocity in cerebral venules is depicted in Table 4. The mean RBC velocity in cerebral venule in sham group did not alter significantly during the period of observation. There was a transient decrease in RBC velocity after I/R injury compared with baseline, with the response being most significant within

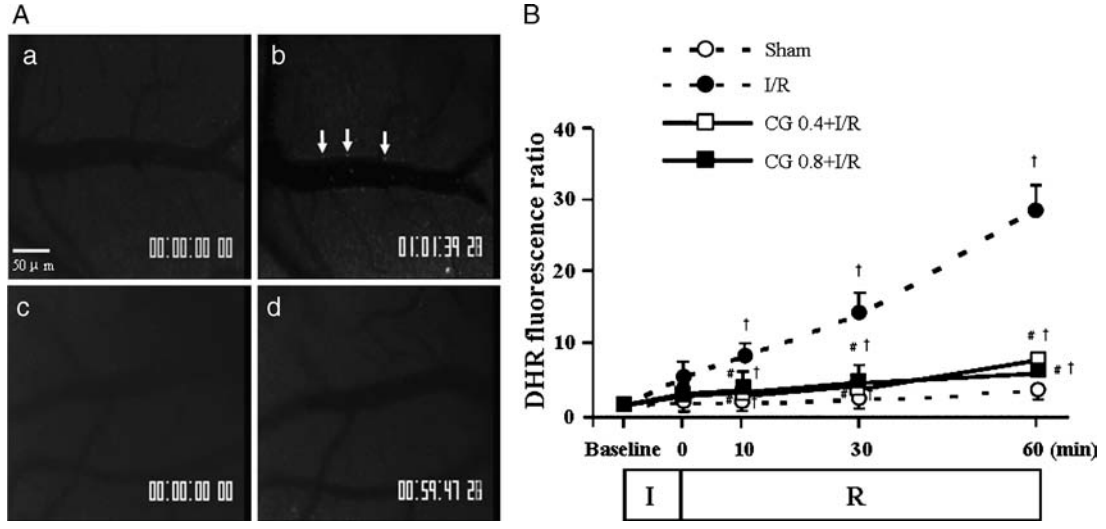

FIG. 2. **The effect of pretreatment with CG on the fluorescence intensity of DHR in venular wall in the cerebral cortex of gerbils**. A, Representative images of I/R group (a and b) and CG 0.8 g/kg + I/R group (c and d) acquired at baseline (a and c) and 60 min after I/R injury (b and d). The DHR fluorescence on the venular wall was diminished by pretreatment with 0.8 g/kg CG at 60 min after I/R injury (d). B, The time course of changes in DHR fluorescence ratio on the venular walls. Sham: sham group; I/R: I/R group; CG 0.4 + I/R: pretreatment with CG 0.4 g/kg + I/R group; CG 0.8 + I/R: pretreatment with CG 0.8 g/kg + I/R group. Data are mean ± SE from six gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

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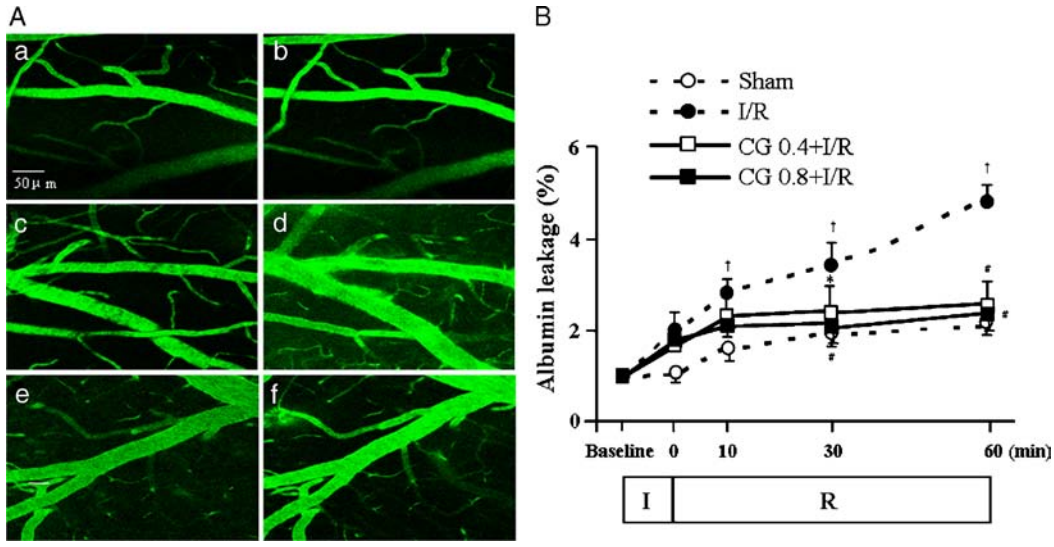

FIG. 3. **Effect of CG on the albumin leakage from the venule in the cortex of gerbils**. A, Representative images of sham group, I/R group, and CG 0.8 g/kg + I/R group. Before I/R (baseline), no obvious albumin leakage was detected in sham group (a), I/R group (c), or CG 0.8 g/kg + I/R group (e). This situation persisted to the end of observation in sham group (b). The albumin leakage from venular wall was observed at 60 min after I/R injury (d). The I/R–induced albumin leakage from cerebral venule was apparently suppressed by the pretreatment with 0.8 g/kg CG (f). B, Time course of changes in albumin leakage from venules. Sham: sham group; I/R: I/R group; CG 0.4 + I/R: pretreatment with CG 0.4 g/kg + I/R group; CG 0.8 + I/R: pretreatment with CG 0.8 g/kg + I/R group. Data are mean ± SE from six gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

the first 10 min of reperfusion and gradually returned to the basal level. The CG treatment had no significant effect on RBC velocity in comparison with I/R group.

Leukocyte adhesion

The time courses of changes in the number of leukocytes adherent to the venular wall under various conditions are shown in Figure 1. In sham group, the number of adherent leukocytes increased slightly. The number of adherent leukocytes in I/R group increased in a similar fashion as that in sham group, but to a much greater extent that the difference between the two groups became significant at 10 min of reperfusion and remained increasing until the end of observation. Pretreatment with CG attenuated the I/R–elicited enhancement of leukocyte adhesion, with the higher dose of CG exerting a more rapid and more significant inhibition effect, although no significant difference was found between the two-dose CG–treated groups being observed. At 60 min of reperfusion, the number of adherent leukocytes (cells/200 μm) in CG 0.4 + I/R group, CG 0.8 + I/R group, and I/R group were 2.43 ± 0.24, 2.28 ± 0.18, and 6.00 ± 0.35, respectively.

Oxidative stress

The fluorescence intensity of DHR, indicative of H_2O_2 production, was measured in cerebral venules during I/R. Figure 2A shows representative images of DHR fluorescence before and 60 min after reperfusion in the nontreated (a, b) and CG-treated (c, d) animals. Obviously, the fluorescence was observed on the wall of venules at 60 min of reperfusion in the nontreated group (b). Cerebralcare Granule treatment apparently attenuated the I/R–induced DHR fluorescence on the cerebral venular walls (d). The time course of changes in DHR fluorescence ratio is presented in Figure 2B. The intensity of DHR fluorescence increased in the I/R group after 10 min of reperfusion compared with sham group, the response persisted for 60 min. Pretreatment with CG (0.4 or 0.8 g/kg) prevented the oxidative response to I/R ($P < 0.05$) significantly, reaching 7.06 ± 0.81, 5.93 ± 0.42, and 28.38 ± 2.70, in CG 0.4 + I/R group, CG 0.8 + I/R group, and I/R group, respectively, at 60 min of reperfusion. But there was no significant difference between the two-dose CG-treated groups.

Albumin leakage

Transvascular flux of FITC-labeled albumin was measured in cerebral venules for different experimental conditions. Representative fluorescent images of fluorochrome-perfused cerebral microvasculature are illustrated in Figure 3A, which

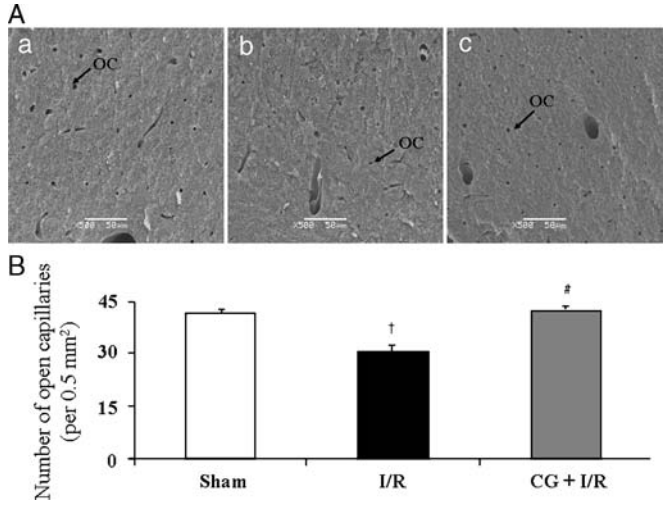

FIG. 4. **Scanning electron microscope examination of fractured face of cerebral cortex**. A, Representative micrographs of sham group (a), I/R group (b), and CG 0.8 g/kg + I/R group (c). OC indicates opening capillaries. B, Quantitative evaluation of opening capillaries in the fractured face of cortex in sham group, I/R group, and CG 0.8 g/kg + I/R group. Data are mean ± SE from three gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

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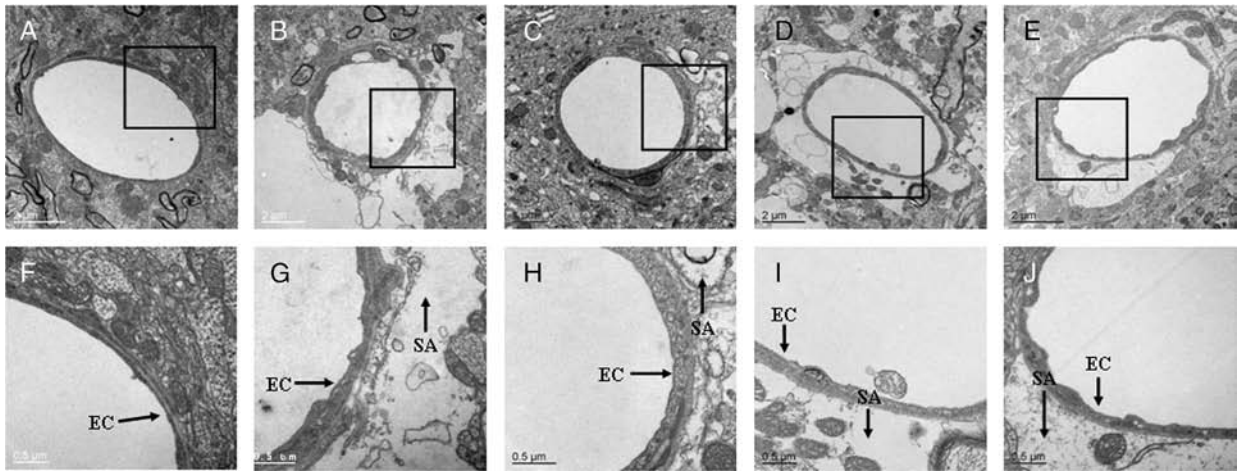

FIG. 5. **The representative transmission electron micrographs of capillaries in the cerebral cortex of gerbils after 60 min or 24 h of reperfusion**. A and F, Sham group. B and G, I/R group at 60 min of reperfusion. C and H, CG 0.8 g/kg + I/R group at 60 min of reperfusion. D and I, I/R group at 24 h of reperfusion. E and J, CG 0.8 g/kg + I/R group at 24 h of reperfusion. EC indicates endothelial cell; SA, swollen astroglial process.

were acquired at 0- and 60-min time point in the sham (a, b), I/R (c, d), and CG + I/R (e, f) groups, respectively. Of notice, reperfusion for 60 min evoked an obvious albumin leakage, and this outcome was attenuated apparently by pretreatment with CG. Figure 3B shows a quantitative evaluation of the leak response over the 60-min time course, presented as percentage of albumin flux. There was no detectable leakage under the basal condition in all the groups. The I/R injury caused albumin leakage, which increased with time and became significant after 10 min of reperfusion. The I/R–induced leak response was diminished significantly in the animals receiving CG, which was 2.51 ± 0.40, 2.33 ± 0.29, and 4.77 ± 0.24, respectively, in CG 0.4 + I/R group, CG 0.8 + I/R group, and I/R group at 60 min of reperfusion, with no difference observed between the two doses examined.

Ultrastructural changes in microvessels

Scanning electron microscopy was performed to examine the microvasculature in the fractured face of cerebral cortex 60 min after reperfusion. A survey at low power revealed that there was a high density of open and uniformly distributed capillaries in sham group (Fig. 4A, a). After I/R injury, by contrast, the number of open capillaries was reduced, and their distribution became heterogeneous (Fig. 4, A and B). The I/R–induced abnormalities were not observed in CG (0.8 g/kg)–treated brains (Fig. 4, A and C). A quantitative evaluation of the number of open capillaries showed that the density of open capillaries was significantly lower in the I/R (30.33 ± 1.67) compared with sham group (41.33 ± 0.67) ($P < 0.05$), while this decline was reversed by treatment with CG (42 ± 0.578) ($P < 0.05$) (Fig. 4B). Figure 5 presents the transmission electron micrographs of the cortical capillaries and perivascular parenchyma 60 min or 24 h after reperfusion in the sham, I/R, and CG + I/R groups. Compared with the sham group (A and F), I/R elicited a remarkable alteration in the cortical capillary manifested as narrowed lumen, rough inner surface, and increased caveolae and intracellular vesicles of various sizes in the endothelial cells. In addition, swollen perivascular astroglial processes containing dilated endoplasmic reticulum were frequently observed (B and G). Twenty-four

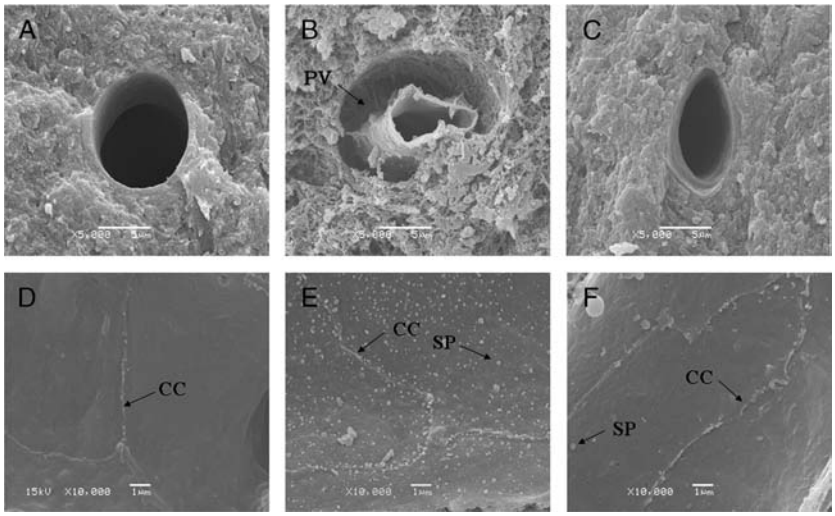

FIG. 6. **The representative scanning electron micrographs of microvessel in the cerebral cortex of gerbils**. The microvessels were fractured both crossly (upper panel) and vertically (lower panel). A and D, Sham group. B and E, I/R group. C and F, CG 0.8 g/kg + I/R group. PV indicates perivascular vacuole; CC, cell-cell contact; SP, surface projection.

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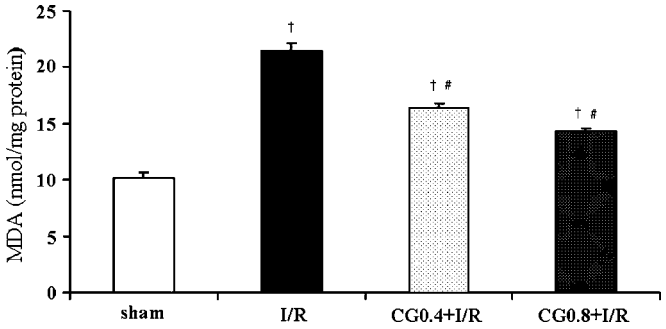

FIG. 7. **Effect of CG on MDA levels in the cerebral cortex of gerbils**. Sham: sham group; I/R: I/R group; CG 0.4 + I/R: pretreatment with CG 0.4 g/kg + I/R group; CG 0.8 + I/R: pretreatment with CG 0.8 g/kg + I/R injury. Data are mean ± SE from six gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

hours after reperfusion, the changes in cortical capillary induced by I/R were similar to those found at 60 min after reperfusion (D and I). These ultrastructural alterations were alleviated by pretreatment with 0.8 g/kg CG, regardless of 60 min (C and H) or 24 h after reperfusion (E and J). A further examination was carried out in the fractured face of cerebral cortex using scanning electron microscopy 60 min after reperfusion. Representative micrographs of capillaries from animals in sham, I/R, and CG + I/R groups are presented in Figure 6. In accordance with the result observed by transmission electron microscopy, the capillary endothelium in the sham group exhibited a smooth inner surface and tightly connected intercellular junctions together with homogeneously and densely distributed neuropil (A and D). The I/R injury challenge caused capillary shrinkage with a rough endothelial surface and numerous pox-like projections into the lumen as well as hypertrophy of the digitations of endothelial cell contacts (B and E). Perivascular vacuole was often observed, which likely resulted from the removal of edematous fluid accumulated in the interstitium or in the astroglial processes. These abnormalities were not observed in CG-treated animals after I/R injury (C and F).

MDA level in the cortex

The level of cortex MDA observed at 60 min of reperfusion was significantly increased in I/R group as compared with sham group (Fig. 7). Pretreatment with CG (0.4 or 0.8 g/kg) significantly reduced the I/R–enhanced MDA level (16.35 ± 0.52 or 14.34 ± 0.68 vs 21.46 ± 0.71 nmol/mg protein). There was no significant difference between the two-dose CG-treated groups.

Cerebral blood flow

Figure 8 shows the time course of changes in CBF in the four groups of animals. The I/R group showed a significant reduction in CBF, starting immediately after the initiation of reperfusion and remaining at a low level over 60 min of reperfusion. Pretreatment with CG (0.4 or 0.8 g/kg) significantly attenuated the I/R–evoked decrease in CBF, with no difference being observed between the two doses used. The values of CBF at 60 min of reperfusion were 0.43 ± 0.07, 0.46 ± 0.02, and 0.17 ± 0.08 in CG 0.4 + I/R, CG 0.8 + I/R, and I/R group, respectively.

DISCUSSION

The present study demonstrates that treatment with CG, a newly developed herb compound containing multiple antioxidants, significantly attenuates the I/R injury–evoked cerebral functional disorders in the cortical microcirculation of gerbils, including the oxidative stress in venular endothelium as evidenced by DHR fluorescence, the reduced red blood cell velocity, the enhanced leukocyte adhesion, albumin leakage, and decreased CBF. The attenuation effect of CG on the cerebral I/R injury in microcirculation was also documented by the ultrastructural examination of the microvessels in the cerebral cortex.

It has long been recognized that I/R injury gives rise to a surge of superoxide anions (O_2^-) in endothelial cells immediately after the onset of reperfusion. The O_2^- are either transformed to H_2O_2 by superoxide dismutase catalyzing or to hydroxyl radical via Haber-Weiss reaction (28, 29). These ROS directly destroys lipid, proteins, and nucleic acids, leading to damages of vascular endothelial cells and the basement membrane (30), and targeting inhibition of ROS is proved to be one of the effective therapeutic interventions for improving microcirculation during I/R injury. It is confirmed previously that ligustrazine, paeoniflorin, ferulic acid, rehmannioside, genistein, and alaternin are able to inhibit *in vitro* oxygen free-radical production significantly (9–14). These compounds are known to be extracted, respectively, from *Rhizoma chuan xiong* (Chuan Xiong), *Radix paeoniae alba* (bai shao), *Radix angelicae sinensis* (Dang Gui), *Radix rehmanniae preparata* (Di Huang), *Caulis spatholobi* (Ji Xue Teng), and *Semen cassiae* (Jue Ming Zi), which are the major ingredients of CG. Because CG is currently in use as a treatment of headache and dizziness associated with cerebrovascular diseases, clarification of the mechanism for the action of CG as a whole, rather than its individual ingredients, would be of more clinical relevance. However, whether the combined recipe of CG has an effect of inhibiting ROS production, especially in *in vivo* conditions, has not been reported. Application of DHR as a probe in the present study enables a dynamic observation of the production of oxygen peroxide in venules of cerebral cortex and provides for the first time the evidence for the antioxidant capacity of CG.

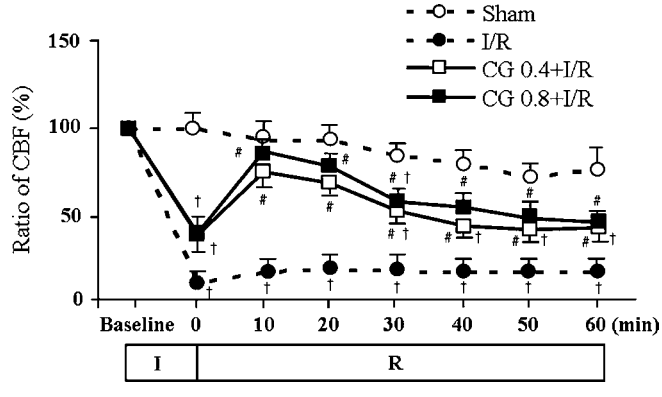

FIG. 8. **Time course of changes in CBF of gerbils**. Sham: sham group; I/R: I/R; CG 0.4 + I/R: pretreatment with CG 0.4 g/kg + I/R group; CG 0.8 + I/R: pretreatment with CG 0.8 g/kg + I/R group. Data are mean ± SE from 6 gerbils. $^{\dagger}P < 0.05$ vs. sham group, $^{\#}P < 0.05$ vs. I/R group.

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This notion is further supported by the finding that I/R–induced elevation in cerebral cortex MDA, an indicator of lipid peroxidation, was attenuated by administration of CG.

I/R evokes the expression of E–selectin and intercellular adhesion molecule–1 (ICAM-1) in vascular endothelium and L–selectin and CD11b/CD18 in neutrophils, respectively (31, 32). The expression of adhesion molecules brings about neutrophils to roll along and adhere to vascular wall. The activated endothelial cells and neutrophils, in turn, release oxygen free radicals and protein enzymes, leading to damage on endothelial cells and basement membrane and consequently increasing vascular permeability that results in albumin leakage and extravascular edema (21, 33, 34). Thus, inhibiting leukocytes adhering to endothelium is considered to be a remedy to improve microcirculatory disturbance induced by I/R. The previous reports have shown that the ligustrazine possesses ability to inhibit the expression of ICAM-1 in endothelium induced by phytohemagglutinin (35, 36). The present study revealed that CG is able to inhibit leukocytes adhering to venular walls in cerebral cortical region undergoing I/R injury. The exact cause for this result is, at present, unknown as the antibody against Mongolian gerbil is not available commercially for the time being. Nevertheless, this result provides a mechanistic rationale for the clinical use of CG, although whether compound(s) other than ligustrazine among the multiple components of CG contributes to this effect remains to be clarified.

Albumin leakage is the sequela of microvessel injury after production of ROS by I/R (37–39). Morphologically, microvessel injury exhibits diverse alterations in both the vessel itself and the surrounding parenchyma, as assessed by electron microscopy in the present study, including rough inner surface of the capillary wall, increased number of caveolae and intracellular vesicles in endothelial cells, hypertrophy of the digitations of endothelial cell contact, and the pronounced swollen perivascular astroglial processes. The protective effect of CG against I/R–induced microvessel injury is anticipated because of its antioxidant potential, and this effect in the cerebral cortex in current condition is corroborated by not only the remarkably rectifying of ultrastructure alterations induced by I/R, but also the attenuation of albumin leakage. Two pathways have been postulated to account for the albumin leakage from microvessels after inflammatory insults, one is mediated by transendothelial transport via caveolae, the other is through interendothelial junctions (40). The finding of ultrastructure examination of the microvessel in the present study suggests that both pathways are probably at work in current situation, although the relative contribution of each pathway to the I/R–enhanced albumin leakage is to be determined. Nevertheless, the mechanism underling the attenuating effect of CG on I/R–induced albumin leakage requires further study.

Consistent with the results from others (41), the present study revealed a diminution in CBF in response to I/R by using laser Doppler perfusion imager. Cerebral blood flow diminution concurred the observed reduction in the velocity of RBC and the number of open capillaries, representing a collective consequence of a variety of insults elicited by I/R injury, such as leukocyte adhesion, rough inner surface of vascular endothelium, and swollen perivascular astroglial processes that impose pressure on the microvessel. It is thus conceivable that CG rectifies the overall microcirculatory disturbance inflicted by I/R injury by attenuating multiple I/R–induced insults.

In conclusion, CG is potentially able to alleviate the overall microcirculatory disturbances in the cerebral cortex of Mongolian gerbil induced by I/R, implicated in the underling mechanism may be its antioxidant capacity that leads to the inhibition of oxygen peroxide production and consequently the attenuation of leukocyte adhesion, injury of the microvessel, and albumin leakage. Further study is required to clarify the relative contribution of the individual ingredient of CG to the effects observed.

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