Ethnopharmacological Communication

Cerebralcare Granule®, a Chinese herb compound preparation, improves cerebral microcirculatory disorder and hippocampal CA1 neuron injury in gerbils after ischemia–reperfusion

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A B S T R A C T

Aim of the study: Cerebralcare Granule® (CG) is a Chinese herb compound preparation that has been used for treatment of cerebrovascular related diseases. However, the effect of post-treatment with CG on ischemia and reperfusion (I/R) induced cerebral injury is so far unclear.

Materials and methods: In present study, cerebral global I/R was induced in Mongolian gerbils by clamping bilateral carotid arteries for 30 min followed by reperfusion for 5 days, and CG (0.4 g/kg or 0.8 g/kg) was administrated 3 h after the initiation of reperfusion.

Results: Post-treatment with CG for 5 days attenuated the I/R-induced production of hydroxide peroxide in, leukocyte adhesion to, and albumin leakage from cerebral microvessels, and, meanwhile, protected neuron from death, reduced the number of caspase-3- and Bax-positive cells, and increased Bcl-2-positive cells in hippocampal CA1 region.

Conclusion: The results suggest that CG given after initiation of reperfusion is able to ameliorate cerebral microvascular dysfunction and hippocampal CA1 neuron damage caused by I/R.

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1. Introduction

Stroke is the third leading cause of mortality in developed countries with serious long-term physical and cognitive disabilities (Sarti et al., 2000; Feigin et al., 2003). Nearly 60–70% of stroke is triggered by ischemia (Silvestrelli et al., 2002). Advanced intravascular procedures and thrombolytic agents effectively reduce functional deficits if given within an optimal time window in stroke patients. However, the challenge remains for this thrombolysis approach is reperfusion injury. Reperfusion after ischemia leads to profound cerebral microcirculatory disturbance and even death of neurons (Aronowski et al., 1997), thus protecting the brain from reperfusion injury after ischemia is an alternative for therapy of stroke. Despite extensive research on the mechanism of reperfusion injury over the past decades, there has been limited success in translating the outcome in basic science to clinical management of cerebral injury (Moustafa and Baron, 2008; Feuerstein and Chavez, 2009).

Cerebralcare Granule® (CG) is a Chinese herb compound preparation derived from a traditional recipe, which consists of 11 Chinese herbs as specified in Table 1. CG has been approved by the China State Food and Drug Administration in 1996 for treatment of headache and dizziness associated with cerebrovascular diseases. The processing of the product was subjected to strict quality control, and the ingredients were subjected to standardization. The composed herbs were manufactured by dynamic cycle extraction and concentration through evaporating and spray drying, resulting in granules of CG as product. Pretreatment with CG has been demonstrated to prevent cerebral microcirculatory disturbance and inflammatory injury caused by ischemia–reperfusion (I/R) (Xue et al., 2009). However, preventive approach is feasible only when the occurrence of ischemia is predictable. Many drugs used in clinic have limited efficacy because they take effect only if given before or immediately after ischemic insults (Jacques et al., 1999). Compared with ischemia, the onset and progress of reperfusion can be better controlled, and drugs able to restrain the progress of reperfusion injury would be of great benefit to the stroke patients.

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The objective of the current study is to test the potential therapeutic effect of CG on I/R-induced cerebral microcirculatory disorders in a Mongolian gerbil model of global ischemia and reperfusion. We administrated CG 3 h after the onset of reperfusion and evaluated its effects on I/R-elicited insults in cerebral microcirculation and hippocampal CA1 neuron 5 days after reperfusion.

2. Materials and methods

2.1. Animals and grouping

A total of 120 male Mongolian gerbils weighing 65–90 g were purchased from the Animal Center of Capital Medical University (Beijing). They were randomly divided into sham ($n = 30$), I/R ($n = 30$), I/R + CG 0.4 g/kg ($n = 30$) and I/R + CG 0.8 g/kg ($n = 30$) groups. The animals were fasted for 12 h before experiment with free access to water. All animals were handled according to the guidelines of the Peking University Animal Research Committee, and the surgical procedures and experimental protocol were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch.

2.2. Cerebralcare Granule® and reagents

CG was produced by Tianjin Tasly Pharmaceutical Co. Ltd. The batch number of the granules used in this experiment was Z10960082. The major identified effective phytochemical compound of each herb included in CG is illustrated in Table 1. CG was packed with aluminum foil composite (4 g/bag). The compound was dissolved in saline to a concentration of 80 mg/ml or 160 mg/ml (I/R + 0.8 group), the gerbils in both groups received the equivalent dose (5 ml/kg) of saline alike. Ninety minutes after drug administration on day 5, the gerbils were re-anesthetized with urethane (2.0 g/kg body weight) intraperitoneally for evaluating various parameters.

2.3. Animal model and drug administration

After being anesthetized with urethane (2.0 g/kg body weight) intraperitoneally, 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. For the animals in I/R group, cerebral ischemia was induced via occluding bilateral common carotid arteries for 30 min, and the occlusion was then released for reperfusion (Choki et al., 1984; Xu et al., 2009). The incision on the neck was closed and the animals were allowed to recover. In sham group, the animals underwent a similar treatment but without occlusion. In CG-treated group, the drug was administrated by gavage 3 h after the beginning of reperfusion for the first time at a dose of either 0.4 g/kg (I/R + CG 0.4 group) or 0.8 g/kg (I/R + 0.8 group), and animals received the same dose of drug every 24 h over the next 5 days. Because CG administrated was dissolved in saline to a concentration of 80 mg/ml (I/R + CG 0.4 group) or 160 mg/ml (I/R + 0.8 group), the gerbils in both groups received the same dose of saline (5 ml/kg). The animals in control group received the equivalent dose (5 ml/kg) of saline alike. Ninety minutes after drug administration on day 5, the gerbils were re-anesthetized with urethane (2.0 g/kg body weight) intraperitoneally for evaluating various parameters.

2.4. Microcirculation observation

The animal’s head was secured in a stereotactic frame and the skull was exposed through a midline incision. A 3 mm × 3 mm craniotomy was performed before the bregma with a hand-held drill. The dura was superfused continuously with 37 °C warm physiologic saline. The cerebral microcirculation was observed using an upright intravital fluorescent microscopy (BX51 WT, Olympus, Japan). Venules with diameter ranging from 30 to 50 μm and length of 200 μm were selected for study (Xu et al., 2009). One venule was selected in the cerebral cortex of each animal for evaluation of a microcirculatory variable and the values from six animals of a group were averaged.

2.4.1. Measurement of adherent leukocytes

The fluorescence tracer Rhodamine 6G (Fluka Chemie AG, Switzerland) and urethane from Tianlian Fine Chemical Co (Shanghai, China).

Table 1

<table>
<thead>
<tr>
<th>Latin name (Chinese name)</th>
<th>Botanical family</th>
<th>The part used</th>
<th>Content (%)</th>
<th>Major effective phytochemical compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica sinensis (Oliv.) Diels (Dang Gui)</td>
<td>Umbelliferae</td>
<td>Root</td>
<td>6.76</td>
<td>Ferulic acid</td>
<td>Xu et al. (2009) and Yi et al. (2009)</td>
</tr>
<tr>
<td>Ligusticum chuanxiong Hort. (Chuan Xiong)</td>
<td>Umbelliferae</td>
<td>Rhizome</td>
<td>6.76</td>
<td>Ligustrazine</td>
<td>Guo et al. (2008) and Xu et al. (2009)</td>
</tr>
<tr>
<td>Paeonia lactiflora Pall (Bai Shao)</td>
<td>Paeoniaceae</td>
<td>Root</td>
<td>5.41</td>
<td>Paeoniflorin</td>
<td>Xu et al. (2009) and Zhou et al. (1998)</td>
</tr>
<tr>
<td>Uncaria sinensis (Oliv.) Havil. (Gou Teng)</td>
<td>Rubiaceae</td>
<td>Branch</td>
<td>13.51</td>
<td>Rynchophylline</td>
<td>Xu et al. (2009) and Wang et al. (2009)</td>
</tr>
<tr>
<td>Spatholobus suberectus Dunn (Ji Xue Teng)</td>
<td>Fabaceae</td>
<td>Vine stem</td>
<td>13.51</td>
<td>Genistein</td>
<td>Xu et al. (2009) and Yoon et al. (2004)</td>
</tr>
<tr>
<td>Prunella vulgaris L. (Xia Ku Cao)</td>
<td>Labiatae</td>
<td>Spica</td>
<td>13.51</td>
<td>Ursolic acid</td>
<td>Lee et al. (2008) and Xu et al. (2009)</td>
</tr>
<tr>
<td>Hyriopsis cumingii (Lee) (Zhen Zhu Mu) Rehmannia glutinosa</td>
<td>Unionidae</td>
<td>Shell</td>
<td>13.51</td>
<td>Water-soluble polysaccharide</td>
<td>Xu et al. (2010) and Xu et al. (2009)</td>
</tr>
<tr>
<td>Libosch Libosch. (Di Huang) Lobo. (Yan Hu Suo)</td>
<td>Scrophulariaceae</td>
<td>Root</td>
<td>5.41</td>
<td>Rehmannioside</td>
<td>Xu et al. (2009) and Zhang et al. (2008)</td>
</tr>
<tr>
<td>Cassia tora L. (Jue Ming Zi).</td>
<td>Leguminosae</td>
<td>Seed</td>
<td>13.51</td>
<td>Naphthopyrone glucosides</td>
<td>Lee et al. (2006) and Xu et al. (2009)</td>
</tr>
<tr>
<td>Corydalis yanhusuo W.T.Wang (Xi Xing)</td>
<td>Fumariaceae</td>
<td>Tuber</td>
<td>6.76</td>
<td>Tetrahydropalmatine</td>
<td>Ou et al. (2006) and Xu et al. (2009)</td>
</tr>
<tr>
<td>Asarum sieboldii Miquel</td>
<td>Aristolochiaceae</td>
<td>Whole plant</td>
<td>1.35</td>
<td>Methyleugenol</td>
<td>Han et al. (2008) and Xu et al. (2009)</td>
</tr>
</tbody>
</table>

The major phytochemical compound of each herb included in CG is illustrated in Table 1. CG was packed with aluminum foil composite (4 g/bag). The compound was dissolved in saline to a concentration of 80 mg/ml or 160 mg/ml before use (Xu et al., 2009). Dihydrorhodamine 123 (DHR) and fluorescein isothiocyanate (FITC) conjugated-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).

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walls for more than 30 s (Kurose et al., 1994; Xu et al., 2009). The number of adherent leukocytes was counted along venules selected from the recorded images, and presented as the number per 200 μm of venule length.

2.4.2. Measurement of oxidative stress in venules
To monitor oxidative stress in venules, the hydrogen peroxide (H2O2)-sensitive fluorescent probe dihydrodihoradamine 123 (DHR) (Sigma–Aldrich, St Louis, MO) was administrated to the superfusate (10 μM) after craniotomy of gerbils in a separate experiment (n = 6 for each group). Fluorescence intensity in the surrounding area (Ia) and in the wall (Iw) of selected microvascular segments was acquired 60 min thereafter at excitation wavelength of 510 nm and emission wavelength of 534 nm, and estimated using Image-Pro Plus 5.0 (Media Cybernetics Inc, US). The difference between Iw and Ia was determined as an indicator of oxidative stress (Harris and Langlois, 1998; Xu et al., 2009).

2.4.3. Measurement of albumin leakage
Fluorescein isothiocyanate (FITC)-albumin (Sigma–Aldrich, St Louis, MO) was infused (50 mg/kg body weight) through femoral vein after craniotomy of gerbils in a separate experiment (n = 6 for each group). The venular images were acquired 60 min thereafter under irradiation at wavelength of 488 nm, and the fluorescence intensities of FITC-albumin were determined in the lumen of selected venules (Iv) and in the surrounding interstitial area of the same size (Iv) using Image-Pro Plus 5.0 software. The ratio of Iv to Iv was calculated as an indicator of albumin leakage (Xu et al., 2009; Han et al., 2009).

2.5. Tissue preparation for histologic examination
Five days after reperfusion, the brains of gerbils from another set of experiments (n = 6 for each group) were perfused first with heparinized phosphate-buffered saline, followed by perfusion with 4% formaldehyde in phosphate-buffered saline for 40 min. The brain was removed and cut into blocks, embedded in paraffin, and sectioned at 10 μm. The sections were deparaffinized and rehydrated, sequentially, and examined by Nissl staining, immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) techniques, respectively, as detailed below.

2.5.1. Nissl staining
The sections were stained with cresyl violet and examined with light microscope (BX512DP70, Olympus, Japan), according to the standard procedure. Five fields of CA1 sector in hippocampus of each animal were randomly selected and the number of surviving pyramidal cells per 1 mm of CA1 region was counted (América et al., 2008).

2.5.2. TUNEL assay
TUNEL assay was applied to assess the apoptotic death neurons in CA1 region of hippocampus of gerbil using an in situ cell death detection kit (Roche, US), and conducted according to the instruction of the manufacturer. The number of total nuclei and TUNEL-positive nuclei in each field of CA1 region was scored with Image-Pro Plus 5.0 software (Chen et al., 2009), using laser confocal microscope (Axiovert 200, Zeiss, Germany) with a 63× objective, and five fields of CA1 region were examined for each animal. As control, a consecutive section was treated similarly except that the primary antibody was omitted. Positive staining was visualized with diaminobenzidine. The images were captured by a digital camera connected to a microscope (BX512DP70, Olympus, Japan) and analyzed with Image-Pro Plus 5.0 software (Chen et al., 2009). Five fields of CA1 region were examined for each animal.

2.6. Western blot analysis
Five days after reperfusion, animals from another set of experiments (n = 3 for each group) were deeply anesthetized and transcardially perfused with saline through the left ventricle. The brain was removed and cerebral hippocampus was homogenized in lysis buffer including protease inhibitors. 30 μg of the supernatant was mixed with 4× sample buffer. The protein samples were separated on Tris–glycine SDS–PAGE in a reducing condition. The primary antibody against Bax (1:500, Sigma–Aldrich, St Louis, MO) or Bcl-2 (1:500, Sigma–Aldrich, St Louis, MO) was used. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology, US) at room temperature for 60 min (Sharma and Mehra, 2008).

2.7. Electron microscopy
Five days after reperfusion, brains of gerbils from a separate experiment (n = 3 for each group) were 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. The fixed brain was removed and processed as routine for transmission electron microscopy (TEM) or scanning electron microscopy (SEM). The instrument used was JEM 1230 (Jeol, Japan) for TEM and JSM-5600LV (Jeol, Japan) for SEM (Xu et al., 2009).

2.8. Statistical analysis
All parameters were expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA followed by Tukey test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

3. Results
3.1. Effect of CG on adhesion of leukocytes to, DHR fluorescence intensity in, and albumin leakage from cerebral cortical venular wall
To address the effect of CG post-treatment on I/R-induced impairment in cerebral cortical microcirculation of gerbils, cortical venules were evaluated with attention being focused on leukocyte adhesion, oxidative stress and permeability of venular wall 5 days after reperfusion. As noticed in Fig. 1A, the number of adherent leukocytes in I/R group increased markedly compared to that in sham group. Post-treatment with CG at both dosages tested significantly attenuated I/R-elicited leukocyte adhesion. Similarly, the fluorescence intensity of DHR, indicative of H2O2 production, increased obviously in I/R group compared to sham group. Post-treatment with CG at both 0.4 g/kg and 0.8 g/kg significantly prevented the oxidative response to I/R (Fig. 1B). The profit of post-treatment with CG on I/R-induced disorder in cerebral cortical microcirculation was also evidenced by the attenuation of vascular permeability, as showed in Fig. 1C, wherein I/R challenge
Fig. 1. The effects of post-treatment with CG on microcirculatory disturbances in cerebral cortex of gerbils. (A) The representative image of leukocyte adhesion (left panel) and the effect of CG on the number of leukocytes adherent to venular wall (right panel). (B) The representative image of DHR fluorescence (left panel) and the effect of CG on the fluorescence intensity of DHR in venular wall (right panel). (C) The representative image of albumin leakage (left panel) and the effect of CG on the albumin leakage from the venule (right panel). Sham: sham group; I/R: I/R group; I/R + CG 0.4: post-treatment with CG 0.4 g/kg after I/R; I/R + CG 0.8: post-treatment with CG 0.8 g/kg after I/R. Data are mean ± SE (n = 6). a p < 0.05 vs. sham group, b p < 0.05 vs. I/R group.

Fig. 2. The effects of post-treatment with CG on hippocampal CA1 structures. Tissues were stained with Nissl. (A) Changes in morphology of hippocampus. a, e: sham group, b, f: I/R group, c, g: I/R + CG 0.4 group, d, h: I/R + CG 0.8 group. CA1 region. Bar = 20 μm. (B) Quantitative evaluation of Nissl-positive neurons in various conditions. Sham: sham group; I/R: I/R group; I/R + CG 0.4: post-treatment with CG 0.4 g/kg after I/R; I/R + CG 0.8: post-treatment with CG 0.8 g/kg after I/R. Data are mean ± SE (n = 6). a p < 0.05 vs. sham group, b p < 0.05 vs. I/R group.
caused albumin leakage was significantly diminished in the animals receiving CG with no difference being observed between the two dosages of CG tested.

3.2. Effect of CG on the histology of CA1 region of hippocampus

The effect of CG post-treatment on morphological changes in CA1 after I/R was assessed by Nissl staining, and the representative images of CA1 sector for various conditions are illustrated in Fig. 2A. In sham group, the pyramidal cells existed in approximately three to four layers and packed regularly with the Nissl bodies being darkly stained (a and e). In contrast, 30 min ischemia followed by reperfusion for 5 days evoked a dramatic alteration in CA1, characterized by thinning of the cell layers, shrinkage and disintegration of neurons (b and f). Post-treatment with CG at both dosages attenuated the morphological alternations (c, g and d, h). Fig. 2B shows a quantitative evaluation of the cell number in the CA1 region in the four groups. Compared to the sham group, the neuron number decreased significantly after I/R challenge, which was relieved by the post-treatment of CG.

3.3. Effect of CG on cell apoptosis

The TUNEL and Caspase-3 positive neurons in the hippocampal CA1 sectors of gerbils were detected. As demonstrated in Fig. 3A, the TUNEL positive cells (arrows) increased significantly in the I/R group (a3) compared to the sham group (a2) and I/R+CG 0.4 group (a4). The TUNEL positive cells significantly decreased in the I/R+CG 0.4 group (a4) as compared to the I/R group (a3). The Caspase-3 positive cells were increased in the I/R group (b3) compared to the sham group (b2) and I/R+CG 0.8 group (b5). The Caspase-3 positive cells significantly decreased in the I/R+CG 0.8 group (b5) as compared to the I/R group (b3).

Fig. 3. The effects of post-treatment with CG on TUNEL-and caspase-3-positive neurons in cerebral hippocampal CA1 of gerbils. (A) Representative photomicrographs of hippocampal CA1 neurons stained by TUNEL. a1: negative control, a2: sham group, a3: I/R group, a4: I/R + CG 0.4 group, a5: I/R + CG 0.8 group. Arrows indicate TUNEL-positive neurons. Bar = 20 μm. (B) Immunostaining for caspase-3 of hippocampal CA1 neurons. b1: negative control, b2: sham group, b3: I/R group, b4: I/R + CG 0.4 group, b5: I/R + CG 0.8 group. Arrows indicate caspase-3-positive neurons. Bar = 100 μm. (C) Quantitative analysis of TUNEL- and caspase-3-positive cells in four groups. Sham: sham group; I/R: I/R group; I/R + CG 0.4: post-treatment with CG 0.4 g/kg after I/R; I/R + CG 0.8: post-treatment with CG 0.8 g/kg after I/R. Data are mean ± SE (n = 6). a p < 0.05 vs. sham group, b p < 0.05 vs. I/R group.

Fig. 4. Expression of Bax and Bcl-2 in cerebral hippocampus 5 days after reperfusion. (A and B) Western blot showing the effects of post-treatment with CG on Bax and Bcl-2 expression. (C and D) Quantification of the Western blot results. Sham: sham group; I/R: I/R group; I/R + CG 0.4: post-treatment with CG 0.4 g/kg after I/R; I/R + CG 0.8: post-treatment with CG 0.8 g/kg after I/R. Data are mean ± SE (n = 3). a p < 0.05 vs. sham group, b p < 0.05 vs. I/R group.
Fig. 5. Ultrastructure of the cerebral cortex of gerbils 5 days after reperfusion. (A) The representative transmission electron micrographs of capillaries in cerebral cortex of gerbils. Note the contraction of the vessel and swelling of perivascular astroglial process (SA) in response to I/R (A2), which was attenuated by post-treatment with CG (0.8 g/kg, A3). (B) The representative scanning electron micrographs of microvessel in cerebral cortex. The I/R challenge caused a roughness of endothelial surface and numerous surface projections (f) into the lumen as well as digitations of endothelial cell contacts (B2), which was alleviated by post-treatment with CG (0.8 g/kg) (B3). Bar = 1 μm. (C) The representative scanning electron micrographs of the fractured face of cerebral cortex. Note that after I/R, the number of open arterioles (a) and venules (v) was reduced and their distribution became heterogeneous (C2) in comparison with sham group (C1). I/R-induced abnormalities were recovered in CG (0.8 g/kg) post-treated group (C3).

Fig. 6. The representative transmission electron micrographs of neurons in the cerebral hippocampal CA1 of gerbils. Compared with sham group (A and D), 30 min ischemia followed by reperfusion for 5 days evoked distinguished changes in neuron morphology, typically exhibiting shrinkage of cytoplasm and nucleus with high electron density (B, rectangle) and swollen rough endoplasmic reticulum (E, rectangle). Post-treatment with CG (0.8 g/kg) recovered the I/R-induced alteration in neuron morphology to a great extent (C and F).
3.3. Effect of CG on hippocampal CA1 neuron death and apoptosis-related proteins

To further specify the effect of post-treatment with CG on the damage of CA1 neurons after I/R, TUNEL assay was performed. The results displayed in Fig. 3A show that a large number of TUNEL-positive cells occurred in the CA1 region of gerbils subjected to I/R challenge (Fig. 3A, a3), while TUNEL-positive cells were hardly observed in the sham group (Fig. 3A, a2). The I/R-evoked increase in the number of TUNEL-positive cells in CA1 region was significantly reduced in CG-treated groups, as shown in Fig. 3A, a4 and a5.

In line with the results of TUNEL assay, the number of caspase-3-positive cells increased considerably in the CA1 region of gerbils after I/R stimulation (Fig. 3B, b3) compared to sham group (Fig. 3B, b2), which was significantly alleviated by CG post-treatment (Fig. 3B, b4 and b5).

Fig. 3C presents the quantitative determination of both TUNEL assay and caspase-3 immunostaining, providing further support for the results above.

Fig. 4 illustrates the result of Western blot, showing a significant increase of Bax (Fig. 4A and C) and a noticeable decrease of Bcl-2 (Fig. 4B and D) in I/R group compared with sham group. CG post-treatment significantly mitigated these changes.

3.4. Effect of CG on ultrastructure of cerebral cortex and hippocampus

TEM was performed to examine the microvasculature in the cerebral cortex 5 days after reperfusion. The capillary endothelium in the sham group exhibited a smooth inner surface and tightly connected intercellular junctions; no edema was observed in perivascular tissues (Fig. 5, A1). In contrast, I/R-elicted obvious perivascular edema, as well as remarkable abnormalities in the cortical capillaries characterized by narrowed lumen and rough inner surface (Fig. 5, A2). These changes were attenuated by CG (0.8 g/kg) post-treatment (Fig. 5, A3).

SEM examination on the inner surface of capillary endothelium confirmed the findings by TEM (Fig. 5, B1–B3). Furthermore, SEM revealed a high density of open and uniformly distributed capillaries in the cortex in sham group (Fig. 5, C1). After I/R, by contrast, the number of open capillaries reduced (Fig. 5, C2). I/R-induced abnormalities were alleviated noticeably in CG (0.8 g/kg)-treated brains (Fig. 5, C3).

Fig. 6 shows the representative images of hippocampal neurons observed by TEM 5 days after reperfusion. As noticed, the neurons in sham group exhibit clear cytoplasm and nucleus (Fig. 6A and D), while the ultrastructural feature of hippocampal neurons in I/R group changed obviously, characterized by shrinkage of both nucleus and cytoplasm with increased electron density (Fig. 6B, rectangle). Also, extensive swollen rough endoplasmic reticulum commonly existed (Fig. 6E, rectangle). Of notice, post-treatment with CG ameliorated I/R-induced alterations in hippocampal neurons (Fig. 6C and F).

4. Discussion

In present study we used a gerbil model of 30 min of carotid artery occlusion with subsequent reperfusion up to 5 days to explore the potential of CG post-treatment as a therapeutic approach for I/R-induced cerebral microcirculatory disorders and neuron injury. The results demonstrated that administration of CG one dose a day for 5 days starting from 3 h after reperfusion significantly attenuated I/R-induced cerebral microvascular injury and hippocampal CA1 neuronal damage.

The preventive effect of pretreatment with CG on I/R-induced insults in cerebral microvasculature has been proved in a previous publication from our group (Xu et al., 2009). In present study, we show that the I/R-induced cerebral impairments may benefit from post-treatment with CG as well. We started administrating CG 3 h after initiation of reperfusion, a time point while extensive cerebral impairments have occurred but still within the time window for clinical intervention. The improved outcomes observed after 5 days of administration of CG feature its ability to either restore the existed cerebral disorders or antagonize against initiating or progressing of delayed impairments, or both, suggesting the potential of CG as an alternative for management of patients with cerebral I/R injury.

Adhesion of leukocytes to the endothelium is one of the major steps leading to microcirculatory disturbance during reperfusion (Jürgen et al., 2007). Adherent neutrophils release proteinases and reactive oxygen species (ROS) that target the endothelium and basement membrane and increase vascular permeability, resulting in albumin leakage and tissue edema (Kurose et al., 1997; Han et al., 2001; Olanders et al., 2002). Moreover, leukocyte extravasation directly damages the brain tissue (Hernandez et al., 1987; Zhang et al., 1994). Thus, inhibiting leukocyte adhesion has been considered one of the anti-inflammatory treatments after I/R challenge. The fact that CG is able to inhibit leukocyte adhesion to venular walls in the cerebral cortex of gerbils subjected to global cerebral I/R accounts for, at least partly, the favorable action of CG on cerebral injury observed in this study. As to the herb responsible for the role of CG in attenuating leukocyte adhesion, *Ligusticum chuanxiong* Hort. (Chuan Xiong), one of the ingredients of CG, was reported to contain a compound, Ligustazine, which is able to inhibit the expression of intracellular adhesion molecule-1 (ICAM-1) in endothelium induced by phytohemagglutinin (Zhao et al., 2000). The contribution of other ingredients of CG in this regard remains unclear and needs further elucidation.

It is well established that free radicals burst immediately after I/R through diverse mechanisms, and persist elevated for various period of time depending on the experimental condition and animal model used (Nelson et al., 1992). These free radicals inflict lipid, proteins, and nucleic acids, leading to damages of vascular endothelial cells and basement membrane (Fellman and Raivio, 1997). On the other hand, increased free radical generation activates nuclear transcription factor NF-κB, up-regulating expression of ICAM-1 on endothelial cells and enhancing leukocyte accumulation in the vessels (Olanders et al., 2002). There may exist a positive feedback between free radical generation and leukocyte adhesion, and, thus, targeting inhibition of free radicals is likely one of the effective therapeutic interventions for improving I/R injury. In agreement with the result from our study of pretreatment with CG (Xu et al., 2009), the present study demonstrated that CG given 3 h after reperfusion also ameliorates oxidative stress, as evidenced by blunting fluorescent intensity of DHR in cerebral venular wall and blocking albumin leakage from venules. The antioxidant property of CG is anticipated in light of the fact that among the 11 herbs composing CG, 6 herbs have been identified to contain compound with antioxidant essence (Choi et al., 1994; Okubo et al., 2000; Patro et al., 2002; Miao et al., 2003; Fan et al., 2006; Liang et al., 2008). The results of present study suggest that the inherent antioxidant nature of CG may contribute, to a great extent, to the beneficial effects of CG, even in the late phase of cerebral I/R injury.

The present study revealed that 5 days of reperfusion following 30 min of ischemia resulted in albumin leakage from venular wall, prominent edema in surrounding of microvessels, and a reduced number of open microvessels in cortex, suggesting that I/R-provoked enhanced leukocyte adhesion and oxidative stress in microvessel eventually damage the vessel wall, leading to increase in vessel permeability, efflux of plasma proteins, and edema in perivascular interstice, which, in turn, deteriorate...
cerebral microcirculation. Noticeably, these alterations were attenuated significantly by CG post-treatment, providing further support for the beneficial effect of CG post-treatment on I/R-induced cerebral microcirculatory dysfunction.

Neuron death is a critical consequence of cerebral I/R injury, which is initiated upon ischemia and aggravated by prolonged reperfusion (Dirmagl et al., 1999; Kirino, 2000), characterized by a long delay, from hours to weeks, depending on the nature of the insult and brain region being affected. The pyramidal cells in CA1 region are widely studied in cerebral global ischemia due to their vulnerability. Consistent with other studies (Urbate et al., 2000; Wang et al., 2005), the present study showed that 30 min cerebral ischemia followed by reperfusion up to 5 days induced extensive hippocampal CA1 neuron injury, as evidenced by Nissl staining and ultrastructural findings. Two major types of neuron death were identified during cerebral I/R insult, i.e., necrosis and apoptosis. Although neuron apoptosis has been proved to occur in cerebral focal ischemia and reperfusion, its existence in cerebral global ischemia and reperfusion has never been demonstrated with certainty. In accord with the results from others, although the ultrastructure examination on CA1 neurons after 5 days of reperfusion following 30 min of ischemia found no cell having typical feature of apoptosis, the results of TUNEL staining suggested the occurrence of CA1 neuron apoptosis in present circumstance. Furthermore, the decrease in the number of positive cells of TUNEL staining, Bax and caspase-3, and concurrent increase in the number of Bcl-2-positive cells by 5 days of treatment with CG strongly favor the notion that CG exerts attenuating effect on I/R-induced neuron injury, at least in part, through interfering in CA1 neuron apoptosis pathway. Nevertheless, the I/R-elicted neuron injury in CA1 region was alleviated obviously by CG post-treatment, suggesting that this Chinese compound medicine may be used as a new strategy for protecting brain from I/R injury.

In conclusion, the present study using a gerbil model of global cerebral I/R demonstrates that post-treatment with CG alleviates I/R-elicted cerebral injury, which is associated with inhibition of leukocytes adhesion and oxidative stress in microvessels, and interference of hippocampal CA1 neuron apoptosis pathway.

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References


