Cerebralcare Granule® attenuates blood–brain barrier disruption after middle cerebral artery occlusion in rats

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A R T I C L E   I N F O
Article history:
Received 3 February 2012
Revised 21 July 2012
Accepted 24 July 2012
Available online 31 July 2012

Keywords:
Blood–brain barrier
Ischemic stroke
Tight junction proteins
Caveolin-1
Chinese medicine

A B S T R A C T
Disruption of blood–brain barrier (BBB) and subsequent edema are major contributors to the pathogenesis of ischemic stroke, for which the current clinical therapy remains unsatisfactory. Cerebralcare Granule® (CG) is a compound Chinese medicine widely used in China for treatment of cerebrovascular diseases. CG has been demonstrated efficacy in attenuating the cerebral microcirculatory disturbance and hippocampal neuron injury following global cerebral ischemia. However, the effects of CG on BBB disruption following cerebral ischemia have not been investigated. In this study, we examined the therapeutic effect of CG on the BBB disruption in a focal cerebral ischemia/reperfusion (I/R) rat model. Male Sprague–Dawley rats (250 to 300 g) were subjected to 1 h middle cerebral artery occlusion (MCAO). CG (0.4 g/kg or 0.8 g/kg) was administrated orally 3 h after reperfusion for the first time and then once daily up to 6 days. The results showed that Evans blue extravasation, brain water content, albumin leakage, infarction volume and neurological deficits increased in MCAO model rats, and were attenuated significantly by CG treatment. T2-weighted MRI and electron microscopy further confirmed the brain edema reduction in CG–treated rats. Treatment with CG improved cerebral blood flow (CBF). Western blot analysis and confocal microscopy showed that the tight junction proteins claudin-5, JAM-1, occludin and zonula occluden-1 between endothelial cells were significantly degraded, but the protein expression of caveolin-1, the principal marker of caveolae in endothelial cells, increased after ischemia, all of which were alleviated by CG treatment. In conclusion, the post-treatment with CG significantly reduced BBB permeability and brain edema, which were correlated with preventing the degradation of the tight junction proteins and inhibiting the expression of caveolin-1 in the endothelial cells. These findings provide a novel approach to the treatment of ischemic stroke.

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Introduction

Ischemic stroke and post-ischemia/reperfusion (I/R) induced by thrombolytic therapy result in the blood–brain barrier (BBB) disruption, leading to the development of brain edema and hemorrhagic conversion (Hackett et al., 1999; Jung et al., 2010). Despite the advances in the overall management of acute stroke, the propensity of ischemic brain tissue to develop edema remains the major cause of death within the first few days of stroke in patients with serious herniation (Davalos et al., 1999; Moulin et al., 1985). Thus, drugs protected the BBB may be a promising management strategy for treatment of ischemic stroke.

The endothelial cells of the BBB are the first-line of the defense between the blood and the brain (Abbott et al., 2010). The permeability of the endothelial barrier is regulated by two different routes, one is the paracellular pathway, which is through interendothelial junctions, and the other is transcellular pathway which is via caveolae-mediated vesicular transport. Tight junction (TJ) between adjacent endothelial cells plays critical role in the BBB disruption during ischemic stroke (Jiao et al., 2011). The TJ consists of three types of integral transmembrane proteins: claudins, occludin, and junction adhesion molecules (JAMs), and several cytoplasm accessory proteins including zonula occludens (ZO)s (Abbott et al., 2010; Wollburg et al., 2009), among which claudin-5, occludin, JAM-1 and ZO-1 play a pivotal role in the ischemic BBB injury (Cosine and Engelhardt, 2011; Sandoval and Witt, 2008; Simard et al., 2007; Zehender et al., 2009).

Caveolae, 50–100 nm plasmaemal vesicles in the cytoplasm, are important for regulation of a range of endothelial cell functions, such...
as production of nitrogen oxide (Bernatchez et al., 2005), signaling along MAP kinase cascade (Engelman et al., 1998), as well as the transcytosis of albumin across endothelial cells (Ghitescu et al., 1986; Schubert et al., 2001). Caveolin proteins represent the principal structural proteins of the caveolae. As the principal marker of caveolae (Rothberg et al., 1992), caveolin-1 is required for the caveolae-mediated transcytosis of albumin (Minshall et al., 2003). Under pathological condition the breakdown of BBB causes the increase in albumin leakage via both paracellular and transcellular pathway.

Cerebralcare Granule (CG; Tasly Pharmaceutical Co Ltd, Tianjin, China) is a newly developed compound Chinese medicine composed of eleven herbs (Xu et al., 2009). The CG was approved in 1996 by the Chinese State Food and Drug Administration (1002004736603642) and had been widely used in China for treatment of headache and dizziness associated with cerebrovascular diseases. Our previous studies have demonstrated that CG can inhibit the production of superoxide in cerebral venular endothelium, albumin leakage across venules, alleviate the overall microcirculatory disturbances and hippocampal neuron damage in global I/R of Mongolian gerbil (Sun et al., 2010; Xu et al., 2009). However, the effect of CG on BBB injury elicited by focal cerebral I/R has not been well explored. Therefore, this study was designed to investigate the role of CG in BBB disruption after I/R, with particularly addressing the involvement of tight junction and caveolae.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 250 to 300 g were purchased from the Animal Center of Peking University Health Science Center (Beijing, certificate no. SCXK 2006-0008). The animals were housed in cages at 22 °C±2 °C and humidity of 40%±5% under a 12-hour light/dark cycle, and received standard diet and water ad libitum. The rats were fasted for 12 h before experiment but allowed free access to water. The experimental procedures were carried out in accordance with the guidelines of the Peking University Animal Research Committee. All animals were handled according to the guidelines of the Peking University Animal Research Committee. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (IA2011-38).

Cerebralcare granule

CG was produced by Tasly Pharmaceutical Co. Ltd (Tianjin, China). The batch number of the CG used in this experiment was 1002004736603642. 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 drugs were manufactured as granules after dynamic cycle extraction and concentrated by evaporating and spray drying. The CG was packed with aluminum foil composite, 4 g per bag. The CG was dissolved in saline to a concentration of 80 mg/ml or 160 mg/ml before use (Xu et al., 2009).

Middle cerebral artery occlusion model and experimental groups

Male Sprague–Dawley rats were subjected to MCAO and reperfusion, as reported previously (Longa et al., 1989). Briefly, after anesthetized with pentobarbital sodium (0.1 g/kg body weight) intraperitoneally, neck vessels were exposed through a midline incision, and branches of the right external carotid artery were isolated and cauterized. A 4.0-monofilament nylon suture was prepared by rounding the tip to approximately 0.35 mm in diameter with a cautery. The right middle cerebral artery was occluded with the suture by inserting it through the right external carotid artery and gently advancing into the internal carotid artery up to a point approximately 18 mm distal to the bifurcation of the carotid artery. Reperfusion was achieved by slowly pulling the suture back after 60 min occlusion. The incision on the neck was closed and the animals were allowed to recover. Rectal temperature was maintained at 37 ± 0.5 °C throughout the procedure from the start of the surgery until the recovery of the animals from anesthesia with a thermostat-controlled heating pad.

A total of 165 rats were included and randomly divided into 5 groups: (1) sham group (n = 33), (2) I/R 3H group (n = 33), (3) I/R 6D group (n = 33), (4) I/R6D + CG0.4 group (n = 33), and (5) I/R 6D + CG0.8 group (n = 33) (see Table 1 for further details). In the sham group, rats were operated in the same way, but without occlusion of middle cerebral artery. In CG post-treated groups, CG was administrated by gavage (5 mL/kg) 3 h after the beginning of reperfusion for the first time at a dose of either 0.4 g/kg (I/R6D + CG 0.4 group) or 0.8 g/kg (I/R6D + CG 0.8 group), and received the same dose of CG every 24 h for 6 days. The animals in sham group and I/R 6D groups were given equivalent volume of saline in the same manner. Determination of the doses of CG used in the present study was based on our previous works (Sun et al., 2010; Xu et al., 2009). All variables in this study were examined at 3 h (I/R 3H group), or on day 6 (remaining groups), after reperfusion, unless otherwise mentioned.

MRI examination

Rat brain edema was examined in a 3.0-Tesla (T) MRI animal scanner (Magnetom Trio with TIM system, Siemens, Erlangen, Germany). The rats in each group (n = 5) were submitted to examine by MRI for two times, the one at 3 h after reperfusion, and the other on day 6. The animal’s head was positioned in a custom-made “birdcage coil” (inner diameter of 30 mm) for signal excitation and detection. MRI parameters were set at TE = 92 ms, TR = 3620 ms, FOV = 8 × 8 cm2, M = 256 × 256, NA = 2, thickness = 2 mm, and gap = 0 mm. After the optimal adjustment of contrast, hemisphere intensity was examined by Image-Pro Plus 5.0 software (Media Cybernetic, Bethesda, MD, USA) using the operation “mean density value.” The intensity percentage of the ipsilateral hemisphere against the contralateral hemisphere was calculated, and statistical analysis was performed (Zhang et al., 2011).

Albumin leakage

The animal’s head was secured in a stereotactic frame. With a hand-held drill, a 4 × 6 mm2 cranial window was performed through an incision 1 mm behind the coronal suture, and 1 mm on the right side of the sagittal suture. This location corresponds to the margin of the MCA territory. The dura was removed and the pia mater was superfused contiguously with 37 °C warm physiological saline.

Table 1

| The number of animals for different experimental groups and various parameters. |
|----------------------------------|---|---|---|---|---|---|---|
| Evans blue extravasation         | 5 | 5 | 5 | 5 | 5 | 25 |
| Albumin leakage                  | 6 | 6 | 6 | 6 | 30 |
| Cerebral blood flow, TTC staining and neurological score | 6 | 6 | 6 | 6 | 30 |
| Confocal                         | 3 | 3 | 3 | 3 | 3 | 15 |
| MRI and brain                    | 5 | 5 | 5 | 5 | 5 | 25 |
| Western blot assay               | 5 | 5 | 5 | 5 | 25 |
| Ultrastructure examination       | 3 | 3 | 3 | 3 | 3 | 15 |
| Total                            | 33 | 33 | 33 | 33 | 165 |

The same animals were used for detection of CBF, TTC staining and neurological score. The same animals were used for detection of MRI and brain water content. Sham: Sham group; I/R 3H: I/R 3 h group; I/R 6D: I/R 6 days group; I/R 6D + CG 0.4: I/R 6 days plus post-treatment with CG 0.4 g/kg group; I/R 6D + CG 0.8: I/R 6 days plus post-treatment with CG 0.8 g/kg group. MRI: Magnetic Resonance Imaging; TTC: triphenyltetrazolium chloride.
Albumin leakage was observed using an upright fluorescence microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a color monitor (20PF5120, Philips, Eindhoven, Netherlands), a video timer (VTG-33, FOR.A, Tokyo, Japan), and a DVD recorder (DVR-560H, Philips, Eindhoven, Netherlands). The cerebral venules ranging from 35 to 45 μm in diameter and 200 μm in length were selected for this study. Only rats whose cranial window was without any bleeding or inflammatory process were included in the study. Ten minutes before observation, the rats (n=6 for each group) was intravenously injected with 50 mg/kg fluorescein isothiocyanate (FITC)-albumin (Sigma-Aldrich, St Louis, MO, USA) through femoral vein. Fluorescence signal (excitation wave length at 420 to 490 nm, emission wave length at 520 nm) was acquired using a super-sensitive CCD camera (USS-301, UNIQ Vision Inc, Santa Clara, CA, USA). The fluorescence intensities of FITC-albumin in the venules (Iv) and in the perivenular interstitial area (Ii) were measured with Image-Pro Plus 5.0 software. Albumin leakage was presented as Ii/Iv (Xu et al., 2009).

Evans blue leakage and brain water content

Evans blue leakage was assessed as previously described (Hu et al., 2009) with some modifications. Evans blue dye (Sigma-Aldrich, St Louis, MO, USA, 4%, 3 ml/kg) in 0.9% saline was injected into the right femoral vein. Three hours thereafter, rats were anesthetized with pentobarbital sodium (0.1 g/kg body weight, i.p.) and transcardially perfused with physiological saline. Brains were removed, each hemisphere were weighed, homogenized in PBS, and centrifuged at 2000 ×g for 15 min at 4 °C. Then, 0.5 ml of the resultant supernatant was added to an equal volume of trichloroacetic acid. After overnight incubation and centrifugation at 2000 ×g for 15 min at 4 °C, the supernatant was taken for spectrophotometric quantification of extravasated Evans blue dye at 620 nm.

Brains water content was measured as described (Hu et al., 2011). Briefly, brains (n=5) were quickly separated into the left and right cerebral hemispheres and weighed (wet weight). Brain specimens were then dried in an oven at 120 °C for 48 h and weighed again (dry weight). The percentage of water content was calculated as [(wet weight-dry weight)/wet weight]×100%.

Cerebral blood flow measurement

Cerebral blood flow (CBF) (n=6) was measured using Laser Doppler perfusion image system (PeriScan PIM3 System; 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 parietal bone window was opened on the right side, 1 mm behind the coronal suture, and 1 mm lateral to sagittal suture, with an area of 4×6 mm. 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 80 pixels. 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 (Paris et al., 2004). The images were acquired after 10 min basic observation.

Infarct size and neurological deficits

Rats were euthanized, decapitated, and the brains were rapidly removed. Infarct volumes (n=6) were measured as described previously (Tsubokawa et al., 2007). In brief, brains were cut into 5 coronal...
brain slices (2 mm thick) with a matrix (Brain Matrix, WPI-Europe, Aston, Stevenage, UK), and stained in 1% TTC (2,3,5-triphenyltetrazolium chloride) in 0.1 mol/L phosphate buffer (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37 °C. The infarcted tissue remained unstained (white), whereas normal tissue was stained red. The infarct zone was analyzed by Image-Pro 5.0 software (Media Cybernetic, Bethesda, MD, USA). The infarct volume was calculated by the equation [(contralateral hemisphere volume-non-infarcted volume of the ipsilateral hemisphere)/contralateral hemisphere volume] to avoid the influence of brain edema (Gao et al., 2006).

Neurological scores were evaluated as previously described (Hunter et al., 2000), according to a graded scoring system: 0 = no deficit; 1 = flexion of contralateral torso and forelimb upon lifting of the whole animal by the tail; 2 = circling to the contralateral side, when held by tail with feet on floor; 3 = spontaneous circling to contralateral side; 4 = no spontaneous motor activity. Each animal's...
scores were estimated within approximately 1 min, and estimation was repeated another 3 times for consistency. Score of 0 corresponds to a normal neurological status, and higher scores correspond to a behavioral deficit.

**Ultrastructure examination**

The rat brain \((n = 3 \text{ for each group})\) was perfused for 40 min with a fixative made of 4% formaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer at a speed of 3 ml/min. For transmission electron microscopy, a coronal slice in the cortex penumbra area approximately 1 mm thick was taken. The slice was placed in fresh prepared 3% glutaraldehyde overnight at 4 °C. After rinsing with 0.1 mol/L phosphate buffer for 3 times, the tissue block was post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 2 h at 4 °C. The samples were dehydrated and then embedded in Epon 812. Ultra-thin 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 into blocks and placed in the fresh prepared glutaraldehyde for 2 h, rinsed with 0.1 mol/L phosphate buffer, and then post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 2 h. The specimens were processed as routing and examined under a scanning electron microscope (JSM-5600LV, JEOL, Tokyo, Japan).

**Immunofluorescence staining and confocal microscopy**

Coronal fresh frozen sections \((n = 3)\) were sliced in 10 μm thick using a cryostat (CM1800, Leica, Bensheim, Germany). After the slides were completely air dried, slices were treated with 0.01 mol/L sodium citrate for antigen retrieval and washed by PBS for three times. After blocking with 3% normal goat serum at room temperature for 0.5 h, slices were then incubated with primary antibodies diluted in PBS overnight at 4 °C. The primary antibodies were as follows: mouse anti-claudin-5 (1:100, Invitrogen, Camarillo, CA, USA), mouse anti-occludin (1:50, Invitrogen, Camarillo, CA, USA), rabbit anti-JAM-1 (1:50, Invitrogen, Camarillo, CA, USA), mouse anti-ZO-1 (1:50, Invitrogen, Camarillo, CA, USA), and rabbit anti-vWF to label endothelial cells (1:100, Millipore, Temecula, CA, USA). After rinsing with PBS, brain sections were then incubated with secondary antibodies, Dylight 488-labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) and Dylight 549-labeled goat anti-mouse IgG (KPL, Gaithersburg, MD, USA), for 2 h at room temperature. Hoechst 33342 (Molecular Probes) was applied to stain all the nuclei as a background staining. The brain sections were mounted

**Fig. 4.** Ultrastructure of microvessels in the ipsilateral cerebral cortex of rats. The representative transmission electron micrographs of capillaries in the cerebral cortex are displayed in a and b, while the scanning electron micrographs of the cerebral cortex fractured face are displayed in c and d. The micrographs in a and d are the high magnification of the area inside the boxes in a and c, respectively. N: neuron; SA: swelling astroglial process; OC: opening capillaries; E: perivascular edema; V: venules Bar = 50 μm.
Author’s personal copy

Western blot assay

Western blot analysis (n = 5) was performed as described previously (Huang et al., 2007). Briefly, whole-cell protein was prepared from the ischemic cortices and contralateral cortices, and extracted by RIPA buffer for determination of protein expression of tight junction proteins and caveolin-1. Protein samples (200 μg) were separated using a 10% Tris–HCl precast gel for polyacrylamide electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 90 to 120 min. The proteins were transferred to polyvinylidene fluoride membranes with 200 mA at 4 °C for 120 min. The membranes were blocked in TBST containing 5% nonfat milk for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against occludin (1:1000, Abcam, Cambridge, UK), Claudin-5, JAM-1 and ZO-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, USA). The membranes were washed with TBST before incubation with the respective horseradish peroxidase-conjugated secondary antibodies at a 1:5000 dilution for 60 min at room temperature. Blots were developed using Chemilucent Detection System Kit (Millipore Chemicon International, Inc. Temecula, CA, USA), and protein bands were visualized on X-ray film. Semiquantitation of the protein was performed with the use of Image-Pro Plus 5.0 software (Media Cybernetic, Bethesda, MD, USA).

Data analysis

All parameters were expressed as mean ± SE. Statistical analysis was conducted by one-way analysis of variance, followed by multiple-comparison procedures using Tukey test. A value of P < 0.05 was considered statistically significant.

Results

CG improves MRI outcome and reduces albumin leakage, Evans blue extravasation, and brain water content

MRI is considered the most promising and noninvasive approach for examining brain edema formation in real time. T2-weighted MRI is frequently used to determine the edema induced by I/R. By using this technique, we evaluated the cerebral edema in each condition, and the representative images are shown in Fig. 1A. Apparently, no edema was detected in the animals of sham group at both time points examined. In contrast, edema was observed at 3 h after reperfusion in I/R6D alone group (420.22 ± 12.45) or I/R6D + CG groups (406.72 ± 17.95 in I/R6D + CG0.4 group and 434.84 ± 16.49 in I/R6D + CG0.8 group) without obvious difference (P > 0.05). The cerebral edematous area in I/R6D alone group remained almost unchanged on day 6 of reperfusion (390.22 ± 15.49), in distinct contrast to I/R6D + CG0.4 group (178.72 ± 12.93) or I/R6D + CG0.8 group (142.84 ± 24.84), wherein cerebral edematous areas were reduced significantly (P < 0.05). Fig. 1B summarizes the quantitative analysis of MRI based on intensity analysis of the images in the ischemic hemisphere against those in the contralateral hemisphere.

Transvascular eflux of FITC-labeled albumin from cerebral venules was detected in all groups as shown in Fig. 2A, with the quantitation of the albumin leakage depicting in Fig. 2B. A faint fluorescence is noted in the interstitial tissue outside the venule in the sham group, representing the background. The result demonstrated that the albumin leakage increased significantly 3 h after reperfusion as compared to that in sham group (58.05 ± 0.03 vs. 40.61 ± 0.03, P < 0.05), and persisted at high level till day 6 (61.63 ± 0.04). The leakage of FITC-labeled albumin after I/R was attenuated apparently by CG post-treatment at two doses tested (48.27 ± 0.02 in I/R6D + CG0.4 group and 45.21 ± 0.03 in I/R6D + CG0.8 group), indicating the effect of CG to protect BBB from disruption.
The amount of Evans blue dye extravasation in the right cerebral hemisphere was markedly increased at 3 h of reperfusion compared with the sham brain (15.22±0.86 vs. 2.77±0.08, P<0.05) and maintained until 6 days (13.52±0.99) after reperfusion without CG treatment. In contrast, post-treatment with CG attenuated the I/R-elicited extravasation of Evans blue dye in the ischemia hemisphere significantly, reaching 9.56±0.53 and 7.73±0.73 in I/R6D+CG0.4 group and I/R6D+CG0.8 group, respectively (Figs. 3A and B). Meanwhile, CG treatment (0.4 or 0.8 g/kg) for 6 days significantly reduced the brain water content in comparison with both I/R 3 h and I/R 6D groups (P<0.05) (Fig. 3C).

Taken together, these findings demonstrate that post-treatment with CG attenuated the I/R-elicited increase in BBB permeability.

CG ameliorates cerebral microvasculature and CBF

Transmission electron microscopy clearly identified the structures of cerebral microvasculature in the cortex, as shown in Figs. 4a and b. The cerebral microvasculature in sham operated group was normal. Of notice, I/R elicited a remarkable alteration in the microvasculature after 3 h of reperfusion, as compared with the sham group, such as narrowed lumen, rough inner surface, as well as evident swelling perivascular astrocyte end feet, a morphological manifestation consistent with brain edema. The intracellular organelles were frequently absent or scarce in such swelling astrocyte end feet (arrows in Figs. 4a2 and b2). These alterations in cerebral microvasculature after I/R remained obvious on day 6 of reperfusion if without interference (Figs. 4a3 and b3), but were alleviated apparently by CG (0.4 g/kg and 0.8 g/kg) post-treatment (Figs. 4a4 and b4, and a5 and b5). A further examination was performed using scanning electron microscopy for different experimental conditions. Consistent with the result observed by transmission electron microscopy, CG post-treatment attenuated the alterations in cerebral microvasculature, particularly the perivascular edema, induced by I/R. Moreover, CG treatment restored the decrease in the number of open capillaries elicited by I/R (Figs. 4Ac and d).

CBF was determined by a laser Doppler perfusion image system in different groups as shown in Fig. 5A, and the quantification of the result is presented in Fig. 5B. Impressively, I/R challenge for 3 h evoked a significant reduction in CBF compared with sham group (243.49±23.73 vs. 475.74±29.78) and this reduction remained for 6 days (301±25.65) after reperfusion. CG post-treatment significantly attenuated the I/R-evoked decrease in CBF with no difference between the two dosages of CG tested (414±27.92 in I/R6D+CG0.4 group and 426.83±17.27 in I/R6D+CG0.8 group).

Taking together, CG post-treatment attenuates the alterations in cerebral microvasculature and perivascular edema after I/R, and thus improves CBF.

CG alleviates degradation of tight junction proteins and reduces number of caveolae and caveolin-1 protein expression in endothelial cells

To gain insight into the rationale behind the role of CG in maintaining BBB integrity, vascular endothelial TJ proteins were examined for various
groups by confocal microscopy and Western blot. Fig. 6 shows the results regarding claudin-5 and JAM-1. Confocal microscopy revealed that both proteins localized between endothelial cells as continuous lines in sham group (Fig. 6a, e, i, and m). These continuous distributions were disrupted apparently after reperfusion for 3 h and 6 days, becoming dotted lines, concomitant with reduction in immune staining, indicating degradation of the tight junction proteins claudin-5 and JAM-1 in response to I/R. Interestingly, this degradation was restored evidently by CG treatment (Fig. 6d, h, l, and p). These results were confirmed by Western blot (Figs. 6B and C). Similar results were obtained for occludin and ZO-1 (Fig. 7).

Caveolae in capillary endothelial cells are another mediator of BBB permeability. Electron microscopy revealed more caveolae in the cytoplasm of capillary endothelial cells in I/R 3H and I/R6D groups than that in sham group. On the other hand, the caveolae in the endothelial cells in I/R6D + CG0.4 or I/R6D + CG0.8 groups were only scarcely observed (Fig. 8A), suggesting involvement of caveolae in regulation of BBB in the present circumstance. This speculation was supported by Western blot analysis of caveolin-1 protein, which showed a marked increase in caveolin-1 protein level in the ischemic hemisphere of MCAO rats at 3 h (138.86 ± 2.67) after reperfusion, and a further increase after 6 days of reperfusion if no treatment was given compared with sham group (144.84 ± 1.12 vs. 100 ± 0.00, \(P < 0.05\)). Impressively, CG treatment significantly attenuated the increases in caveolin-1 protein expression caused by I/R (111.80 ± 2.65 in I/R6D + CG0.4 group and 107.51 ± 2.31 in I/R6D + CG0.8 group) (Fig. 8B).

This result suggests that CG attenuates the impairment in BBB after I/R by interference in both interendothelial and transendothelial pathway.

**CG reduces infarction volume and neurological deficits**

TTC staining was used to reveal cerebral infarct, whereby normal brain tissue is stained red, while the infarct lesion remains unstained (white color) (Fig. 9A). As expected, brain infarct size significantly increased in both I/R 3H and I/R6D groups when compared with sham-operated group (41.07 ± 1.41 or 39.64 ± 1.52 vs. 0 ± 0.00, \(P < 0.05\)). Noticeably, these I/R-elicited cerebral infarcts were reduced by CG treatment significantly and dose-dependently (27.26 ± 1.51 in I/R6D + CG0.4 group vs. 10.98 ± 0.86 in I/R6D + CG0.8 group, \(P < 0.05\)) (Fig. 9B). To evaluate the outcome of neurological impairment in different groups, a neurological score was conducted, and the result is presented in Fig. 9C. Animals in I/R 3H group exhibited severe neurological deficits compared to the sham group (1.33 ± 0.21 vs. 0 ± 0.00, \(P < 0.05\)). After 6 days of reperfusion without any treatment, the neurological deficits stood unchanged (1.5 ± 0.22), while CG treatment significantly prevented neurological impairment evoked by I/R (0.83 ± 0.17 in I/R6D + CG0.4 group and 0.67 ± 0.21 in I/R6D + CG0.8 group).
All in all, CG post treatment is not only beneficial for attenuation of BBB impairment but also relieves the cerebral infarct and neurological deficit after I/R.

Discussion

To cope with the brain edema after ischemic stroke and post-I/R following thrombolytic therapy remains a challenge for clinicians (Arnold et al., 2003; Cruz-Flores et al., 2001). The results of the present study revealed CG to be a promising management in these circumstances, demonstrating that post-treatment with CG significantly reduced the BBB damage and cerebral edema after I/R, and more importantly, relieved brain infarction and neurological deficits. Furthermore, CG was found to attenuate I/R-induced degradation of the tight junction proteins and increase in the expression of caveolin-1 in the endothelial cells.

A number of clinical studies demonstrated that CG attenuates chronic cerebrovascular insufficiency (Gu et al., 2005; Li, 2003), migraine (Feng et al., 2004) and cerebral arteriosclerosis (Wang et al., 2004). Our group and others have reported that CG ameliorates cerebral microcirculatory disturbance, neuron damage and cognitive impairment elicited by ischemia (Sun et al., 2010; Xiong et al., 2011; Xu et al., 2009). However, to date the effect of CG on BBB disruption associated with cerebral I/R has not been reported.

Ischemic stroke and subsequent reperfusion cause numerous severe clinical complications including brain edema (Jung et al., 2010). Likewise, in the MCAO model of present study, an obvious brain edema was observed after 3 h of reperfusion following 60 min of ischemia, which maintained till 6 days of reperfusion, as evidenced by both T2-weighted MRI and brain water determination. Interestingly, we observed that if CG was continuously administrated starting from 3 h of reperfusion, the I/R-elicited brain edema was alleviated significantly on day 6, indicating the efficiency of CG as a therapeutic strategy for this insult.

Brain edema occurs commonly after a longer period of ischemia, e.g., 30–60 min, like the case in present study, and is mostly vasogenic, in which BBB breakdown is an initial episode. The BBB is a highly specialized structure between the brain and blood circulation that maintains the homeostasis of the neural microenvironment and protects the brain from undesirable penetration by external compounds, cells or excess fluid. It is composed of three components: highly specialized endothelial cells, basal lamina, and surrounding astrocytic end-feet and pericytes (Ballabh et al., 2004; Hawkins and Davis, 2005). In the present work, we observed by electron microscopy the attenuation of the structures of endothelial cells and astrocytic end-feet associated with cerebral I/R has not been reported.

Fig. 8. CG post-treatment decreases the number of caveolae and inhibits caveolin-1 protein expression in endothelium. A: Ultrastructure of microvascular endothelium in the ipsilateral hemisphere in different groups. Arrows indicate the caveolae in the endothelial cells. Bar = 250 nm. a: Sham group; b: I/R 3H group; c: I/R 6D group; d: I/R 6D + CG 0.4 group; e: I/R 6D + CG 0.8 group. B: Representative Western blots and quantitative analysis of caveolin-1. Values are the mean ± SEM. *P < 0.05 vs. Sham group, †P < 0.05 vs. I/R3H group, #P < 0.05 vs. I/R6D group, n = 5.
which may act as a primary shock-absorber, mediating TJ responses to acute changes in vascular dynamics. Claudin-5, another TJP which has four transmembrane domains, is specifically important in the active regulation of paracellular permeability for small solutes at the BBB (Nitta et al., 2003). Drugs that increase claudin-5 expression have been demonstrated to increase transendothelial resistance and decrease BBB permeability (Honda et al., 2006). JAM-1 is a family of immunoglobulin protein localized within the intercellular cleft of TJ, which has a single transmembrane domain. JAM-1 plays a similar role in regulating the integrity of the BBB (Yeung et al., 2008), and is also involved in the leukocyte transendothelial migration (Weber et al., 2007). Occludin, claudin-5 and JAM-1 are known to interact with ZO-1 (Gonzalez-Mariscal et al., 2000), which in turn binds to the actin cytoskeleton, connecting and anchoring the transmembrane proteins to the actin cytoskeleton, and recruiting signaling molecules to TJs (Bazzoni and Dejana, 2004). Although a great deal has been learned about TJP, the exact mechanism of CG on TJP is not explicit. Our experiments with immune confocal microscopy, Western blot and electron microscopy demonstrated that both pathways are implicated in the BBB permeability modulation by CG. The role of basal lamina in the action of CG on BBB permeability is not clear at present, and needs further clarification.

Oxidative stress is one of the most important mechanisms responsible for the BBB disruption and neuronal death in acute ischemic stroke (Sugawara and Chan, 2003). It is well known that leukocytes adherent to vascular wall release oxygen free radicals, resulting in endothelial cells damage and perivascular edema (Han et al., 2001; Kuhlmann et al., 2009). Our previous work showed that CG inhibited leukocyte adhesion to the vessel wall and alleviated perivascular edema and microcirculation disturbance (Xu et al., 2009). In support of these results, other studies reported that chemicals included in CG play an essential role in suppression of reactive oxygen species production (Liang et al., 2008; Lin et al., 2008). Therefore, the protective action of CG on BBB permeability is probably linked to its favorable antioxidant capacity. Nonetheless, the details of the mechanism underlying the role of CG in attenuation of BBB permeability after I/R requires further elucidation.

Brain edema brings about the risk of a critical rise in intracranial pressure with subsequent deteriorative complications (Marmarou, 1992; Marmarou, 2007). Decompressive surgery has currently been applied to cope with this condition, but the outcome is frequently less than optimal (Bratton et al., 2007; Stiver, 2009; Timofeev et al., 2008). The results of the present study showed that the administration of CG resulted in an apparent improvement in cerebral blood flow and cerebral infarction, as demonstrated by CBF, TTC staining, and neurological score, in addition to the attenuation of brain edema. It is not clear if any relationship exists among these results. It is most likely, however, that the beneficial effect of CG starts with restoration of BBB permeability, followed by alleviation in brain edema, which reduces the intracranial pressure, leading to an improvement in the supply of cerebral blood and energy, and, finally, the attenuation of infarction and neurological outcome. Another possibility is that CG exerts action by attenuating both BBB breakdown and proapoptotic pathway in neuron, and the attenuation of infarction and neurological outcome observed is independent of improvement in brain edema. Nonetheless, much more works are needed to clarify this issue.

In conclusion, the present study demonstrated that the compound Chinese medicine CG is able to attenuate I/R-induced brain edema by interference in tight junction protein degradation and caveolin-1 expression in vascular endothelial cells, accompanying by an improvement in cerebral infarction and neurological score, suggesting CG as a promising alternative approach for the patients at risk to develop severe brain edema.

Disclosure

The authors have no financial conflict of interest.