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RESEARCH ARTICLE

Neuroprotective effect of Cerebralcare Granule after cerebral ischemia/reperfusion injury

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Graphical Abstract

Cerebralcare Granule exerts specific neuroprotective effects against cerebral ischemia/reperfusion injury

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Abstract

Cerebralcare Granule (CG) improves cerebral microcirculation and relieves vasospasm, but studies investigating its therapeutic effect on cerebral ischemia/reperfusion injury are lacking. In the present study, we administered CG (0.3, 0.1 and 0.03 g/mL intragastrically) to rats for 7 consecutive days. We then performed transient occlusion of the middle cerebral artery, followed by reperfusion, and administered CG daily for a further 3 or 7 days. Compared with no treatment, high-dose CG markedly improved neurological function assessed using the Bederson and Garcia scales. At 3 days, animals in the high-dose CG group had smaller infarct volumes, greater interleukin-10 expression, and fewer interleukin-1β-immunoreactive cells than those in the untreated model group. Furthermore, at 7 days, high-dose CG-treated rats had more vascular endothelial growth factor-immunoreactive cells, elevated angiopoietin-1 and vascular endothelial growth factor expression, and improved blood coagulation and flow indices compared with untreated model animals. These results suggest that CG exerts specific neuroprotective effects against cerebral ischemia/reperfusion injury.

Key Words: *nerve regeneration; Cerebralcare Granule; cerebral ischemia/reperfusion; angiogenesis; inflammatory response; blood coagulation; hemorheology; neural regeneration*

Introduction

Cerebralcare Granule (CG) is a compound Chinese medicine that improves brain microcirculation, relieves vasospasm, and is usually used to treat dizziness, headache, chronic cerebral circulation insufficiency, hypertension and insomnia (Wang et al., 2013; Qu et al., 2014). The major components of CG are *Radix Angelica Sinensis* (Qu et al., 2014), *Tetramethylpyrazine, Radix Paeoniae Alba* (Xu et al.,

2009), *Radix Rehmanniae Preparata, Concha Margaritifera Usta, Spica Prunellae, Herba Asari, Rhizoma* (Wang et al., 2012), *Ramulus Uncariae cum Uncis, Caulis Spatholobi* and *Semen Cassiae*. The active components of CG comprise a number of acids (protocatechuic, caffeic, chlorogenic, rosmarinic, and ferulic) and paeoniflorin (Wang et al., 2013). These substances mainly exist in *Radix Angelica Sinensis, Tetramethylpyrazine* and *Radix Paeoniae Alba* (Wang et al., 2013), and all components are detectable in the blood of rats after oral administration of CG.

Prognosis following cerebral ischemia/reperfusion (I/R) injury is improved by promoting angiogenesis, reducing inflammation, inhibiting blood coagulation and reducing blood viscosity (He et al., 2007; Cuenca-López et al., 2010). Despite the beneficial effects of CG on the cerebrovascular system, reports addressing its effects on focal cerebral I/R injury are lacking, and its mechanisms remain elusive. The aim of the present study was to determine the effects of CG on angiogenesis, inflammation, hemorheology and coagulation, neurological function, and infarct area after cerebral I/R injury. We established rat models of transient middle cerebral artery occlusion (MCAO) and evaluated the potential of CG as a treatment for acute cerebral infarction.

Materials and Methods

Ethics statement

The experimental procedures were approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology, China, performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and reported following the ARRIVE guidelines (Kilkenny et al., 2010). Precautions were taken to minimize suffering and the number of animals used in each experiment.

Experimental groups

125 male specific-pathogen-free Sprague-Dawley rats, 40–50 days old and weighing 200–240 g, were purchased from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China (license No. SCXK 2010-0009). The rats were allowed free access to standard food and water and were maintained under a 12-hour light/dark cycle in temperature- and humidity-controlled rooms. The rats were randomly divided into five groups: sham, I/R, 0.3 g/mL CG, 0.1 g/mL CG, and 0.03 g/mL CG (n = 25 per group).

Establishment of MCAO models

The intraluminal filament technique was used to establish models of MCAO (Ma et al., 2013) with slight modifications. Rats were anesthetized with 10% chloral hydrate (3 mL/kg, intraperitoneally). The right internal, external and common carotid arteries and vagus nerve were isolated *via* a midline incision in the skin. The common carotid artery was threaded with three suture lines. One suture was used to ligate the proximal end of the common carotid artery. Another suture at the distal end was pulled tight using hemostatic forceps to prevent bleeding. A bulldog clamp was used to obstruct the external carotid artery. Polylysine-coated MCAO monofilament (Beijing Sunbio Biotech Co., Ltd., Beijing, China; the tape is 2432-A4), was inserted through a small incision in the common carotid artery. The third suture was placed in the middle of the common carotid artery and tied gently to prevent bleeding. The monofilament was advanced gently into the internal carotid artery, to a point approximately 18 mm distal to the carotid bifurcation. The bulldog clamp was removed, the common carotid artery was ligated, and the incision was closed. Reperfusion was achieved by slowly pulling the monofilaments back after 2 hours of occlusion. A similar procedure was carried out in the sham-operated rats, but without the use of monofilaments.

Drug administration

CG (batch No. 121145; Tasly Pharmaceutical Co., Ltd., Tianjin, China) was dissolved in saline to 0.3, 0.1 and 0.03 g/mL and administrated by gavage in a volume of 10 mL/kg for 7 days before I/R. After surgery, the rats continued receiving CG, 2 hours after reperfusion, and once daily thereafter for 3 or 7 days. The rats in the sham and I/R groups received normal saline on the same schedule.

Neurological function assessment

Neurological function was assessed 3 hours after I/R, and daily thereafter for 7 days, using the Bederson scale (0–3; Bederson et al., 1986) and the Garcia scale (0–18; Garcia et al., 1995). In the Bederson scale, the higher the score, the greater the degree of impairment was, whereas in the Garcia scale, lower scores indicate greater impairment. Rats that were unconscious or that had died during the period of observation were removed from the experiment. To increase the homogeneity of the models, we removed rats with no functional damage and those that recovered fully within the first 24 hours after surgery. Our success rate for obtaining 125 satisfactory rat models was approximately 60%.

Measurement of infarct volume

Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) staining. Three days after I/R, five rats from each group were euthanized, decapitated and the brains removed rapidly and cut into five coronal slices (2 mm thick). The slices were placed in a Petri dish containing 1% TTC for 15–20 minutes at 37°C. The tissue was then fixed in 4% formaldehyde for 24 hours and photographed using a digital camera (Nikon S6200, Tokyo, Japan). The infarct areas were measured using Photoshop CS5 v12.0 (Adobe Systems Inc, San Jose, CA, USA). The infarct volume was calculated by multiplying the area of unstained tissue by the slice thickness (2 mm), and expressed as a percentage of the contralateral hemisphere, as follows: infarct volume (%) = (area of the contralateral hemisphere − area of the normal part in the ipsilateral hemisphere)/area of the contralateral hemisphere × 100%.

Immunohistochemistry

Three days (for interleukin-1β, IL-1β) or seven days (for

Table 1 Effect of CG on Bederson score following I/R

	Time after surgery							
Group	3 hours	1 day	2 days	3 days	4 days	5 days	6 days	7 days
CG								
0.3 g/mL	2.24±0.72	1.72±0.83	1.24±0.68*	1.00±0.58*	0.90±0.53*	0.70±0.56*	0.60±0.52	0.60±0.53
0.1 g/mL	2.48±0.71	1.88±0.83	1.40±0.67	1.24±0.50	1.00±0.50*	0.90±0.53	0.70±0.47	0.70±0.47
0.03 g/mL	2.48±0.79	1.84±0.84	1.44±0.69	1.20±0.53	1.20±0.53	1.00±0.54	1.00±0.48	0.90±0.48
I/R	2.52±0.85	2.08±0.85	1.76±0.72	1.60±0.55	1.60±0.55	1.60±0.55	1.40±0.49	1.40±0.49

Higher scores indicate more severe impairment. Data are expressed as the mean ± SD (n = 15 for 0–3 days and n = 10 for 4–7 days). *P < 0.05, *vs.* I/R group (one-way analysis of variance followed by Dunnett's or the least significant difference test). CG: Cerebralcare Granule; I/R: ischemia/reperfusion.

Table 2 Effect of CG on modified Garcia score following I/R

	Time after surgery							
Group	3 hours	1 day	2 days	3 days	4 days	5 days	6 days	7 days
CG								
0.3 g/mL	13.16±2.50*	14.24±2.65*	14.44±2.67*	14.72±0.73	15.5±3.57*	15.70±3.65*	16.20±3.65	16.40±3.71*
0.1 g/mL	12.76±2.49	13.60±2.65	13.96±2.68	14.24±2.73	14.90±3.58	15.20±3.66	15.70±3.66	15.90±3.72
0.03 g/mL	12.56±3.14	13.42±3.30	13.44±3.40	13.64±3.45	14.00±4.40	14.70±4.50	15.00±4.56	15.30±4.63
I/R	12.56±3.63	13.04±3.77	13.28±3.86	13.44±3.92	13.7±4.80	14.00±4.89	14.50±5.00	14.60±5.07

Lower scores indicate more severe impairment. Data are expressed as the mean ± SD (n = 15 for 0–3 days and n = 10 for 4–7 days). *P < 0.05, *vs.* I/R group (one-way analysis of variance followed by Dunnett's or least significant difference test). CG: Cerebralcare Granule; I/R: ischemia/reperfusion.

Table 3 The number of VEGF- and IL-1β-immunoreactive cells per 400× field

Group	VEGF (7 d after surgery)	IL-1β (3 d after surgery)
Sham	0	0
I/R	37.14±4.53	55.88±11.3
CG		
0.3 g/mL	49.37±6.27*	41.56±4.41*
0.1 g/mL	45.47±5.92*	44.60±6.48
0.03 g/mL	38.78±4.94	51.08±7.33

Data are expressed as the mean ± SD (n = 5 per group). *P < 0.05, *vs.* I/R group (one-way analysis of variance followed by Dunnett's or least significant difference tests). CG: Cerebralcare Granule; VEGF: vascular endothelial growth factor; IL: interleukin; I/R: ischemia/reperfusion; d: days.

Table 4 Effect of CG on blood coagulation function at 7 days after I/R

Group	PT (second)	INR	Fbg (g/L)	TT (second)
Sham	18.36±1.06	1.63±0.04	2.81±0.38	23.01±3.05
I/R	18.21±0.65	1.57±0.07	3.54±0.44#	21.83±3.35
CG				
0.3 g/mL	20.63±1.55*	1.80±0.16*	2.74±0.38*	29.35±3.41*
0.1 g/mL	19.21±0.52*	1.67±0.07	3.15±0.49	25.64±2.77
0.03 g/mL	18.10±0.74	11.56±0.09	3.49±0.70	21.55±3.91

Data are expressed as the mean ± SD (n = 8 for each group). *P < 0.05, *vs.* I/R group; #P < 0.05, *vs.* sham group (one-way analysis of variance followed by Dunnett's or least significant difference tests). PT: Prothrombin time; INR: international normalized ratio; TT: thrombin time; Fbg: fibrinogen; CG: Cerebralcare Granule; I/R: ischemia/reperfusion.

Table 5 Effect of CG on hemorheology 3 days after I/R

	Whole blood viscosity (mPA•s)						
Group	1/s	5/s	50/s	100/s	200/s	Plasma viscosity (mPA•s)	EAI
Sham	24.89±3.76	11.82±1.41	5.02±0.49	4.09±0.35	4.93±0.86	1.31±0.11	5.49±0.88
I/R	31.74±5.31	15.54±1.30#	6.75±0.32#	5.86±0.12#	6.26±0.19#	1.41±0.12	6.11±0.99
CG							
0.3 g/mL	23.22±3.22*	10.57±0.94*	4.99±0.12*	4.68±0.05*	4.36±0.55*	1.04±0.10*	5.11±0.54*
0.1 g/mL	24.69±4.38	10.91±1.30*	5.33±0.43*	4.84±0.52*	4.51±0.55*	1.09±0.97*	5.72±0.36
0.03 g/mL	31.98±4.65	14.75±0.41	6.58±0.11	5.75±0.49	5.17±0.13*	1.19±0.13*	6.11±1.09*

Data are expressed as the mean ± SD (n = 8 per group). *P < 0.05, *vs.* I/R group; #P < 0.05, *vs.* sham group (one-way analysis of variance followed by Dunnett's or the least significant difference test). EAI: Erythrocyte aggregation index; CG: Cerebralcare Granule; I/R: ischemia/reperfusion.

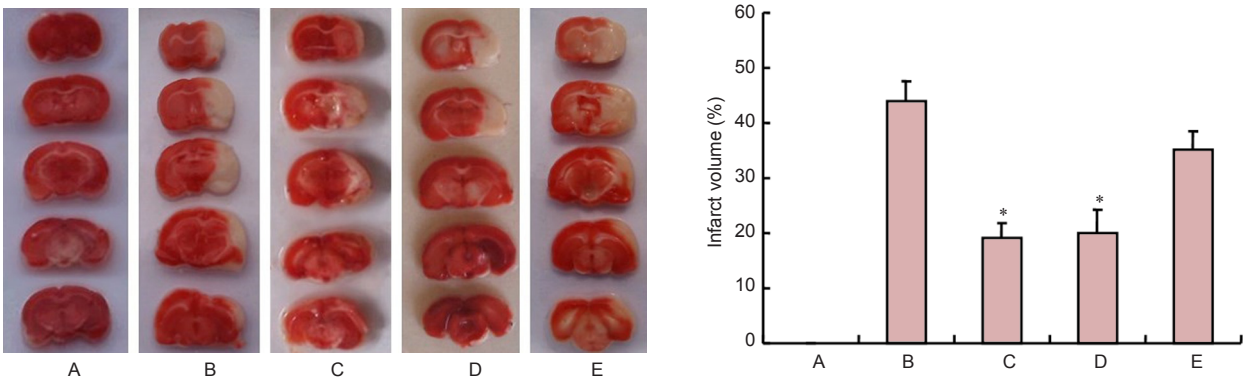


Figure 1 Effect of CG on infarct volume 3 days after I/R (2,3,5-triphenyltetrazolium chloride staining).
White regions in the coronal sections represent cerebral infarct. Data are expressed as the mean ± SD (n = 5 per group). *P < 0.05, *vs.* I/R group (one-way analysis of variance followed by Dunnett's or least significant difference tests). A: Sham group; B: I/R group; C: 0.3 g/mL CG group; D: 0.1 g/mL CG group; E: 0.03 g/mL CG group; CG: Cerebralcare Granule; I/R: ischemia/reperfusion.

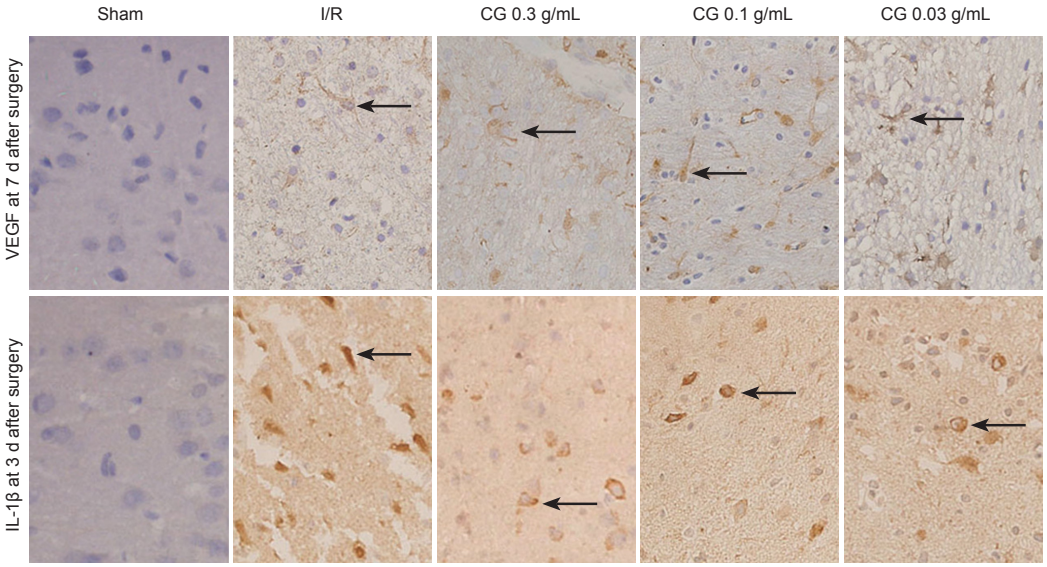
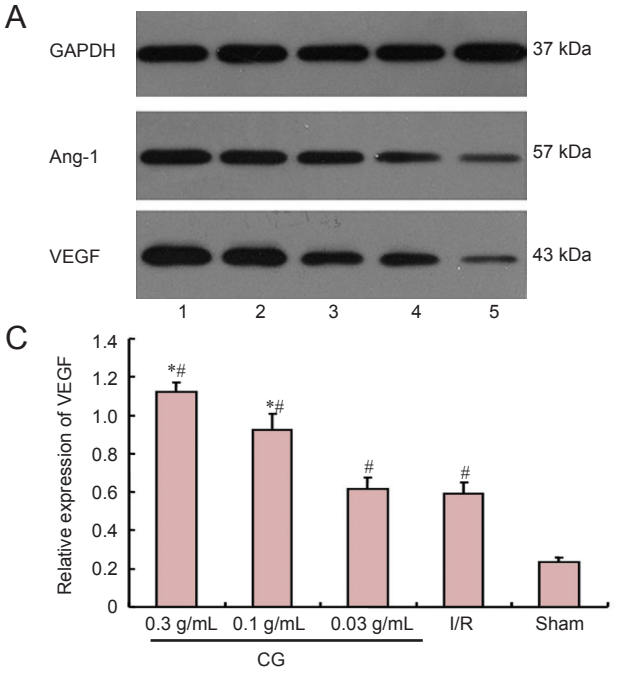


Figure 2 Effect of CG on VEGF and IL-1β immunoreactivities after I/R (immunohistochemistry, × 400).
VEGF- and IL-1β-immunoreactive cells were observed mainly in the peri-infarct regions on the ischemic side. Most were glial-, epithelial- and neuron-like cells. Strong IL-1β-immunoreactivity was visible mainly in the cytoplasm. Arrows indicate VEGF- or IL-β-immunoreactive cells. CG: Cerebralcare Granule; VEGF: vascular endothelial growth factor; IL: interleukin; I/R: ischemia/reperfusion; d: days.

vascular endothelial growth factor, VEGF) after I/R, five rats from each group were perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde overnight and then embedded in paraffin. Coronal brain sections (4 μm) were cut for immunohistochemical staining using the standard avidin-biotin-peroxidase complex method. Sections were incubated with rabbit anti-VEGF (1:200; Abcam, Cambridge, UK) or anti-IL-1β (1:200; Santa Cruz Biotechnology, Dallas, Texas, USA) polyclonal antibodies at 4°C overnight, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200; KPL, Gaithersburg, Maryland, USA) at 4°C for 1 hour. Proteins were visualized using 3,3′-diaminobenzidine and counterstained with hematoxylin. Negative controls were processed in parallel, but the primary antibody was replaced with phosphate-buffered saline. VEGF- and IL-1β-immunoreactive cells were counted in five representative fields of view per section, under an optical microscope at 400× magnification (Olympus, Tokyo, Japan) using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA).

Western blot assay

Seven days after I/R, cerebral cortices were collected and homogenized in lysis buffer. Equal amounts of proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding was blocked by soaking the membranes in 5% non-fat milk for 1 hour, before incubating them with rabbit anti-angiopoietin-1 (Ang-1; 1:200; Abcam) or anti-VEGF (1:200; Abcam) polyclonal antibodies overnight at 4°C. Rabbit anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (1:1,000; Abcam) served as the control. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200; KPL) at room temperature for 30 minutes. Immunoactive bands were visualized using an enhanced chemiluminescence detection system and X-ray film. Optical density of the bands was calculated using Quantity One software (Bio-Rad, Hercules, CA, USA). Expression of the target protein was represented by the optical density ratio of target protein to GAPDH.



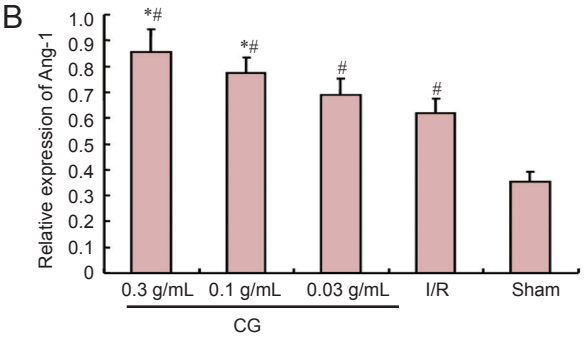


Figure 3 Effect of CG on Ang-1 and VEGF expression after I/R (western blot assay).
(A) Western blot assay for Ang-1 and VEGF. 1: Sham group; 2: I/R group; 3: CG 0.3 g/mL group; 4: CG 0.1 g/mL group; 5: CG 0.03 g/mL group. (B, C) Quantification of the optical density for Ang-1 and VEGF, normalized to GAPDH. Data are expressed as the mean ± SD (n = 5 per group). *P < 0.05, *vs.* I/R group; #P < 0.05, *vs.* sham group (one-way analysis of variance followed by Dunnett's or least significant difference tests). CG: Cerebralcare Granule; Ang-1: angiopoietin-1; VEGF: vascular endothelial growth factor; I/R: ischemia/reperfusion; GAPDH: glyceraldehyde phosphate dehydrogenase.

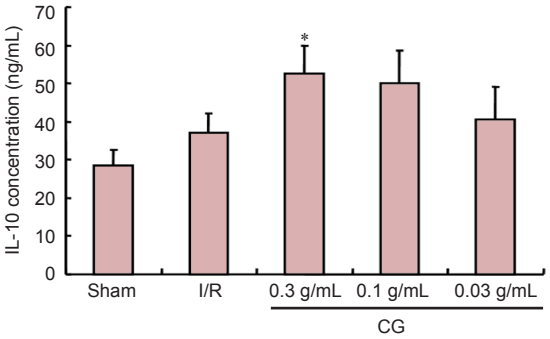


Figure 4 Effect of CG on the expression of IL-10 in the ischemic cerebral cortices after I/R (ELISA).
Data are expressed as the mean ± SD (n = 5 per group). *P < 0.05, *vs.* I/R group (one-way analysis of variance followed by Dunnett's or the least significant difference test). CG: Cerebralcare Granule; IL: interleukin; I/R: ischemia/reperfusion; ELISA: enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay (ELISA)

Three days after I/R, five rats from each group were decapitated and the brains removed. Ischemic cerebral cortices were harvested and homogenized. The supernatant was collected and IL-10 levels were measured using anti-rat IL-10 ELISA kits (Jingmei Biotech., Shenzhen, Guangdong Province, China). The results were representative of at least three independent experiments.

Blood coagulation and hemorheology measurements

Three days after I/R, eight rats from each group were anesthetized, the chest and heart exposed, and 4 mL of blood taken from the heart and placed in heparin-lithium anticoagulant tubes for automated hemorheology (Succeeder, Beijing, China). Seven days after I/R, 2 mL of blood was taken in the same way from the remaining eight rats from each group, and added to sodium citrate anticoagulant tubes for automated blood coagulation analysis (Stago, Paris, France).

Statistical analysis

All data are expressed as the mean ± SD and analyzed using SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Means were compared by one-way analysis of variance followed by Dunnett's test for unequal variances, or the least significant difference test for normally-distributed data. A value of P < 0.05 was considered statistically significant.

Results

CG improved neurological function after I/R

Bederson scale

No neurological deficits were observed in sham-operated rats, and rats in the I/R group showed poor neurological function as expected. In the CG treatment groups, rats showed improved functional outcomes compared with the I/R group; in particular, the 0.3 g/mL CG group had significantly lower Bederson scores than the I/R group at 2–5 days after I/R (P < 0.05). Similarly, rats in the 0.1 g/mL CG group had significantly lower scores than the I/R group at 4 days after surgery (P < 0.05; **Table 1**).

Modified Garcia score

Sham-operated rats showed no neurological deficits. Rats in the 0.3 g/mL CG group had significantly higher Garcia scores than the I/R group at all but one time points (**Table 2**).

CG reduced brain infarct volume in I/R rats

No infarction was observed in the sham-operated rats, and the I/R group showed a large region of infarction. Infarct volumes in the 0.3 and 0.1 g/mL CG groups were significantly smaller than in the I/R group (**Figure 1**).

CG increased whole brain VEGF and IL-1β expression after I/R

The number of VEGF-immunoreactive cells was significantly greater in the 0.3 and 0.1 g/mL CG groups, and the number of IL-1β-positive cells significantly lower in the 0.3 g/mL CG group, than in the I/R group (**Figure 2**; **Table 3**).

CG increased Ang-1 and VEGF expression in ischemic cerebral cortex

Western blot assay showed that protein levels of Ang-1 and VEGF 7 days after I/R were significantly higher in all model groups than in the sham group. Furthermore, Ang-1 and VEGF protein levels in the 0.3 and 0.1 g/mL CG groups were significantly higher than those in the I/R group (**Figure 3**).

CG increased IL-10 levels in the ischemic cerebral cortex after I/R

ELISA showed that IL-10 protein levels of the 0.3 g/mL CG group were significantly greater than in the I/R group 3 days after surgery. IL-10 protein levels of the I/R group were greater than in the sham group, but the difference did not reach statistical significance ($P > 0.05$; **Figure 4**).

CG improved blood coagulation function after I/R

Seven days after I/R, plasma prothrombin time, international normalized ratio and thrombin time were significantly greater, and fibrinogen concentration significantly lower, in the 0.3 g/mL CG group than in the I/R group. Prothrombin time was markedly higher in the 0.1 g/mL CG group than in the I/R group. The fibrinogen concentration was higher in the CG 0.3 g/mL group than in the sham group (**Table 4**). Prothrombin time, international normalized ratio and thrombin time in the 0.3 and 0.1 g/mL CG groups were higher than in the sham group, but the difference did not reach statistical significance.

CG improved hemorheology after I/R

Whole blood and plasma viscosities in the CG groups were significantly greater than in the I/R group 3 days after I/R. The erythrocyte aggregation index was significantly lower in the 0.3 g/mL CG group than in the I/R group, and the whole blood viscosity was greater than in the sham group. Plasma viscosity and erythrocyte aggregation index were greater in the I/R groups than in the sham group, but the results did not reach statistical significance (**Table 5**).

Discussion

The mechanism of cerebral I/R injury is complex; contributing factors include mitochondrial dysfunction, oxidative stress, inflammation, blood-brain barrier damage, brain edema, blood coagulation and hemorheology dysfunction, angiogenesis, and neural stem cell regeneration. In the focal cerebral I/R injury rat model, CG administration protects the blood-brain barrier and reduces brain edema (Zhang et al., 2013), attenuates cerebral microcirculatory disturbances, and ameliorates apoptosis in hippocampal CA1 neurons (Shehadah et al., 2010). Here, we explored the possible mechanisms underlying the neuroprotective effects of GC after focal cerebral I/R injury, using a rat model of transient MCAO.

We used an improved procedure in which a nylon monofilament was inserted through the right common carotid artery with the help of three suture lines. This technique involves less manipulation and results in less bleeding compared with inserting the monofilament through the right external carotid artery. We used a bulldog clamp to obstruct the external carotid artery, a less traumatic procedure than the commonly used method of isolating and ligating the external carotid artery.

Angiogenesis plays a vital role in functional recovery after stroke. VEGF and angiopoietin are essential factors for angiogenesis. VEGF increases microvascular permeability (Zheng et al., 2011). Members of the angiopoietin family, including Ang-1 and Ang-2, act by binding to the tyrosine kinase receptor Tie-2. Ang-1 promotes the maturity and stability of blood vessels, while Ang-2 competitively inhibits this effect, reducing the stability of blood vessels and promoting budding (Zan et al., 2011). Angiogenesis aids recovery of neurological function after cerebral ischemia. VEGF protein expression increases during the first 6 hours after I/R, peaking at 12 hours and remaining high for the following 12 hours, then decreasing slightly but remaining elevated until 14 days after ischemia (Zan et al., 2011). VEGF-positive cells are primarily located in the ischemic penumbra region and present mainly as glial-like and neuron-like cells. Ang-1 protein expression decreases to a minimum at 1 day, and then increases gradually until 7 days after I/R. Ang-1-expressing cells are also primarily located in the ischemic penumbra region and present as glial-like, endothelial-like and neuron-like cells (Zan et al., 2011). Niacin, a B vitamin, increases the expression of VEGF and PI3K/AKT after I/R in rats, compared with non-treated I/R controls (Shehadah et al., 2010). In the present study, most VEGF immunoreactivity was found in glial-like, endothelial-like and neuron-like cells in the ischemic penumbra. Our results also suggest that CG promotes angiogenic factors, such as VEGF and Ang-1, after cerebral I/R.

Inflammation is involved in the mechanism of cerebral I/R injury and is an important factor influencing prognosis. Microglial cells are activated quickly after cerebral I/R, which stimulates the synthesis and release of a series of cytotoxins, inflammatory mediators and cytokines such as IL-1β, tumor necrosis factor-α and IL-10. IL-1β is considered to be an initiating factor for the inflammatory cascade, increasing the expression of adhesion molecules and participating in the inflammatory response after ischemia together with tumor necrosis factor-α, interferon-γ and nuclear factor-κB (Fan et al., 2012). IL-10 is the cytokine that inhibits the inflammatory response. An increase and decrease in pro- and anti-inflammatory cytokines, respectively, is associated with poor prognosis after massive cerebral infarction. Liu et al. (2013) reported that IL-1β levels are elevated in ischemic brain tissues and serum after cerebral infarction, but that ginsenoside Rb1 reduced this overexpression (Liu et al., 2013). Liu et al. (2009) demonstrated in rats that the expression of IL-10 mRNA and protein was elevated at 1

and 7 days after cerebral infarction, and that bone marrow mesenchymal stem cells which were injected into the lateral ventricle upregulated IL-10 expression at both time points (Liu et al., 2009). In the present study, we observed that CG could upregulate IL-10 and downregulate IL-1β expression after I/R. Poor blood coagulation and platelet hyperfunction are important mechanisms of thrombosis, consistent with our results.

Poor blood coagulation and hemorheology play an important role in the development and pathogenesis of atherosclerosis and cerebral infarction (Zhu et al., 2013). Our results demonstrate that CG improves blood coagulation and blood flow parameters after cerebral I/R. We chose two widely used scales for the assessment of neurological function. In rats, the Bederson score is used to evaluate motor function, and the improved Garcia score describes both sensory and motor function. Compared with functional assessment, TTC staining is a direct measure of infarct volume. In our experiment, the infarct volumes and neurological function scores were consistent with each other. CG improved neurological defects and decreased the cerebral infarction size in rats after focal cerebral I/R.

In summary, our results indicate that CG improves neurological defects, promotes angiogenic factors, regulates inflammatory mediators, and improves hemorheology and coagulation indices after focal cerebral I/R. CG is an effective adjuvant therapy in the treatment of cerebral infarction. However, its specific mechanism warrants further study. We plan to quantify new vessels and inflammatory cells to further investigate the effects of CG on angiogenesis and inflammation. In addition, the actions of each contributing component in the CG compound, and any synergistic effects between them, remain unclear. Finally, the effect of CG on neural stem cell regeneration after focal I/R also remains to be determined.

Author contributions: *XXZ, GLY and YLM participated in animal surgery and data processing. DL and NX were responsible for immunohistochemistry and statistics. XXZ and GLY were in charge of western blot assay and statistics. GLY and FW performed the test of blood coagulation function and hemorheology. XXZ and FC had full access to all data and participated in analysis of both data integrity and data accuracy. FC, FFH, XXZ and HNL participated in study design, study supervision and paper preparation. All authors approved the final version of the manuscript.*

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