Calycosin-7-O- β -D-Glucoside Treatment Promotes Axonal Regeneration via Rho/ROCK Pathway After Ischemia/Reperfusion Injury

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Research

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Calycosin-7-*O*- β -D-glucoside treatment promotes axonal regeneration via Rho/ROCK pathway after ischemia/reperfusion injury

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Abstract

Background: As a medical component in Astragalus(AR), Calycosin-7-*O*- β -D-glucoside (CG) defends ischemia/reperfusion(I/R) injury in cerebral ischemia due to its anti-oxidative and anti-inflammatory effects. However, whether CG can facilitate I/R injury by stimulating neuroregeneration and its specific mechanism is remained to be elucidated.

Methods: In this study, an animal model of ischemic stroke was established by middle cerebral artery occlusion (MCAO). Seven days after CG, triphenyltetrazolium chloride (TTC) staining was performed to examine the ischemic volume, accompanied by behavioral tests to assess neurological function. Nissl staining and Bielschowsky's silver staining were used to observe nerve cell damage and axonal loss, while immunofluorescence was used to evaluate axonal regeneration.

Results: The expression of proteins associated with the Rho/ROCK pathway was detected by using western blot (WB) and quantitative real-time polymerase chain reaction (qRT-PCR). We showed that CG significantly reduced ischemic volume, facilitated axonal regeneration, improved neurological function, and regulated expression of RGMa, Rho, ROCK, and CRMP2.

Conclusions: Our results suggested that CG promotes axonal regeneration by limiting activation of the Rho/ROCK pathway to promote recovery after cerebral ischemia.

Keywords: Calycosin-7-*O*- β -D-glucoside, ischemia/reperfusion, Rho/ROCK pathway, axonal regeneration

1. Introduction

Ischemic stroke (IS) is a rapidly-developing brain disorder that accounts for the vast majority of strokes [1]. In the early stages of stroke, brain damage is often accompanied by impaired axonal atrophy resulting in neurotransmission defects. Therefore, stroke patients commonly have disabilities that involve severe language, cognitive, and physical dysfunctions [2]. Exercise rehabilitation training for IS patients or animal models can fix the nerve fibers that control muscle movements, suggesting that promoting prolongation of atrophic axons can significantly improve recovery of neurological function [3].

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The Ras homolog gene /Rho-associated protein kinase (Rho/ROCK) pathway is involved in maintaining microtubule and cytoskeletal stability, which is important for growth cone formation during axonal regeneration [4]. Surface receptors of the growth cone can recognize various growth signals received in the neurons and guide the axon to extend in the correct direction. Repulsive guidance molecule a (RGMa) is a potent inhibitor of axonal regeneration that is expressed in the developing and mature central nervous system. After a stroke, up-regulated RGMa binds to the specific surface receptor on the growth cone and activates the Rho/ROCK pathway, eventually destabilizing the cytoskeleton and causing the growth cone to collapse [5]. Inhibiting RGMa or treatment with ROCK inhibitors can stimulate recovery of motor function, which is possibly due to the promotion of axonal regeneration [6, 7]. Therefore, the inhibition of axonal regeneration is dependent on activation of this signaling pathway.

As a basic herb in traditional Chinese medicine, AR has been proven to have various bioactivities. Such as anti-inflammatory、anti-oxidative、immunoregulatory and certain neuroregeneration effect et.al [8, 9, 10]. CG is a compound of Flavonoids [11], which is mainly exist on *Leguminosae sp.*, as a medicinal ingredient of AR. Our previous researches had confirmed that CG can reduce oxidative stress and neuronal apoptosis[12], and improved expression of GAP43 in PC12 cell oxygen-glucose deprivation/reperfusion (OGD/R) model, suggesting that CG can promote axonal regeneration.[13].Nevertheless, how CG exert protection for I/R injury is unclear. This shows CG is a promising candidate in stimulating neuroregeneration for I/R injury treatment.

In this study, we established an animal model of middle cerebral artery occlusion (MCAO) to investigate the effects and mechanism of CG on improving axonal regeneration and functional outcomes after I/R injury. We demonstrated that CG promoted axonal regeneration and neurological recovery after IS by inhibiting Rho/ROCK pathway. Our study provides insight into the mechanism for promoting stroke rehabilitation and empirical evidence for its use in the clinic.

2. Materials and Methods

2.1 Drugs and chemicals

CG is isolated from the ethanol extract of AR, with a purity greater than 98% detected by high pressure liquid chromatography (Figure 1), provided by Chengdu Chroma-Biotechnology Co., Ltd (Chengdu, China). Hydrochloride Fasudil (HF) was supplied by Neuraxpharm (German). Triphenyltetrazolium chloride (TTC) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Hematoxylin and Eosin (HE) Staining Kit, Nissl Staining Kit and BCA Kit, Radio immunoprecipitation assay (RIPA) buffer and antifade mounting medium were purchased from Beyotime Biotechnology (Shanghai, China). Optimal cutting temperature compound (OCT) was obtained from Sakura Finetek USA Inc. (Torrance, USA). Primary antibody against NF-200, GAP43, RGMa and Rho were supplied by Abcam (Cambridge, UK); MAP2 and MLC2 were from CST (Boston, USA); GFAP, CSPG4, CSPG5, ROCK, p-MLC2, CRMP2, p-CRMP2, β -actin were from Affinity (Cincinnati, OH, USA). Alexa Fluor 594, FITC and DAPI were obtained from Affinity (Cincinnati, OH, USA). Glodenstar™ RT6 cDNA Synthesis Mix Kit and 2× T5 Fast qPCR Mix (SYBR Green I) Kit were from Tsingke Bio-Technology (Guangzhou, China).

Figure 1. structure and chemical formula of CG

2.2 Animals and induction of I/R injury

Sprague-Dawley rats (male, 250-280 g, permission number: SCXK, Cantonese, 2013-0034) provided by the Experimental Animal Centre of Guangzhou University of Chinese Medicine (Guangzhou, China). Rats were raised in animal rooms with suitable temperature and humidity with a 12 h-light and dark cycle for seven days to acclimatize the rats to laboratory conditions. Animal care was conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised in 1978).

The animals were fasted for 12 h, but allowed to drink water ad libitum before middle cerebral artery occlusion (MCAO) was established using a modified version of the method published by Longa et al. [14]. Briefly, the rats were fixed on the operating table where covered with electrical heating blankets to keep the anus temperature at 37°C after anesthesia. The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed, so the CCA and ECA were ligated to prevent bleeding. After making a small cut in the distal region of CCA, a 2.66 mm nylon filament suture, coated with polylysine at the head end, was inserted into the ICA along the CCA. This was inserted until the 18~20 mm marker reached the fork, blocking blood flow of the middle cerebral artery for 2 h. Penicillin was used to prevent infection. Animals in the sham group were treated similarly, except the nylon filament suture used to block blood flow.

2.2 Experimental groups and drug administration

Neurological function in the rats after recovery from anesthesia was assessed to eliminate unsuccessful modeling rats using a modified version of the protocol published by Bederson et al. [15]. Rats with scores between 1 to 3 points were randomly grouped to ensure the consistency of neurological deficit in the groups. Rats were randomly divided into six groups with twenty rats each for intraperitoneal injection: sham control, I/R, CG low-dose (CG-L, 15 mg/kg), CG middle-dose (CG-M, 30 mg/kg), CG high-dose (CG-H, 60 mg/kg) and HF (positive drug control, 10 mg/kg). CG and HF was dissolved with DMSO and diluted by sterilized normal saline containing 0.5% Tween-20. The animals were administered with the drugs or vehicle (sham and I/R groups) daily for 7 days.

2.3. Neurological function scoring

Behavioral testing was performed at day 1, 3, 5 and 7 after the MCAO procedure to assess the effect of CG on neurological impairment. For modified neurological severity scoring (mNSS), neurological function scores with regards to spontaneous activity, movement of the four limbs, forepaw extension, climbing, response to touch, and vibrissal touch were obtained for each group according to a method published by Garcia[16]. The final score was the total score of the six evaluation items, with a maximum score of 18 points, and a minimum score of 3 points. A lower score is associated with more severe neurological damage.

For balance beam test (BBT), was aiming to assess motor balance and coordination. The narrow beam (80cm long wooden beam, 1.5cm wide) was used for the experiment. The beam was placed 15cm above the soft platform. Rats should be trained every day, enabling them to pass the beam smoothly before making the model. Total score of BBT was 6-point [17], the higher the final score, the more severe the damage represented.

2.4. TTC staining

The rats were anesthetized and sacrificed, then brains were quickly removed and frozen at -20°C for 30 min. Each brain tissue was cut into 6 pieces which were then immersed in 2% TTC solution in the dark at 37°C for 30 min. After the brain slices were removed from TTC and washed with phosphate buffered saline (PBS, pH 7.4), the tail side of each slice was taken for image analysis using Image J software (Rawak Software, Inc. Germany). The total infarct volume and the infarct rate were calculated as described in a previous study [18], and the formula for calculating the infarct rate is: $(\text{volume of non-infarcted hemisphere} - \text{volume of non-infarcted volume in infarcted hemisphere}) / \text{volume of non-infarcted hemisphere} \times 100\%$

2.5. Brain water content

After the last administration, the brain was quickly removed and was made into a coronal section, each about 3 mm thick, and immediately weighed with an electronic balance to obtain the wet weight of the brain. The brain slices were placed on the baked tin foil, dried in an oven at 102°C for 12 h, and weighed to obtain the brain stem weight. The calculation formula of brain water content is as follows: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$

2.6. Pathological assessment

After 7 days of dosing, brain tissue and nerve cell damage were evaluated by HE staining and Nissl staining. Briefly, the animals were anesthetized and perfused with cold saline followed by 4% paraformaldehyde. The brain tissues were then quickly removed and frozen. Frozen tissues were sliced into 10 µm sections with a freezing microtome (Thermo, USA) post-fixation. The sections were washed with distilled water and stained with a HE Staining Kit or Nissl Staining Solution according to the manufacturer's instructions. The cortex and hippocampus of the infarcted hemisphere were imaged on a microscope (Olympus, Japan), and neurons with a clear nucleolus and a sufficient number of endoplasmic reticulum were analysed by Image J software [19].

2.7. Bielschowsky's silver staining (BSSM)

Axonal degeneration was evaluated by BSSM. The frozen sections were immersed in AgNO₃ solution, then Glee's solution, followed by conventional dehydration, transparency, and mounting using a modified protocol by Segura-Anaya et al. [20]. A semi-quantitative method was used to evaluate axonal loss according to Zeinali et al. [21]: 0 = no loss, 1 = superficial loss of a few lesions containing less than 25% of tissue, 2 = deep axonal loss of lesions including more than 25% of tissues, 3 = diffuse and extensive axonal loss including more than 50% of tissues.

2.8. Immunofluorescence (IF)

Immunofluorescence staining of NF-200, MAP2 and GAP43 was used to assess the growth of neuritis; GFAP and CSPGs to analyse the inhibition of axons. Frozen sections were washed with PBS, followed by incubation with 0.30% Triton X-100. The brain sections were treated with the following steps: washed by PBS, blocked with 10% goat serum, and incubated overnight at 4°C with primary antibodies NF-200 (1:50), MAP2 (1: 250), GAP43 (1: 500), GFAP (1:100), CSPG4 (1:100) and CSPG5 (1:100). On the next day, the sections were washed with PBS containing 0.1% tween-20 (PBST), and then incubated with the secondary antibodies Alexa Fluor 594 (1: 200) or FITC (1: 250). Sections were then stained with DAPI (5 µg/mL). The slices were mounted with an anti-queching media with an anti-fluorescence quencher and observed under a laser confocal microscope (Carl Zeiss, Germany).

2.9. Western blot (WB)

Proteins extracted from brain tissue samples were fully cleaved by radio immunoprecipitation assay (RIPA) buffer. The samples were centrifuged and the amount of protein in the supernatant was measured using a BCA kit. The total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with Tris-buffered saline and Tween-20 (TBST) containing 5% skim milk, the membranes were incubated overnight at 4°C with RGMa (1:15000), Rho (1:2500) and CRMP2 (1:5000) primary antibodies. β-actin (1:1000) was used as the loading control. On the following day, the membranes were incubated with IgG-HRP goat anti-rabbit secondary antibody (1:5000). Proteins on membranes were visualized using an ECL reagent kit and a developer (Tanon, China). The density of each band was analysed by Image J software.

2.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

After 7 days of CG administration, total mRNA was extracted from brain tissues with TRIzol and cDNA was generated with GlodenstarTM RT6 cDNA Synthesis Mix Kit. The qRT-PCR reaction was performed using a 2× T5 Fast qPCR Mix (SYBR Green I) Kit. mRNA expression levels were analysed using the relative quantification method ($2^{-\Delta\Delta C_t}$) with β-actin as an internal control. Primer sequences are shown in Table 1.

Table 1: Sequences of primers

	Primer sequences (5'-3')	Length of product
β-actin	F: AGGGAAATCGTGCGTGACAT R: GAACCGCTCATTGCCGATAG	150 bp
RGMa	F: TCCAGACATGTAAGGTGCAA R: ACTTTCTGGTCCACACACTCT	160 bp
Rho	F: TATTGAAGTGGACGGGAAGCA R: AACTATCAGGGCTGTCGATGGA	140 bp
ROCK	F: GCTCAAGACATGCTCAATCA R: ACATGGCAACAGACTTTGC	178 bp
CRMP2	F: ACCAACGCAGCCAAAGTCTT R: GAGCACTGTTGTGCGTCTTG	130 bp

2.11. Data analysis

All experimental statistics were analysed using SPSS 22.0 software (IBM, USA) and GraphPad Prism 7.0 software (GraphPad Software, USA). One-way analysis of variance (ANOVA) was used to determine significance between more than two groups of data, while the student's *t*-test was used between two groups. The results were displayed as means $\pm$ standard deviation (SD), and a *P* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. CG promotes neurological recovery after I/R injury

The neurological function scores of the sham, I/R, CG, and HF groups are summarized in Figure 2. A higher mNSS score was achieved in the rats that received CG-H and HF by day 3 ($*P < 0.05$), while no obvious differences in BBT were observed among the I/R, CG, and HF groups. In contrast to the I/R group, MCAO rats in the CG-M, CG-H and HF group showed higher mNSS score and lower BBT score ($**P < 0.01$) at day 5 and 7. These findings suggested that CG treatment improved neurological deficits, and promoted neurological recovery after stroke.

Figure 2. Effect of CG on neurological function. (A) Analysis of mNSS scores. Rats in the CG group had higher mNSS scores at days 3, 5, and 7 compared to the I/R group ($*P < 0.05$, $**P < 0.01$), $n=5$. (B) Analysis of BBT scores. Rats in the CG group had lower BBT scores at day 5 and 7 compared to the I/R group ($*P < 0.05$, $**P < 0.01$), $n=5$.

3.2. CG reduces cerebral infarction and edema after I/R injury

TTC staining results showed that there were no infarcts in the coronal brain sections in the sham group, while a white infarct area clearly defined in the normal tissue, appeared in the I/R model group (Figure 3A). Treatment with different doses of CG and HF could markedly attenuate cerebral infarction compared to the untreated IS group ($***P < 0.001$, Figure 3B). Brain edema was also remarkably reduced after 7 days of CG and HF administration. The best anti-I/R effect was shown in the CG-H group with the minimum infarction rate ($15.63 \pm 1.63\%$) and cerebral water content ($77.69 \pm 1.65\%$). The results demonstrated that CG was protective against cerebral ischemic injury after stroke.

Figure 3. Images of cerebral infarction and cerebral edema after seven days of CG administration. (A) Image of TTC staining. Non-infarcted areas were stained in red. (B) The relative size of infarct volume (% total volume of the contralateral brain) of each group. Rats from CG and HF group had lower infarct volume compared to the I/R group ($***P < 0.001$), $n=5$. (C) Analysis of cerebral edema of each group. Rats from CG and HF group had lower water content compared to the I/R group ($**P < 0.01$, $***P < 0.001$), $n=5$.

3.3. CG attenuates histology and neuron damage after neurological impairment

Since CG had a positive effect on recovery after stroke, we next used HE and Nissl staining to assess changes in cerebral pathology. The images of HE staining (Figure 4A) showed that the cortical and hippocampal tissues from the I/R group had manifested severe lesions with condensed nucleus, pyramidal cell loss and unclear cell stratification when compared with the sham group. There was obviously less cell body pyknosis and neurons necrosis in different doses of CG treatment.

Damaged neurons can be identified by Nissl body, a basophilic substance found in the cytoplasm of neurons. As shown in Figure 4B, the neurons from the sham group were densely and regularly arranged. Compared with the sham group, there was severe cortical and hippocampal neuronal loss in I/R rats, while tissues from rats post-CG and post-HF treatment showed less tissue damage and reduced neuronal apoptosis. Meanwhile, an increased number of neurons can be observed in the cortex and hippocampus in the CG group (Figure 4C-F).

Figure 4. Images of cerebral pathology after HE and Nissl staining showing histological and neuronal damage in rats sacrificed at day 7 after I/R injury. (A) HE staining images. Scale bar = 50 μ m. (B) Nissl staining images. Scale bar = 50 μ m. (C) Quantitative analysis of cortical neurons. (D) Quantitative analysis of hippocampus CA1 neurons. (E) Quantitative analysis of hippocampus CA3 neurons. (F) Quantitative analysis of hippocampus DG neurons. CG increased the number of nerve cells compared to the I/R group, * P <0.05, ** P <0.01, *** P <0.001, n=5

3.4. CG alleviates axonal degeneration after I/R injury

BSSM can be used to evaluate neurofibropathy in axons because of their argyrophilic properties. Neuronal axons are stained dark brown showing the arrangement of filamentous nerve fibers. Lots of axonal retraction balls were observed (red arrows), along with axonal swelling (yellow arrows) and nerve fiber retraction in the I/R group (Figure 5A). These pathological structures at the synapses in the CG and HF group were improved compared with those of the I/R group. The CG groups also scored lower revealing less axonal loss (Figure 5C), without significant difference in comparison with HF group.

Figure 5. BSSM staining and IF staining in tissues from rats sacrificed at day 7 after I/R injury. (A) Images of neurons after BSSM staining. Treatment with CG promoted nerve fiber elongation, reduced axonal retraction balls (red arrows), and axonal swelling (yellow arrows). Scale bar = 50 μ m. (B) Representative IF images showing NF-200, MAP2, and GAP43 localization and expression. Scale bar = 20 μ m. (C) Semi-quantitative analysis of axonal loss. (D-F) Semi-quantitative analysis of fluorescence intensity of NF-200, MAP2 and GAP43. * P <0.05, ** P <0.01, *** P <0.001, compared to the I/R group, n =5.

3.5. CG facilitates axonal regeneration after I/R injury

IF was used to observe the expression of proteins involved in axonal structure, NF-200, MAP2 and GAP43, reflexes of axonal regeneration. As shown in Figure 5B, expression was easily detected in the sham group, but barely detectable in the I/R model group, suggesting the axonal structure was seriously damaged after I/R injury. Protein expression was significantly increased after 7 days of CG administration (Figure 5B, $***P<0.001$) in a dose-dependent manner. The results of both BSSM and IF analyses suggest that CG promotes axonal regeneration and remodeling during the I/R recovery process.

3.6. CG ameliorate axonal regeneration environment

GFAP is an important material basis for the formation of glial scars, so the proliferation of astrocytes can be determined by observing the expression of GFAP. Down regulation of GFAP in CG-M、CG-H groups (Figure 6B, compared to the I/R group) indicated that CG middle and high dosage can inhibit the formation of glial scars. CSPGs (including CSPG4 and CSPG5) are significantly up-regulated after cerebral ischemia under common situation, forming an inhibitory environment in the extracellular matrix, restricting further axon extension. NG2 in rat is equal to CSPG4 in human. Both CG groups shows that CG can decrease expression of CSPGs (Figure 6C-D), suggesting that CG may improve environment for axon repairment.

Figure 6 IF staining in tissues from rats sacrificed at day 7 after I/R injury. (A) Compared to the I/R group, CG-M、CG-H groups, the expression of GFAP increased significantly ($***P<0.01$). Expression of NG2 and CSPG5 were declined compared to the I/R group. Scale bar

301 = 40 μ m (B-D)Semi-quantitative analysis of fluorescence intensity of GFAP、NG2、CSPG5.
302 * P <0.05, ** P <0.01, *** P <0.001,compared to the I/R group,n=5

303 **3.7. CG promotes axonal regeneration via Rho/ROCK signaling pathway after I/R** 304 **injury**

305 Our results show that CG can improve recovery after neurological damage caused by IS,
306 where axonal regeneration plays a vital role during this process. To address whether the
307 Rho/ROCK signaling pathway participates in this process, WB and qRT-PCR were carried
308 out to detect the expression of RGMa, Rho, ROCK, MLC2 and CRMP2. Semi-quantitative
309 analysis of WB (Figure7A-E) showed that protein expression of RGMa, Rho and ROCK
310 decreased dramatically in the CG and HF group compared with the I/R group, so do the
311 phosphorylation of MLC2 and CRMP2. In addition, a markedly reduction of RGMa, Rho,
312 ROCK, MLC2 and CRMP2 mRNA levels was presented in CG and HF group in contrast to
313 the I/R group (Figure 7F-J). Taking the results of WB and qRT-PCR together, it reveals that
314 CG promoted axonal regeneration partly by inhibiting the Rho/ROCK signaling pathway
315 after IS.

Figure 7. CG dependence on the Rho/ROCK signaling pathway after MCAO injury. Rats were sacrificed at day 7 after MCAO. β -actin was used as an internal loading control. (A-D) Protein expression of RGMa, Rho, ROCK, MLC2, when compared with the I/R group, $n=3$. (E-J) mRNA levels of MLC2, RGMa, Rho, ROCK, MLC2 and CRMP2, when compared with the I/R group compared with the I/R group, $n=5$.

4. Discussion

In this study, we described the mechanism behind how CG can reduce cerebral infarction volume and promote recovery of neurological function in IS rats. Necrosis cannot be reversed in the central infarct area, even after a short period of time post-cerebral infarction [22]. However, ischemic tissue surrounding the infarct centre, known as the ischemic penumbra (IP), is short of electrical activity, but can still maintain normal transmembrane potential and voltage. If the supply of blood to the brain can be repaired in time, IP can be recovered to prevent further expansion of the infarct [23]. Therefore, timely and effective neuroprotection of the IP is now the main method of ischemic stroke treatment [24]. We suggest that CG

promotes blood circulation to restore blood supply to IP, thereby reducing the cerebral infarction rate.

Cerebral ischemic injury causes irreversible neuronal death. It is estimated that 1 h of ischemia can cause damage to 120 million neurons, 830 billion synapses, and 714 km of myelinated fibers [25]. In the first few days after cerebral infarction, synaptic structure of the IP and other functional areas that are distant from the injured area are also damaged, which may be related to adjacent tissue edema, blood flow reduction, and metabolic disorders [26]. Therefore, we observed fewer nerve cells accompanied by axonal retraction balls in the brain tissue of I/R rats, which indicated that neuronal axons were damaged during cerebral infarction causing neurological dysfunction. Studies have found that acute injury caused by IS attenuates proliferation of neural stem cells in the subventricular zone and striatum [27, 28]. Although neural stem cells are renewable, it is difficult for them to mature and establish connections, since ischemia causes deficiencies in energy and nutrients [29]. Therefore, stroke recovery is dependent on normal axons around the injured area producing a new branch and rapidly extending to the damaged area to compensate for damaged fiber bundles [30, 31]. Moreover, axons near the primary motor cortex injury area sprout and establish new connections with the distal region, such as the contralateral hemisphere and the spinal cord [32, 33]. We show that axonal retraction and axonal swelling were reduced, extending nerve fibers and restoring neurological function. Our results suggest that CG promotes axonal sprouting and extension to re-establish connections among nerve fibers, thereby accelerating the recovery of nerve function.

The growth cone at the front end of the axon, whose cytoskeleton is composed of microtubules, microfilaments, and myosin, is sensitive to environmental signals [34]. Cytoskeletal proteins are degraded after cerebral infarction [35]. NF-200, an intermediate filament that is abundant in the axons of nerve cells, is the main component of the axon skeleton. It is a marker of axonal elongation, and thus can reflect the morphology of neurons after injury [36, 37]. CG upregulated expression of NF-200, suggesting axonal extension occurs after CG treatment. MAP2 is a signature microtubule-associated protein in neurons that is distributed in cell bodies, dendrites, and axons [38]. MAP2 is necessary for microtubule polymerization and stability, facilitating microtubule assembly, repair, and regeneration of damaged axons [39, 40]. Upregulation of MAP2 expression in the CG-treated group indicated increased axonal formation. GAP43 is a neuronal protein that is located in axonal growth cones during the development of the nervous system and during regeneration [41]. After nerve damage, GAP43 expression is upregulated rapidly and transferred from the cell body to the axon until a complete synaptic connection is established [42]. Therefore, GAP43 can be used as a marker for axonal sprouting and axon regeneration. Our study confirmed that CG upregulated expression of NF-200, MAP2, and GAP43. Therefore, we conclude that CG can promote neuronal skeleton stability and facilitate axonal regeneration after stroke.

The Rho/ROCK pathway plays an important role in maintaining cytoskeletal stability. Therefore, activation of this signaling pathway is important in regulating axonal regeneration [43, 44]. RGMa functions upstream of the Rho/ROCK pathway, and inhibiting the Rho/ROCK pathway with Rho inhibitor or ROCK inhibitor can completely prevent RGMa-induced growth cone collapse [45]. Axonal growth and motor function improvement is observed after inhibiting RGMa with anti-RGMa antibody after spinal cord injury [46]. CG can inhibit the RGMa expression, revealing its potential as a RGMa inhibitor. CRMP-2 is mainly localized to neuronal cell bodies, axons, and dendrites, participating in important

stages of the neurodevelopmental process downstream of Rho/ROCK [47]. It can promote microtubule assembly and regulate microtubule dynamics during axonal growth by binding to tubulin heterodimers [48]. After ischemic injury, activation of ROCK prevents CRMP2 from binding to tubulin dimers and inactivates axonal elongation, which is a major contributor to neuronal death [49]. CG may reduce neuronal apoptosis by upregulating CRMP2 expression. Our study showed that CG downregulated the RGMa and Rho expression, but upregulated expression of CRMP2 in MCAO rats. Therefore, we hypothesized that inhibition of the Rho/ROCK pathway may be the potential underlying mechanism for CG-induced axonal regeneration and improved neurological outcomes.

5. Conclusions

Our study indicated that CG can promote axonal regeneration and neural functional recovery after the cerebral ischemia. In rats treated by CG, behavioral scores, nerve fiber fracture, and axonal degeneration were significantly improved. Expression of NF-200, MAP2, and GAP43 were significantly upregulated. These beneficial mechanisms in neurons are dependent on the Rho/ROCK signaling pathway inhibition after ischemic stroke. These findings extend our understanding of CG in terms of neural regeneration and provides further evidence for efficacy as a therapeutic treatment for ischemic stroke.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Authors' contributions

LW and AY conceived the idea and design the experiment. NZ and AY performed the experiment, analysed the data and draft the manuscript. GW, HZ and XS performed the experiment. NZ, YH, LZ revised the manuscript and gave some suggestions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was performed in accordance with the approved protocol by the Animal Experimental Ethics Committee of Guangzhou University of Chinese Medicine (Guangzhou, China). Animal care was conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised in 1978).

Consent for publication

Not applicable

Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures

Figure 1

structure and chemical formula of CG

Figure 2

Effect of CG on neurological function. (A) Analysis of mNSS scores. Rats in the CG group had higher mNSS scores at days 3, 5, and 7 compared to the I/R group (* $P < 0.05$, ** $P < 0.01$), $n=5$. (B) Analysis of BBT scores. Rats in the CG group had lower BBT scores at day 5 and 7 compared to the I/R group (* $P < 0.05$, ** $P < 0.01$), $n=5$.

Figure 3

Images of cerebral infarction and cerebral edema after seven days of CG administration. (A) Image of TTC staining. Non-infarcted areas were stained in red. (B) The relative size of infarct volume (% total volume of the contralateral brain) of each group. Rats from CG and HF group had lower infarct volume compared to the I/R group ($***P < 0.001$), $n=5$. (C) Analysis of cerebral edema of each group. Rats from CG and HF group had lower water content compared to the I/R group ($**P < 0.01$, $***P < 0.001$), $n=5$.

Figure 4

Images of cerebral pathology after HE and Nissl staining showing histological and neuronal damage in rats sacrificed at day 7 after I/R injury. (A) HE staining images. Scale bar = 50 μ m. (B) Nissl staining images. Scale bar = 50 μ m. (C) Quantitative analysis of cortical neurons. (D) Quantitative analysis of hippocampus CA1 neurons. (E) Quantitative analysis of hippocampus CA3 neurons. (F) Quantitative analysis of hippocampus DG neurons. CG increased the number of nerve cells compared to the I/R group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 5$

Figure 5

BSSM staining and IF staining in tissues from rats sacrificed at day 7 after I/R injury. (A) Images of neurons after BSSM staining. Treatment with CG promoted nerve fiber elongation, reduced axonal retraction balls (red arrows), and axonal swelling (yellow arrows). Scale bar = 50 μ m. (B) Representative IF images showing NF-200, MAP2, and GAP43 localization and expression. Scale bar = 20 μ m. (C) Semi-quantitative analysis of axonal loss. (D-F) Semi-quantitative analysis of fluorescence intensity of NF-200, MAP2 and GAP43. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the I/R group, $n = 5$.

Figure 6

IF staining in tissues from rats sacrificed at day 7 after I/R injury. (A) Compared to the I/R group, CG-M and CG-H groups the expression of GFAP increased significantly (** $P < 0.01$). Expression of NG2 and CSPG5 were declined compared to the I/R group. Scale bar = 40 μm (B-D) Semi-quantitative analysis of fluorescence intensity of GFAP, NG2, CSPG5. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the I/R group, $n=5$

Figure 7

CG dependence on the Rho/ROCK signaling pathway after MCAO injury. Rats were sacrificed at day 7 after MCAO. β -actin was used as an internal loading control. (A-D) Protein expression of RGMa, Rho, ROCK, MLC2, when compared with the I/R group, n=3. (E-J) mRNA levels of MLC2, RGMa, Rho, ROCK, MLC2 and CRMP2, when compared with the I/R group compared with the I/R group, n=5.