

Neuroprotection by cattle encephalon glycoside and ignotin beyond the time window of thrombolysis in ischemic stroke

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Graphical Abstract A neuroprotective effect of cattle encephalon glycoside and ignotin (CEGI) injection on ischemic stroke



Abstract

Cattle encephalon glycoside and ignotin (CEGI) injection is known as a multi-target neuroprotective drug that contains numerous liposoluble molecules, such as polypeptides, monosialotetrahexosyl ganglioside (GM-1), free amino acids, hypoxanthine and carnosine. CEGI has been approved by the Chinese State Food and Drug Administration and widely used in the treatments of various diseases, such as stroke and Alzheimer's disease. However, the neuroprotective effects of CEGI beyond the time window of thrombolysis (within 4.5 hours) on acute ischemic stroke remain unclear. This study constructed a rat middle cerebral artery occlusion model by suture-occluded method to simulate ischemic stroke. The first daily dose was intraperitoneally injected at 8 hours post-surgery and the CEGI treatments continued for 14 days. Results of the modified five-point Bederson scale, beam balance test and rotaric test showed the neurological function of ischemic stroke rats treated with 4 mL/kg/d CEGI improved significantly, but the mortality within 14 days did not change significantly. Brain MRI and 2,3,5-triphenyltetrazolium chloride staining confirmed that the infarct size in the 4 mL/kg/d CEGI-treated rats was significantly reduced compared with ischemic insult only. The results of transmission electron microscopy and double immunofluorescence staining showed that the hippocampal neuronal necrosis in the ischemic penumbra decreased whereas the immunopositivity of new neuronal-specific protein doublecortin and the percentage of Ki67/doublecortin positive cells increased in CEGI-treated rats compared with untreated rats. Our results suggest that CEGI has an effective neuroprotective effect on ischemic stroke when administered after the time window of thrombolysis. The study was approved by the Animal Ethics Committee of The Third Military Medical University, China.

Key Words: apoptosis; brain; cattle encephalon glycoside and ignotin; central nervous system; neurological function; plasticity; rat; regeneration; stroke

Chinese Library Classification No. R453; R741; Q546

Introduction

A recent report by the American Heart Association found that acute ischemic stroke is a leading cause of death and disability around the world, costing billions of dollars in health care expense per year (Powers et al., 2018). Although therapeutic strategies for intravenous thrombolysis have been developed,

novel neuroprotective and neuro-regenerative treatments are still required because the window for therapeutic thrombolysis is only 4.5 hours (Donnan et al., 2011; Tan et al., 2015a). Other existing neuroprotective agents, such as calcium channel blockers (Gelmers et al., 1988), glutamate receptor antagonists (Eltzschig and Eckle, 2011), γ -aminobutyric acid

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agonists (Lodder et al., 2006) and antioxidant agents (Shuaib et al., 2007), can interrupt the cascade of neuron injuries caused by ischemia in animal experiments. Unfortunately, stroke clinical trials of these agents have repeatedly failed to show neuroprotection (O'Collins et al., 2006). Therefore, none of the above drugs has been approved by the US Food and Drug Administration for ischemic stroke.

Normally the neurovascular network is maintained by an adequate blood supply with oxygen and nutrients, but after a stroke, cessation of cerebral blood flow followed by revascularization induces neuronal injury and loss (Zerna et al., 2016). Complex, coordinated and interrelated cascades of molecular events take place in the ischemic core and penumbra area. With reduced oxygen supply, the generation of ATP and transportation of Na^+/K^+ is disrupted, resulting in cellular depolarization and Ca^{2+} influx (Chen et al., 2017). Eventually, Ca^{2+} overload in the neurons leads to the activation of the intrinsic apoptosis pathway. The complexity of these pathophysiological processes and neurovascular network dysfunction have led to the use of multi-target and multi-level treatments to improve clinical outcomes (Lapchak, 2011).

Cattle encephalon glycoside and ignotin (CEGI) injection is a known multi-target neuroprotective reagent that is composed of numerous liposoluble molecules, such as polypeptides, monosialotetrahexosyl ganglioside (GM-1), free amino acids, hypoxanthine and carnosine (Ma et al., 2018). Our previous study revealed that CEGI injection could alleviate white matter injury and the occurrence of hydrocephalus induced by intracerebral hemorrhage in rats (Li et al., 2016b). GM-1, one of the main components of CEGI, is a critical regulator in cell membrane synthesis and is involved in nervous system development/regeneration (Schnaar et al., 2014). Clinical trials showed that GM-1 improved stroke patients' neurological status, while CEGI was also proved effective in treating acute stroke patients (Lenzi et al., 1994; Zhang et al., 2020). Another component, carnosine, is also proven to be protective against oxidant stress and excessive inflammation by inhibiting the formation of advanced glycation and the end-products of lipid oxidation (Boldyrev et al., 2013). CEGI was approved by the Chinese State Food and Drug Administration in 2011 (Approval No. H22025046) and is widely used in China for the treatments of various diseases, such as stroke, Alzheimer's disease, diabetic peripheral neuropathy and neonatal hypoxic-ischemic encephalopathy (Boldyrev et al., 2013; Gao et al., 2015). However, the neuroprotective effects of CEGI administered beyond the time window of thrombolysis, within 4.5 hours of an acute stroke, remain unclear.

In this study, GM-1 was used as a positive control to determine the minimal effective dosage of CEGI on mortality and recovery of neuronal function after middle cerebral artery occlusion (MCAO) in a rat model. The infarct volume of the brain, necrosis of hippocampal neurons and the regeneration of neurons in the ischemic penumbra were examined to evaluate the efficacy of CEGI therapy on post-stroke recovery.

Materials and Methods

Animals

In the present study, to avoid the influence of the female hormonal cycle, a total of 244 adult male Sprague-Dawley rats (age 8 weeks, weight 280–320 g) were purchased from the Animal Center of The Third Military Medical University, China (license No. SCXK (Yu) 2018-0009). The rats were routinely housed in controlled environments at $21 \pm 1^\circ\text{C}$, $60 \pm 10\%$ humidity with a 12-hour dark/light cycle. They were supplied with water and food *ad libitum*. The animal experiments were undertaken under the China Animal Welfare Legislation with the approval of the Animal Ethics Committee of The Third

Military Medical University, China.

Establishment of MCAO rat model

The rat model of MCAO was generated as previously described (Yang et al., 1994). Briefly, rats were anesthetized using sodium pentobarbital (50 mg/kg, intraperitoneal injection). The right common carotid artery, together with the internal carotid artery, was exposed, and the right external carotid artery was ligated. A nylon monofilament (diameter 0.25–0.28 mm) coated with silicone resin was gently inserted into the right internal carotid artery to block the middle cerebral artery. The cerebral blood flow was monitored concurrently using laser Doppler flowmetry (PeriFlux 5000; Perimed, Stockholm, Sweden) to ensure the successful occurrence of ischemia. After 1 hour of occlusion, reperfusion was established. Sham-operated rats underwent the same surgery without the insertion of a nylon monofilament. Body temperature was maintained at $37 \pm 1^\circ\text{C}$.

Drug intervention

The first dose was intraperitoneally injected at 8 hours post-surgery and the daily treatments of CEGI or GM-1 continued for 14 days. CEGI was obtained from Jilin Sihuan Pharmaceutical Co. Ltd. (Meihekou, Jilin Province, China) and GM-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A 1-mL CEGI injection contains 0.925 mg total nitrogen, mainly as polypeptides (3.2 mg), GM-1 (0.24 mg) and free amino acids (1.65 mg), and 12 μg hypoxanthine.

Experimental protocols

Experiment I

According to the previous study (Li et al., 2016b), acute stroke rat model should be injected with 5–20 mL of CEGI per day. To optimize the minimal effective dosage of CEGI in the MCAO rat model, 1 or 4 mL/kg/d of CEGI was used in Experiment I. A total of 178 rats were randomly assigned into six groups: sham ($n = 27$), sham + 4 mL/kg/d CEGI ($n = 27$), MCAO (normal saline, 4 mL/kg/d, intraperitoneal injection; $n = 31$), MCAO + 1 mL/kg/d CEGI ($n = 31$), MCAO + 4 mL/kg/d CEGI ($n = 31$) and MCAO + 50 mg/kg/d GM-1 (Li et al., 2016a) (positive control; $n = 31$) groups. The mortality rates were recorded on day 14 post-surgery. The neurological dysfunction was evaluated on days 3, 7 and 14 following MCAO induction using the Bederson score scale, beam balance score and rotarod performance test (Figure 1A).

Experiment II

Based on the results of Experiment I (Figure 1B), CEGI (4 mL/kg/d) was used in Experiment II. A total of 66 rats were randomly assigned into three groups: sham ($n = 18$), MCAO (normal saline, 4 mL/kg/d, intraperitoneal injection; $n = 24$) and MCAO + 4 mL/kg/d CEGI ($n = 24$) groups. Brain MRI and 2,3,5-triphenyltetrazolium chloride (TTC) staining ($n = 6$ /group in each time point for both MRI and TTC staining) were performed to determine the ischemic volume on days 7 and 14 post-MCAO. Electron microscopy was used to observe hippocampal neuronal necrosis induced by ischemia/reperfusion ($n = 6$) and double immunofluorescence staining of doublecortin (DCX) and Ki67 was performed ($n = 6$) on day 14 following MCAO.

Evaluation of mortality

All animals were kept under the same conditions before surgery. The deaths in each group were recorded within 14 days post-MCAO induction. The mortality rate (%) for each group was calculated as the number of dead rats/the total number of rats in each group $\times 100$.

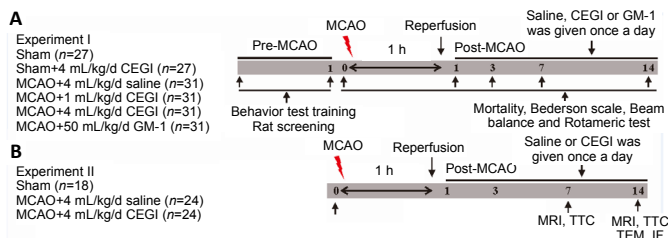


Figure 1 | Study flow chart.

(A) Experiment I; (B) Experiment II. CEGI: Cattle encephalon glycoside and ignotin; GM-1: monosialotetrahexosyl ganglioside; IF: immunofluorescence; MCAO: middle cerebral artery occlusion; TEM: transmission electron microscopy; TTC: 2,3,5-triphenyltetrazolium chloride.

Assessment of neurological deficiency

The neurological dysfunction of rats in each group was evaluated on days 3, 7 and 14 post-MCAO by two independent investigators using the following methods: modified five-point Bederson scale (Bederson et al., 1986), beam balance test (Müller et al., 2008) and rotameric test (Ishrat et al., 2009). The modified five-point Bederson scale was defined as having no deficit (0); forelimb flexion (1); decreased resistance to lateral push (2); unidirectional circling (3); longitudinal spinning (4); or no movement (5). Beam balance performance was conducted and graded on a six-point scale: balancing with steady posture (0); grasping the side of the beam (1); hugging beam with one limb, falling down from beam after 60 seconds elapsed; (2); falling down from beam or spinning on beam before 60 seconds elapsed (3); attempting to balance on the beam but falling down before 40 seconds elapsed (4); attempting to balance on the beam but falling down within 20 seconds (5); and falling down, no attempts to balance or hang on to beam within a timeframe of 20 seconds (6). The rotameric test was carried out using an accelerating rotarod, which started at 4 r/min and accelerated gradually to 30 r/min within 5 minutes. The latency to fall off the rotarod was then determined before ischemia (pre-surgery) and on days 3, 7 and 14 post-MCAO. The average value of the three measurements prior to the surgery was recorded as the baseline performance. For each rat tested, the mean value of two longest of three falling latencies was used for data analysis. Rats not falling off within 5 minutes were given a maximum score of 300 seconds.

Measurement of infarct size

In this study, MRI and TTC staining were performed to determine the infarct size as previously described (Wells et al., 2015). On days 7 and 14 post-MCAO, six experimental animals from each group were anesthetized with 2% isoflurane and scanned using a 7.0 T Varian MRI scanner (Bruker, Karlsruhe, Germany). The imaging protocols consist of a T2 fast spin-echo sequence with a view field of 35 mm × 35 mm and 20 coronal slices (thickness = 1.0 mm). The radiological infarct volume was calculated as a percentage of the entire brain on coronal sections using the T2-weighted images, corrected for edema using a modified Swanson calculation (Swanson et al., 1990).

After the acquisition of brain MRI, all rats were sacrificed immediately by decapitation under deep anesthesia. Brains were removed and sliced into five coronal sections (thickness = 2 mm) with a brain matrix (Zivic Instruments, Pittsburgh, PA, USA). Then brain slices were immersed in 0.1% TTC at 37°C for 20 minutes in darkroom and fixed using 4% paraformaldehyde. The infarct volume was calculated as a percentage of the entire brain corrected for edema using a modified Swanson calculation and analyzed by Image-J software (National Institutes of Health, Bethesda, MD, USA).

Transmission electron microscopy

Under deep anesthesia using sodium pentobarbital (Merck & Co, Kenilworth, Kenilworth, NJ, USA), rat brain tissues were taken from the infarcted hippocampal CA1 region on day 14 following MCAO, sectioned into ~1 mm³ cubes and fixed using 2% glutaraldehyde at 4°C overnight. The brain samples were immersed in propylene oxide, embedded in Epon resin and then sectioned into ultra-thin slices (70–90 nm). The slices were then placed on 200-mesh grids and stained with uranyl acetate and Reynold’s lead citrate. Finally, the ultra-structure of the ischemic hippocampus was observed using a Philips CM 100 transmission electron microscope (Hillsboro, OR, USA) and digitally recorded by a Hamamatsu ORCA-HR camera (Hamamatsu, Shizuoka, Japan). Between 25 and 35 non-overlapping photomicrographs (2550× magnification) of each zone from each animal were obtained for counting necrotic neurons in the hippocampal CA1 region by two blinded investigators according to the previous study (Liang et al., 2017).

Immunofluorescence staining

Different groups of rats were used on day 14 post-stroke. Immunofluorescence staining was performed as previously described (Tan et al., 2015b). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection) and transcardially perfused using 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brain samples were collected from the ipsilateral hippocampal dentate gyrus (DG) region and post-fixed in 4% paraformaldehyde for 24 hours and then dehydrated using a 30% sucrose solution at 4°C for 72 hours. The tissues were then embedded in an optimal cutting temperature compound (Sakura, Japan) and coronally sectioned into 18-µm-thick slices using a Leica CM1860 Cryostat (Wetzlar, Germany). The brain slices were immersed in 0.3% Triton X-100 in phosphate-buffered saline for 30 minutes and blocked with 3% bovine serum albumin in phosphate-buffered saline for 30 minutes at room temperature. The slices were further incubated with primary anti-DCX (anti-doublecortin, a marker for new neurons; 1:200; Proteintech Biotechnology, Rosemont, IL, USA) and anti-Ki67 (a proliferation marker; 1:200; Sigma-Aldrich, St. Louis, MO, USA) antibodies at 4°C overnight. They were then incubated with appropriate secondary antibodies (DCX: goat anti-mouse IgG Alexa Fluor®555, 1:200, Invitrogen, Carlsbad, CA, USA; Ki67: donkey anti-rabbit IgG Alexa Fluor®488, 1:200, Invitrogen) at 37°C for 2 hours. After washing, the nuclei were counterstained with 0.1 mg/mL 4',6-diamidino-2-phenylindole (Sigma-Aldrich) for 10 minutes and visualized by a confocal laser scanning system (LSM780, Zeiss, Jena, Germany).

Statistical analysis

All the statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and presented as the mean ± standard error of mean (SEM). Mortality rates were analyzed by the chi-square test. Other data were analyzed using the Kruskal-Wallis one-way analysis of variance followed by Dunn’s *post hoc* test. A *P* value < 0.05 was considered to indicate a statistically significant difference.

Results

CEGI prevents neurological dysfunction without affecting the mortality rate in MCAO rats

To optimize the dosage of CEGI in the treatment of MCAO rats, their neuro-behavior deficit and mortality induced by ischemic stroke were evaluated. The results revealed that the mortality rate of MCAO rats was about 3%, which was significantly elevated compared with that in sham group (*P* < 0.05). However, there was no significant difference

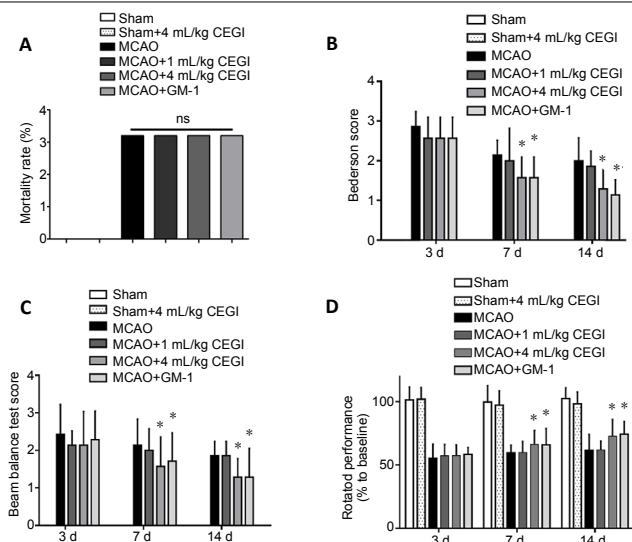


Figure 2 | Assessment of mortality and neurological deficiency in CEGI-treated MCAO rats.

(A) The mortality remained unchanged among each group on day 14 after surgery. (B–D) Modified Bederson score, beam balance test and rotarod test at 3, 7 and 14 days post-MCAO. Data are presented as the mean \pm SEM ($n = 9$ per group at each time point). * $P < 0.05$, vs. MCAO group (Kruskal-Wallis one-way analysis of variance followed by Dunn's *post hoc* test). CEGI: Cattle encephalon glycoside and igitonin; GM-1: monosialotetrahexosyl ganglioside; MCAO: middle cerebral artery occlusion.

between the MCAO and treatment groups ($P < 0.05$; **Figure 2A**). Further experiments were conducted to investigate whether neurotrophic drugs could alleviate MCAO-induced neurological deficits. The results of the modified five-point Bederson scale, beam balance test and rotarod test indicated that treatments with a high dose of CEGI or GM-1 significantly improved the neurological score of MCAO rats on days 7 and 14 ($P < 0.05$; **Figure 2B–D**). The therapy with 4 mL/kg/d CEGI on sham-operated animals exhibited no effects on mortality rates or neuro-behavior scores.

Treatment with CEGI reduces the infarct volume induced by MCAO

Because a high dosage of CEGI exhibited neuroprotective functions, the effects of CEGI on infarct volume were further evaluated using a brain 7.0 T MRI scan and TTC staining. Significant hemispheric infarction, as determined by T2-weighted images and corrected for the space-occupying effects of vasogenic edema formation, was revealed in the brain of MCAO rats. The MRI scan demonstrated CEGI could significantly decrease the infarct brain volume of MCAO rats on days 7 and 14 post-MCAO (**Figure 3A**). Subsequent quantification suggested that the infarct volume was significantly reduced to 28.2% on day 7 and 17.7% on day 14 by CEGI treatment compared with 37.3% on day 7 and 25.6% on day 14 in MCAO groups ($P < 0.05$; **Figure 3B**). The TTC staining results showed that the improvement in the CEGI treated group was mostly observed in the ischemic penumbra. MCAO rats also exhibited TTC pallor in the right caudate nucleus and cortex that are also supplied by the MCA (**Figure 4A**). The quantitative analysis revealed that the percentage of infarction in the MCAO + 4 mL/kg/d CEGI group had significantly decreased compared with the MCAO group on days 7 and 14 post-MCAO ($P < 0.05$; **Figure 4B**).

CEGI reduces the number of necrotic hippocampal neurons

The above data indicate that CEGI treatment could improve ischemia-induced neuro-dysfunction and reduce the infarction size. Thus, we investigated whether it could prevent ischemia-induced neuronal death. The ultrastructural alterations in

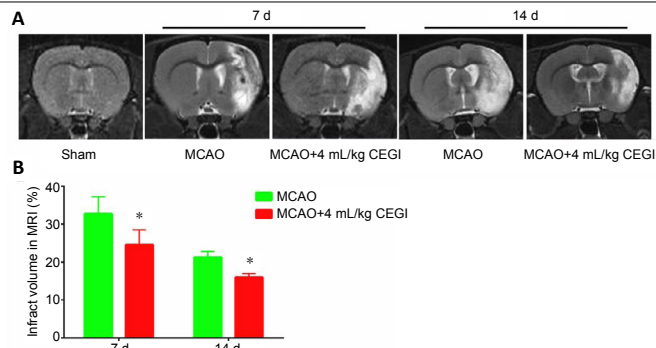


Figure 3 | Effect of CEGI on the cerebral infarction induced by MCAO detected by MRI.

(A) Differences in residual infarct volume revealed by MRI T2-weighted images on day 7 and 14 post-MCAO. (B) Quantitative analysis of infarct size in brain tissues. The percentage of infarction in the MCAO + 4 mL/kg/d CEGI group was significantly decreased compared with that in the MCAO group on days 7 and 14 post-MCAO ($P < 0.05$). Data were presented as the mean \pm SEM ($n = 6$ per group at each time point). * $P < 0.05$, vs. MCAO group (Kruskal-Wallis one-way analysis of variance followed by Dunn's *post hoc* test). CEGI: Cattle encephalon glycoside and igitonin; MCAO: middle cerebral artery occlusion.

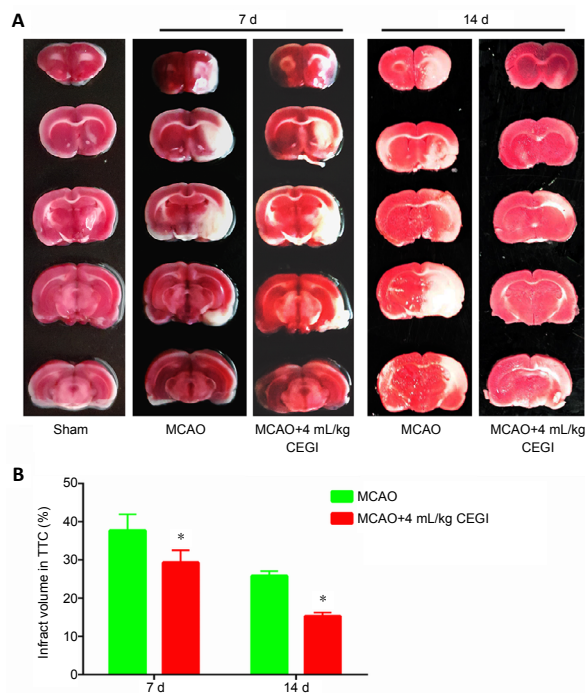


Figure 4 | Effect of CEGI on the infarct volume of MCAO rats detected by TTC staining.

(A) Cerebral infarction in the representative coronal brain slices. (B) Quantitative analysis of infarct size in brain tissues. The percentage of infarction in the MCAO + 4 mL/kg/d CEGI group was significantly decreased compared with that in the MCAO group on days 7 and 14 post-MCAO ($P < 0.05$). Data are presented as the mean \pm SEM ($n = 6$ per group at each time point). * $P < 0.05$, vs. MCAO group (Kruskal-Wallis one-way analysis of variance followed by Dunn's *post hoc* test). CEGI: Cattle encephalon glycoside and igitonin; MCAO: middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride.

the infarcted hippocampal CA1 area induced by ischemia/reperfusion injury were evaluated using a transmission electron microscope on day 14. The images presented in **Figure 5A** show that a proportion of CA1 pyramidal neurons were necrotic, with dark cytoplasm, wrinkled cell membranes, flocculent mitochondria and chromatin clumping. The quantitative analysis suggested that the percentage of necrotic neurons in MCAO rats was significantly reduced following the CEGI treatment ($P < 0.05$; **Figure 5B**).

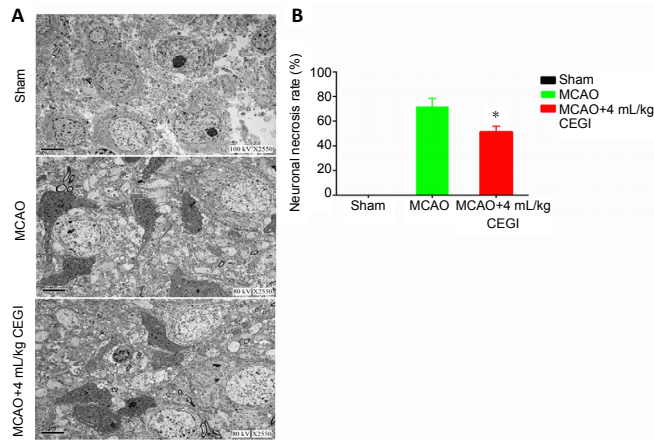


Figure 5 | Effect of CEGI on the ultrastructural alterations in infarcted hippocampal CA1 area at 14 days post-MCAO. (A) Electron microscopic images of neuronal necrosis in the hippocampal CA1 region (2550x original magnification). Scale bars: 5 μ m. (B) Quantitative analysis of identified necrotic hippocampal neurons. The percentage of necrotic neurons was remarkably reduced in the CEGI-treated rats ($P < 0.05$). Data are presented as the mean \pm SEM ($n = 6$ per group at each time point). * $P < 0.05$, vs. MCAO group (Kruskal-Wallis one-way analysis of variance followed by Dunn's *post hoc* test). CEGI: Cattle encephalon glycoside and ignotin; MCAO: middle cerebral artery occlusion.

CEGI stimulates neurogenesis in the subgranular zone of the hippocampal DG

To investigate the effects of CEGI on neural regeneration in the subgranular area of the hippocampal DG region (ischemic penumbra) on day 14, co-staining of Ki67 and DCX was performed. The number of DCX-positive clusters induced by MCAO was notably increased by CEGI treatment on day 14 (Figure 6A). The immunopositivity of new neuronal-specific protein DCX was significantly elevated in MCAO+4 mL/kg/d CEGI group compared with that in MCAO rats ($P < 0.05$; Figure 6B). Additionally, the percentage of Ki67/DCX positive cells, which indicates proliferating neurons, in the subgranular zone of the infarcted hippocampus was significantly increased in CEGI-treated rats compared with that in MCAO-only rats ($P < 0.05$; Figure 6C).

Discussion

Based on previous studies on translational stroke research, more preclinical data and well-designed clinical trials are required for the development of neuroprotective agents. Improvements in brain infarction size and neurobehavioral function are essential indices in animal experiments (Turner et al., 2016). Translational stroke studies are limited by blood-brain barrier penetration, therapeutic dosage, side-effect profiles and drug bioavailability (Zhang et al., 2017). Clinical trials of most single-target neuroprotective agents have not shown improvement in the outcomes of stroke patients, therefore, thrombolytic/reperfusion strategies combined with multi-target neuroprotective drugs could be a novel approach for the treatment of acute ischemic stroke (Sun et al., 2014). However, the efficacy of thrombolysis therapy is restricted by the secondary injuries caused by vascular recanalization, including oxidative stress, activation of excitatory amino acids receptors and blood-brain barrier breakdown (Krueger et al., 2015).

In the present study, the short- and long-term effects of CEGI treatment on functional recovery were evaluated in the ischemia/reperfusion rat model and compared with GM-1 treatment. CEGI-treated rats (4 mL/kg/d) exhibited improved performance in behavioral tests compared with the control on days 7 and 14 post-MCAO. This dose of CEGI was chosen

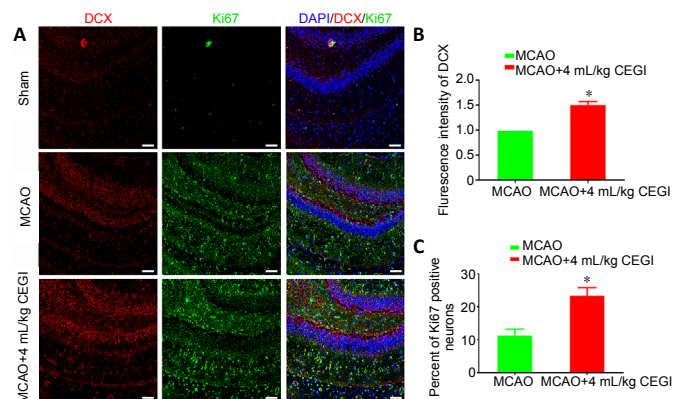


Figure 6 | Immunofluorescence staining of newborn neuron-specific DCX and Ki67 in hippocampal dentate gyrus region on day 14 post-MCAO treatment with CEGI. (A) Double staining of DCX (red, stained by Alexa Fluor®555) and Ki67 (green, stained by Alexa Fluor®488) in the ipsilateral subgranular zone of the hippocampal dentate gyrus region on day 14 post-MCAO. Scale bars: 50 μ m. (B) Temporal changes in density of newborn neurons (DCX⁺). (C) The percentage of proliferating neurons (Ki67⁺/DCX⁺). The fluorescence intensity of DCX was significantly elevated in MCAO + 4 mL/kg CEGI group compared with that in MCAO rats ($P < 0.05$). The percentage of Ki67/DCX positive cells was significantly increased in CEGI-treated rats ($P < 0.05$). Data are presented as the mean \pm SEM ($n = 6$ per group at each time point). * $P < 0.05$, vs. MCAO group (Kruskal-Wallis one-way analysis of variance followed by Dunn's *post hoc* test). CEGI: Cattle encephalon glycoside and ignotin; DAPI: 4',6-diamino-2-phenylindole; DCX: doublecortin; MCAO: middle cerebral artery occlusion.

for further experiments; the infarct volume, necrosis and neural regeneration in MCAO and CEGI-treated groups were examined using brain MRI scanning, transmission electron microscopy and immunofluorescence, respectively. The results demonstrated the beneficial effects of long-term CEGI therapy against MCAO-induced neuronal damage. CEGI treatment exerted neuroprotective functions by promoting neurogenesis and neurorepair at the chronic stage of stroke.

The acute insult is initially due to hypoxia-ischemia and then extends into the reperfusion phase, suggesting the process of ischemia/reperfusion injury was involved in the development of ischemic stroke (Zhang et al., 2016). The cellular mechanisms underlying neuronal death following acute ischemic stroke are complex, involving calcium overload, cortical spreading depolarization, increased oxidative stress, inflammation, apoptosis and impaired energy metabolism. Stroke is a multifactorial disorder, therefore, multi-target treatment such as CEGI might improve the prognosis of patients with ischemic stroke. Previous research on Alzheimer's disease (Gao et al., 2015) has revealed that CEGI could attenuate neuronal damage in the hippocampus area of mice with Alzheimer's disease and upregulate the ratio of Bcl-2/Bax. This is consistent with our findings of CEGI-mediated neuroprotection on necrotic neurons in the infarcted hippocampal CA1 area.

Spontaneous recovery from stroke-induced neuro-dysfunction occurs in human and animal models. The underlying mechanisms, involving angiogenesis, activation of cellular genesis and repair mechanisms, alterations in the existing neuronal pathways and stimulation of neuronal or synaptic plasticity (Cramer, 2008; Martí-Fàbregas et al., 2010; Ergul et al., 2012), require further investigation. Following ischemia, neural stem/progenitor cells proliferate in the ipsilateral subventricular zone. Then the cells migrate towards the ischemic penumbra in the brain, where they differentiate into mature neurons or glial cells and participate in functional neuronal circuits (Ge et al., 2015). Neuroblasts that migrated from the subventricular zone cannot enter the ischemic core

to replace the dead neurocytes. Instead, the locally derived stem/progenitor cells from the peri-infarct region, such as the subgranular zone of the hippocampal DG, migrate into the infarction core. They subsequently differentiate into neurons, astrocytes and oligodendrocytes to restore the neural network (Shimada et al., 2010). Therefore, the development of novel cellular and pharmacological strategies to promote neurogenesis is crucial for stroke recovery.

As critical regulators of cellular synthesis and functions, numerous neurotrophins serve essential roles in mediating axonal regeneration and neural stem/progenitor cell proliferation. For instance, the transfer of mRNAs of essential cytoskeletal elements into the neurons for localized translation is assisted by neurotrophins such as growth-associated protein-43 (Shimada et al., 2010). Furthermore, *in vitro* studies with primary neurons indicated that the expression levels of axonal proteins, their receptor subunits and scaffolding proteins were upregulated by a brain-derived neurotrophic factor (Schäbitz et al., 2004). Because GM-1 is able to permeate blood-brain barrier, it was considered as an essential exogenous ganglioside supplement to exert neuroprotective functions against brain injury and neurodegenerative disease (Li et al., 2016a; Fukami et al., 2017). As reported by Li et al. (2016a), GM-1 treatment immediately after MCAO could contribute to alleviate autophagy activation, neurobehavioral dysfunctions and infarcted brain volume. Unfortunately, it is challenging to give immediate therapy following ischemic insult in clinical practice (Li et al., 2016a). However, in the present study CEGI was intraperitoneally injected 8 hours after a stroke, and the rats were treated for a further 14 days. At 7 and 14 days post-MCAO, the infarcted brain volume, neurological function score and neuronal necrosis in hippocampal CA1 were significantly improved in MCAO rats treated with CEGI exhibited the almost same improvements on neuro-behavioral dysfunctions comparable to those in GM-1 treated rats on days 7 and 14 post-MCAO.

When translating to clinical medicine, treatment with exogenous gangliosides in patients with acute ischemic stroke exhibited no beneficial effects according to a meta-analysis incorporating 12 trials (Candelise and Ciccone, 2002). Gangliosides form the lipid matrix of neuronal cell membranes and play essential roles in neuronal development and neuroplasticity (Palmano et al., 2015). However, the patients treated with GM-1 in large doses or for a longer duration were reported to develop Guillain-Barré syndrome (Nobile-Orazio et al., 1994). Another essential component of CEGI, carnosine (β -alanyl-L-histidine), is a natural pleiotropic dipeptide associated with various deleterious mechanisms, including mitochondrial dysfunction and autophagy induced by ischemic stroke (Baek et al., 2014). Carnosine treatment reduced infarct volume by 29.4% in the animal models of ischemic stroke but its efficacy was dramatically decreased when the treatment lasted for more than 6 hours following ischemia (Davis et al., 2016), which limited its clinical application. Alternatively, combined treatment with neuroprotective agents at low dosages following ischemia/reperfusion could minimize the side effects and broaden the therapeutic window, which was consistent with our findings when the first dose was given to MCAO rats at 8 hours post-ischemia/reperfusion. The antioxidant/anti-lipid oxidant effects of carnosine therapy (Yilmaz et al., 2017) might provide a beneficial microenvironment for ischemic penumbra, reinforcing the neuroprotective roles of GM-1 on proliferation and differentiation of locally derived neural stem/progenitor cells (Wang et al., 2016). This is consistent with our findings on neural regeneration and hippocampal neurogenesis. However, there were a few limitations to our present study. Our data showed that CEGI treatment dramatically reduced the infarcted brain volume

and promoted the neuro-function of the MCAO rats. However, the mechanism by which CEGI stimulates neural regeneration in the ischemic penumbra was not explored in this study. This study was conducted in rats, therefore, a further trial on stroke patients is still required to confirm the efficacy of CEGI. Only male rats were used in this study to exclude the neuroprotective effect of estrogen and fluctuations in its level.

In conclusion, the present study revealed that CEGI treatment could promote functional recovery and reduce the infarcted brain volume of MCAO rats by stimulating neural regeneration in the ischemic penumbra. Together with our previous findings (Li et al., 2016b), these results indicate that CEGI injection might function as a multi-target neuroprotective agent at the early stage of stroke rehabilitation. Further investigations are required to evaluate the underlying mechanisms.

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