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Rapid Report

Herbal decoction of *Gastrodia*, *Uncaria*, and *Curcuma* confers neuroprotection against cerebral ischemia *in vitro* and *in vivo*

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"Tianma" (*Gastrodia*) and "gouteng" (*Uncaria*) are both widely used to treat cerebral ischemia. At the same time, "ezhu" (*Curcuma longa*) or turmeric, is derived from the dried roots of *C. longa*.It is a polyphenol known for its anti-inflammatory effects and its promotion of blood vessel endothelial function. This study explored the neuroprotective effects of a water extract of "tianma", "gouteng", and "ezhu" against ischemic injury. Flow cytometry analysis showed that *Gastrodia*, *Uncaria*, and *Curcuma* reduced the proportion of apoptotic cells in $CoCl_2$ induced B35 ($P = 0.0027$) and SH-SY5Y ($P = 0.0006$) cell sample relative to the respective control group. Western blot indicated that *Gastrodia*, *Uncaria*, and *Curcuma* upregulated the expression of Bcl-2 and inversely downregulated Bax and Caspase-3 ($P < 0.001$). The infarct volume observed in the *Gastrodia*, *Uncaria*, and *Curcuma* group was also decreased compared with the control group ($P < 0.05$). Immunofluorescence detection revealed a lower expression of Caspase-7 in the *Gastrodia*, *Uncaria*, and *Curcuma* group than in the control group, while expression was negligible in the sham group. *Gastrodia*, *Uncaria*, and *Curcuma* confer neuroprotective effects in $CoCl_2$ induced B35/SH-SY5Y cells and a rat model of ischemia by way of its anti-apoptotic effects.

Keywords

Alternative medicine; Chinese herbal medicine; Gastrodia; Uncaria; Curcuma; cerebral ischemia; apoptosis

1. Introduction

Stroke is characterized by interrupted blood flow in the brain leading to the acute neurological deficit, and it represents a significant contributor to global morbidity and mortality (Benjamin et al., 2018). Herein, we focus on ischemic stroke, the pathogenesis of which is usually due to cerebral artery occlusion or severe stenosis, although cerebral sinus or cortical vein thrombosis are also contributors. Risk factors strongly associated with ischemic stroke include advanced age, smoking, hypertension, diabetes, combined heart disease, carotid artery stenosis, and dyslipidemia (Boehme et al., 2017; Poorthuis et al., 2017; Shindo and Tomimoto, 2014; Tsai et al., 2015). The core problem of stroke is the death of neurons, and mechanisms responsible for neuronal death include mitochondrial related deaths, free radical injury, excitotoxicity, apoptosis, autophagy, and aseptic inflammation (Sekerdag et al., 2018). Intravenous recombinant tissue plasminogen activator (rtPA) is currently recommended for acute treatment options by clinical guidelines as a therapy for stroke in eligible patients. Although it is efficacious, it carries a risk of hemorrhagic complications, and its administration is limited due to the effective time window.

Traditional Chinese medicine (TCM), the most widely used form of herbalism around the world, is a sophisticated system of medical theory and practice that is quite different from modern medicine. The decoction "tianma gouteng yin", which includes the herbs "tianma" (*Gastrodia*) and "gouteng" (*Uncaria*), is deemed in TCM as a treatment for wind disorder, calming liver wind, as well as activating blood and clearing heat. It is also used in the treatment of ischemic stroke and brain-related disease due to the mechanisms of antioxidant and anti-apoptotic (Liu et al., 2015; Xian et al., 2016; Zhang et al., 2004). "Ezhu" (*Curcuma*) is used to the treatment of stagnation of "qi" (the body's vital energy), blood stasis induced chest and abdominal pain, dysmenorrhea, and limb pain. Previous studies have demonstrated its neuroprotective effects against cerebral ischemia-reperfusion injury (Huang et al., 2018; Li et al., 2017). Since "tianma", "gouteng" and "ezhu" may have a synergistic therapeutic effect on cerebral ischemic injury, in the present study, we want to verify it through *in vivo* and *in vitro* experiments.

2. Materials and methods

2.1 Extraction methods

Crude *Gastrodia* (product ID. ECN000234), *Uncaria* (product ID. ECN000233) and *Curcuma* (product ID. ECN000195) (GUC)

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were purchased from Bencaopu online Chinese medicine shop, Laoruilin Pharmacy Chain Co., Ltd. (Zhangzhou, P. R. China). The herbs were authenticated according to the methods recorded in The Pharmacopoeia of P. R. China 2015 Edition. Voucher specimens of *Gastrodia*, *Uncaria*, and *Curcuma* were collected at the Shanghai Museum of Traditional Chinese Medicine. The GUC extract was prepared as follows: *Gastrodia* (105.0 g) was immersed in 2.5 L distilled water for one hour and then boiled for 40 min. *Uncaria* (140.9 g) and curcumin (105.5 g) were then added. Heat the mixture at reflux for 15 minutes and then cooled to room temperature and filtered. The resulting filtrate was rotary evaporated and lyophilized for further study (Fig. 1).

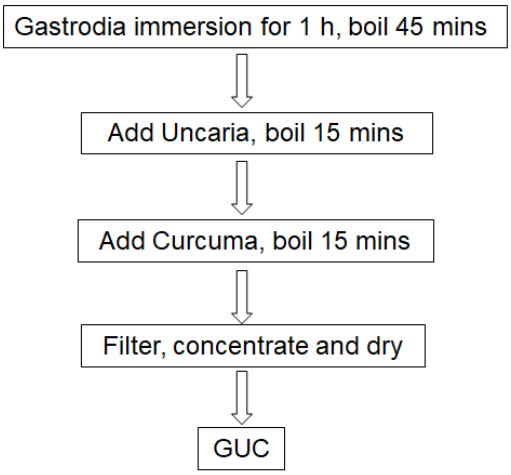

Fig. 1. Extraction protocol of "*tianma gouteng ezhu*" (*Gastrodia*, *Uncaria*, and *Curcuma*) water extracts (GUC).

2.2 Cell lines and $CoCl_2$ induced hypoxia

The rat neuroblastoma B35 and the human neuroblastoma SH-SY5Y cell lines (purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were grown in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO_2. 250 μmol/L $CoCl_2$ for 12 h was used to mimic ischemia/hypoxia.

2.3 GUC treatment followed flow cytometry analysis and western blot

B35 and SH-SY5Y cells treated with $CoCl_2$ were seeded at a density of 2 $\times$ 104 cells/well in 6-well culture plates (Corning). Both cell lines were divided into experimental (GUC) and control check (CK) groups, with three wells per group. The experimental group was treated with GUC extract (sterilized by autoclaving) at a concentration of 4000 μg/mL for 24 hours. The control group was treated with normal saline for 24 hours. Apoptosis was analyzed by an Annexin and DAPI apoptosis detection kit (BD Biosciences). Briefly, cells were harvested and washed with cold PBS, and the supernatant was removed by centrifugation. Then, the cells were resuspended in Annexin V-FITC and DAPI for half an hour in darkness. After that, Cell Quest software on a FACSAria Flow Cytometer (BD Biosciences) was used to analyze apoptosis. A fluorescence signal with an excitation wavelength of 480 nm was captured. To detect apoptotic markers, western blot was conducted, as previously described (Dai et al., 2019). Antibodies used in the present study were as follows: (1 : 500 dilution), Bcl-2 antibody (ProteinTech Group, Inc.), Bax antibody (Abcam), Caspase-3 antibody (Abcam), and β-actin antibody (Abcam). Horseradish peroxidase-conjugated anti-rabbit antibody (1 : 10,000 dilution; Sigma-Aldrich) was used as the second antibody. Detection was finally determined using western blot detection reagents (Odyssey; LI-COR Biosciences).

2.4 Animals

Male Sprague-Dawley (SD) rats (Animal experiment center, School of Medicine, Shanghai Jiao Tong University), weighting 220-250 g, were housed in cages of capacity 45 $\times$ 30 $\times$ 20 cm^3, on a 12-h light/dark cycle with arbitrary access to chow and water. Studies were conducted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guideline. All animal procedures were performed with the approval of the Ethics Committee of Shanghai Pudong Hospital, Fudan University, with the IRB number of WZ-006.

2.5 Middle cerebral artery occlusion

The 18 rats were divided into three groups arbitrarily: sham, control (MCAO-CK), and GUC treatment (MCAO-GUC). Rats in the MCAO-CK and sham groups were administered with distilled water after middle cerebral artery occlusion (MCAO) or a sham operation. In contrast, rats of the MCAO-GUC group were fed with 250 mg/kg GUC once daily for 7 days. The MCAO operation was executed as follows: SD rats were anesthetized intraperitoneally with 350 mg/kg of chloral hydrate (VWR International Ltd), then fixed to a surgical plate. We isolated and ligated the external carotid artery (ECA) and its branch vessels. Then, a 4-0 nylon suture was inserted into the internal carotid artery (ICA) through the ECA and forward to the anterior cerebral artery to block the middle cerebral artery (MCA). After 2 hours, reperfusion was achieved by visual removal of the nylon filament. In sham rats, operations were conducted analogously without the usage of nylon filament.

2.6 Infarct volume assessment

Brain slices of rats were stained by 2,3,5-triphenyl tetrazolium chloride (TTC). Brains were harvested and immersed in ice-cold saline for 20 minutes, then cut into 2 mm-thick slices. After that, brain slices were stained with 2% TTC solution at room temperature without light for 20 minutes, and images were captured subsequently. Infarct regions were analyzed using Adobe Photoshop CC 2018 (Adobe Systems Inc). The infarct area of each slice was multiplied by 2 (mm) to estimate infarct volume per slice. Similarly, the total infarct volume was the summation of infarct volumes of all slices.

2.7 Immunofluorescence

Rats were euthanized, and the brains were isolated and cut into 6-μm slices on the 7th day after MCAO. These slices were blocked with FBS (10% goat serum and 1% BSA), then co-incubated with rabbit polyclonal anti-Caspase-7 (1 : 200; Abcam) at 4 °C overnight. Then, washed with PBS three times and incubated

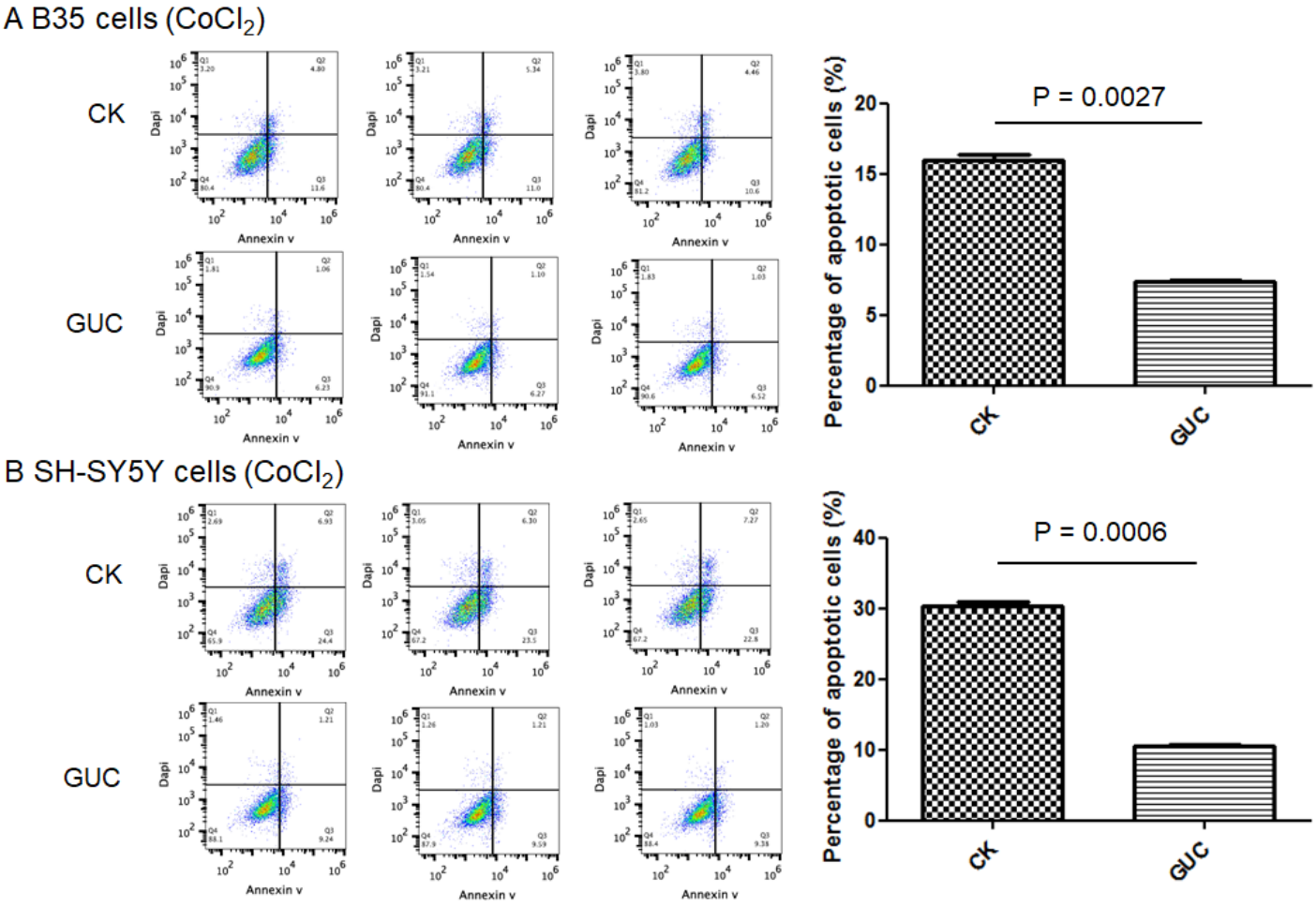

Fig. 2. "*Tianma gouteng ezhu*" protects neuronal survival under hypoxia. GUC reduced the percentage of apoptotic B35 and SH-SY5Y cells after $CoCl_2$ management. CK: control check; GUC: *Gastrodia*, *Uncaria*, and *Curcuma*.

with Alexa Fluor® 555 goat anti-rabbit IgG (1 : 400, Invitrogen Life Technologies) for 1 h to achieve anti-Caspase-7 binding. After that, the slices were rinsed with PBS and counterstained with DAPI. ApoTome. 2 system (Carl Zeiss AG) was used to capture fluorescence images.

2.8 Statistical analysis

The data for each study group were given as mean ± standard deviation (SD). A one-way analysis of variance with Tukey's test was used to compare multiple groups. SPSS for Windows v. 17.0 was used. A two-tailed $P < 0.05$ was the threshold for statistically significant differences.

3. Results

3.1 "Tianma gouteng ezhu" protects neuronal survival under hypoxia

In the B35 cell line, the percentage of apoptotic cells was significantly lower in the group treated with $CoCl_2$ followed by GUC, compared to the control group ($P = 0.0027$) (Fig. 2A). This was also true in the SH-SY5Y cell line ($P < 0.0001$) (Fig. 2B). Further analysis of the apoptotic proteins, the B35 cell line revealed that Bcl-2 and Caspase 3 were reduced ($P < 0.0001$) in the GUC group compared to the control group. At the same time, Bax was distinctly upregulated ($P = 0.0005$) in the GUC group compared to the control group (Fig. 3A). These findings were almost identical in the SH-SY5Y cell line (Fig. 3B).

3.2 The model of MCAO is successfully constructed in the SD rats

Cerebral infarct caused by MCAO was apparent in both the control group and the GUC group, occurring in the right cerebral hemisphere, the cortex, basal ganglia, and hippocampus. GUC treatment was associated with a dramatic reduction of infarct volume of 40% when compared with the control group ($P = 0.0256$) (Fig. 4).

3.3 GUC promotes protection after cerebral ischemia in rats by inhibiting Caspase-7

Immunofluorescence was applied to examine the expression of Caspase-7. The results showed that the expression of Caspase-7 was evident in the MCAO-CK groups (Fig. 5), while hardly evident in the sham group. Furthermore, the expression of Caspase-7 was much lower in the MCAO-GUC group than that of the control group, which suggested that GUC helped to reduce the expression of Caspase-7 *in vivo*.

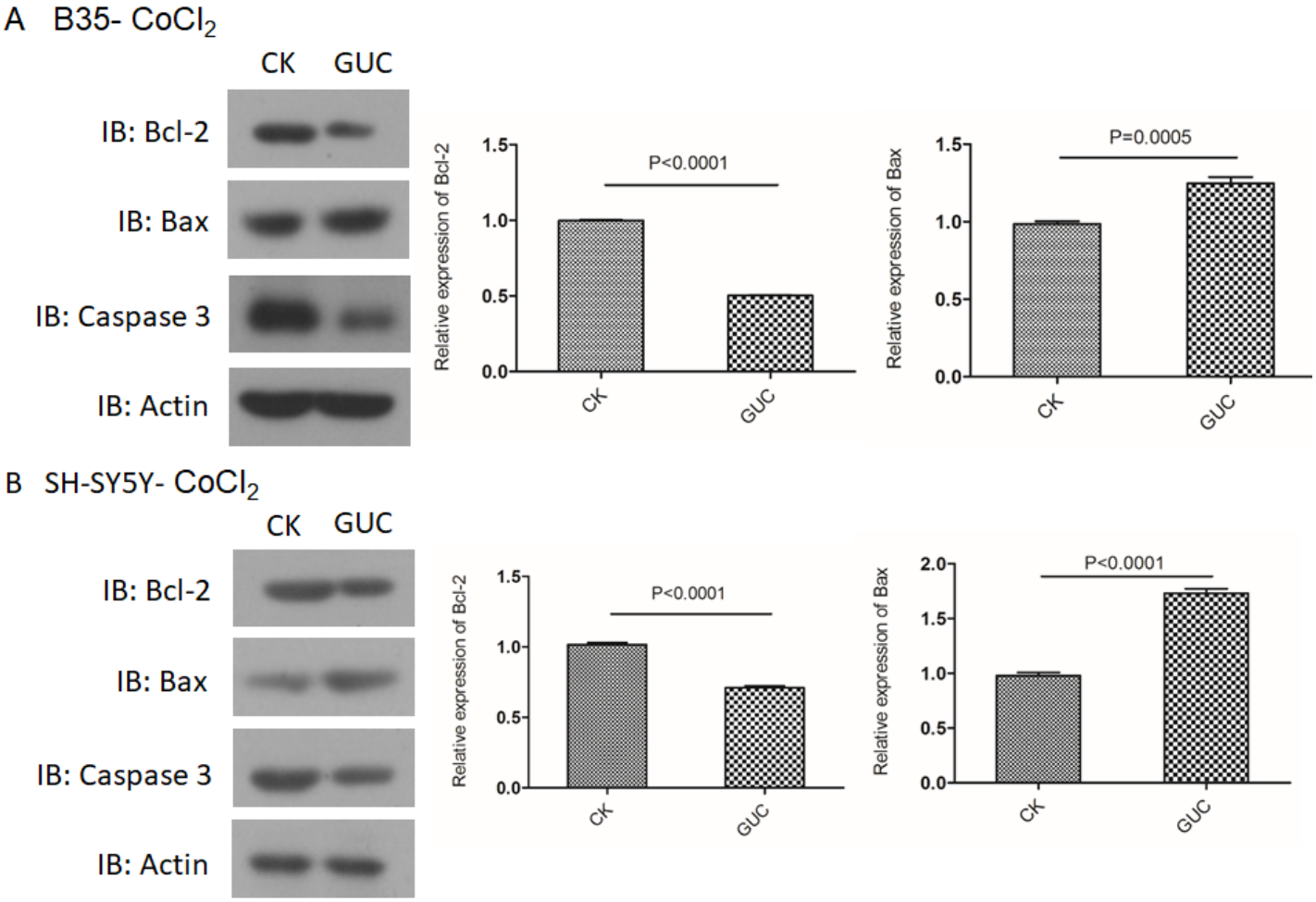

Fig. 3. "*Tianma gouteng ezhu*" reduced apoptotic proteins under hypoxia. (A) Protein bands of Bcl-2, Bax, Caspase-3, and Actin of $CoCl_2$ induced B35 cells treated with or without GUC and relative expression of Bcl-2, Bax, Caspase-3 of $CoCl_2$ induced B35 cells treated with or without GUC. (B) Protein bands of Bcl-2, Bax, Caspase-3, and Actin of $CoCl_2$ induced SH-SY5Y cells treated with or without GUC and relative expression of Bcl-2, Bax, Caspase-3 of $CoCl_2$ induced SH-SY5Y cells treated with or without GUC. CK: control check; GUC: *Gastrodia*, *Uncaria*, and *Curcuma*.

4. Discussion

The application of traditional Chinese medicine in cerebral ischemia is often used in the form of prescriptions. The so-called "Jun Chen Zuo Shi" compatibility method, which emphasizes the combination of therapeutic herbs, has demonstrated prominent effects in stroke, although exact mechanisms of action are unclear (Chen, 2012; Jayakumar et al., 2015). The present study extracted crude *Gastrodia*, *Uncaria*, and *Curcuma* and explored the benefits of their combination in the immediate post-stroke period. According to our results, GUC could protect the B35 and SH-SY5Y cells from $CoCl_2$ induced hypoxia injury by reducing the expression of apoptotic markers. Using an MCAO model to investigate the efficacy of GUC *in vivo*, we showed that the infarct area was reduced with GUC treatment relative to controls. This suggested that more neurons in the penumbra were rescued in the GUC group.

Furthermore, the apoptosis protein Caspase-7 was significantly downregulated with GUC treatment, indicating a restrained onset of the apoptosis pathway. Our results have confirmed the protective effect of water extract of GUC on cerebral ischemia and cell hypoxia. However, the underlying mechanism needs to be further elucidated. Existing data can only indicate that the therapeutic effect is related to the inhibition of the apoptotic pathway. As is known, the apoptosis regulatory network is complex. Experiments have found that active components of *Gastrodia*, *Uncaria*, and *Curcuma* function by different mechanisms to improve neuro-hypoxia/ischemic injury. The phenolic components of *Gastrodia* can relieve cerebral ischemia/reperfusion damage and improve prognosis in rats, and the effects seem to occur via activation of a Nrf2-mediated cell protection system (Alfieri et al., 2013; Kam et al., 2011; Kim et al., 2007; Shi et al., 2018; Yu et al., 2010).

Moreover, 4-hydroxy benzyl alcohol (4-HBA), an important phenolic constituent of *Gastrodia* confers antioxidative, anti-inflammatory and anti-apoptotic effects in neurons and microglia, and that the molecular mechanism underpinning this is anti-Zn^{2+} toxicity (Luo et al., 2018). In another study conducted in rats, the methanol extract of *Uncaria* rhynchophylla was found to protect neurons by inhibiting the induction of cyclooxygenase-2 and the production of TNF-α and NO (Suk et al., 2002). Also, partially purified components of *Uncaria* Sinensis were found to prevent ischemic damage by blood-brain barrier protection by signif-

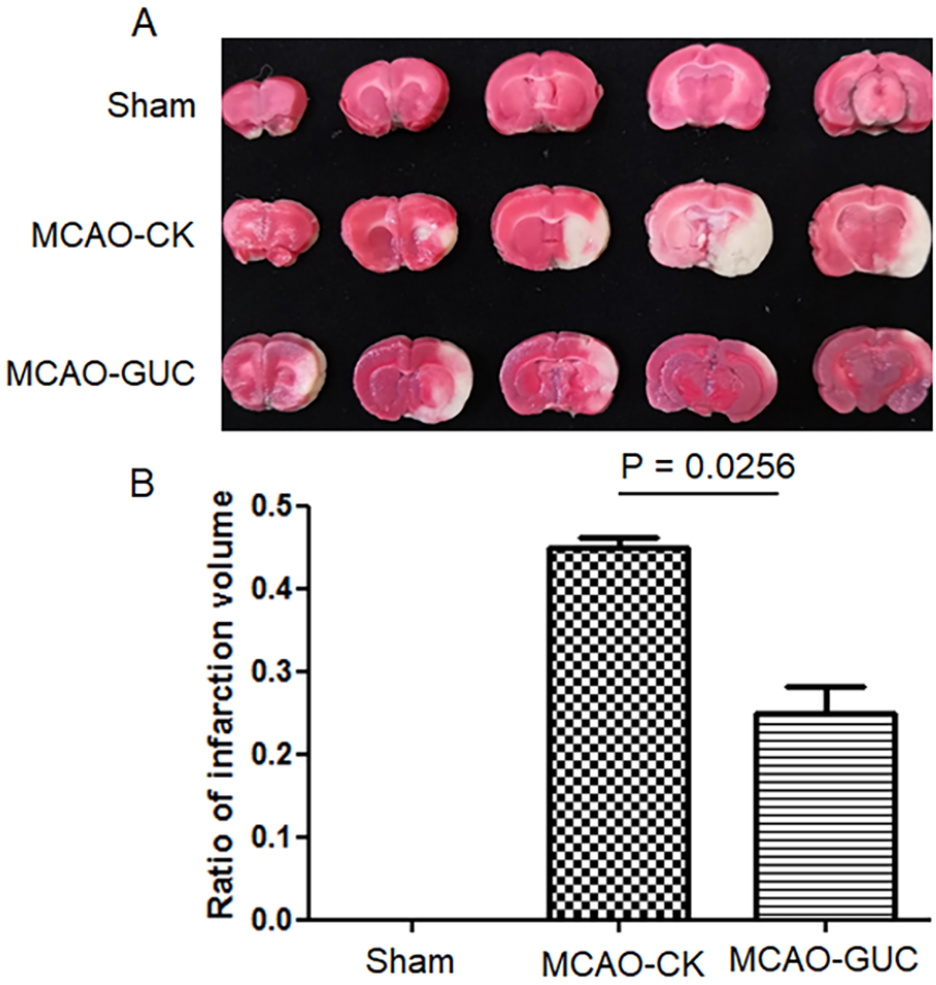

Fig. 4. "*Tianma gouteng ezhu*" enhanced neuronal survival in MCAO models. (A) TTC staining of slices of brains. (B) Statistical analysis of sham, MCAO-CK, and MCAO-GUC groups by oneway ANOVA. MCAO: middle cerebral artery occlusion; CK: control check; GUC: *Gastrodia*, *Uncaria*, and *Curcuma*.

icantly reducing ischemia-induced degradation of tight junction proteins and elevation of matrix metalloproteinase-9 (Seo et al., 2015). One of *Uncaria*'s active constituents, rhynchophylline, has also been found to protect against ischemic damage, possibly via the activation of PI3K/Akt/mTOR signaling and the inhibition of the TLRs/NF-κB pathway (Huang et al., 2014; Yuan et al., 2011). Curcumin has been shown to upregulate expressions of Nrf2 and HO-1 and to protect against focal ischemia in rats (Yang et al., 2009).

Furthermore, curcumin could shift the microglial phenotype from the pro-inflammatory M1 state to the anti-inflammatory and tissue reparative M2 type, as evidenced in mouse (Liu et al., 2017). Indeed, previous studies have confirmed that *Gastrodia*, *Uncaria*, and *Curcuma* alone have protective effects on ischemic brain damage. The essence of TCMs is compatibility, emphasizing the role of prescriptions. Studies of "Tianma Gouteng Yin" in cerebral ischemia support the effectiveness of TCM treatment of cerebral infarction (Xian et al., 2016).

While our research has experimentally demonstrated that GUC has a therapeutic effect on hypoxic-ischemic brain damage via the reduction of apoptosis. In fact, except for GUC, traditional Chinese medicine with the anti-apoptosis effects is commonly practiced in cerebral ischemia. The TCM preparation "Naoshuantong" capsule, Cerebralcare Granule, and Astragalus injection, three traditional Chinese medicine preparations that are widely used clinically in China, are also thought to inhibit the activation of apoptosis, their mechanisms may be associated with the ERK activation, upregulated AKT phosphorylation or down-regulated JNK phosphorylation (Sun et al., 2015).

5. Conclusions

GUC protects B35, and SH-SY5Y cell lines treated with $CoCl_2$ and reduce the infarct volume of brains of SD rats, by decreasing the rate of apoptosis and the expression of apoptotic proteins. The combined use of GUC, therefore, shows promise as a potential treatment for ischemic stroke and hypoxic encephalopathy.

Author contributions

LR and ZW conceived and designed the research. ZHW and BC conducted most of the experiments. YL and JX contributed new reagents or analytical tools and analyzed data and helped with some of the experiments. BC wrote the manuscript. YL ana-

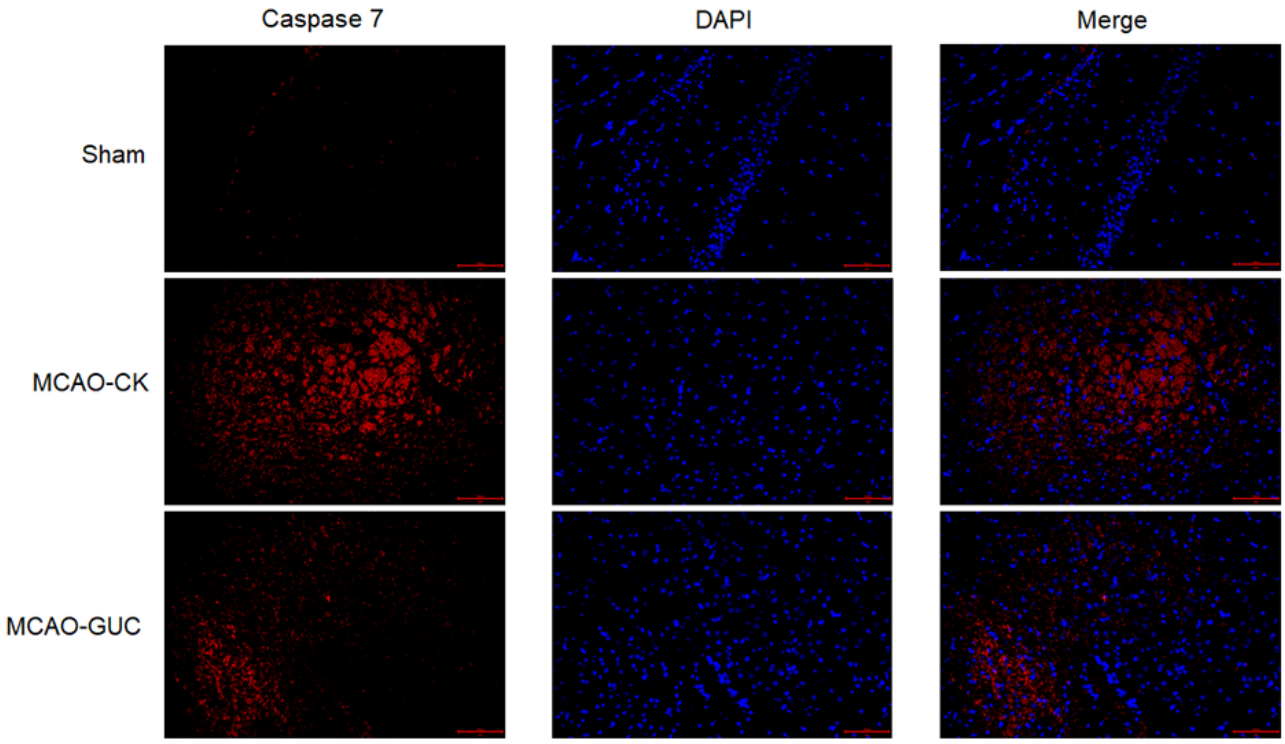

Fig. 5. "*Tianma gouteng ezhu*" reduced Caspase-7 expression in MCAO models. The expression of Caspase-7 was examined by immunofluorescence. The MCAO-GUC group showed a downregulation of the level of Caspase-7 compared to the MCAO-CK group, while in the sham group Caspase-7 was negligible. MCAO: middle cerebral artery occlusion; CK: control check; GUC: *Gastrodia*, *Uncaria*, and *Curcuma*.

lyzed the data and helped revise the draft. All authors read the manuscript carefully, agree with the manuscript content, and are willing to take responsibility for the research contents.

Ethics approval and consent to participate

The animal studies were approved by the Ethics Committee of Shanghai Pudong Hospital, Fudan University.

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Conflict of Interest

The authors declare no conflict of interest.

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