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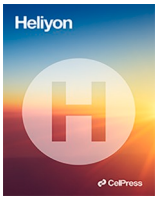

Research article

Tibetan golden acupuncture inhibits JNK/caspase-3 signaling pathway to alleviate neuronal apoptosis in cerebral ischemia-reperfusion injury

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ABSTRACT

Background: Apoptosis induced by cerebral ischemia-reperfusion is one of the key pathological processes of nerve injury. Tibetan golden acupuncture (GA) is a common treatment for ischemic brain injury in Tibetan. The aim of this study was to explore whether GA prevents cerebral ischemia-reperfusion-induced apoptosis in mice by blocking the JNK/caspase-3 pathway.

Methods: In experiment I, 36 mice were randomly divided into a Sham group, CI/RI group, CI/RI + GA group. Morris water maze tests, TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining and flow cytometry (FCM) were used to evaluate the effect of the GA intervention on CI/RI. In experiment II, 30 mice were randomly divided into a Sham group, CI/RI group, CI/RI + GA group, CI/RI + SP group and CI/RI + SP + EA group. Western blotting was used to detect protein expression of key factors in the JNK signaling pathway in the hippocampus.

Results: After 7 and 14 interventions, behavioral evaluations in CI/RI + GA group was significantly different from those in CI/RI groups ($p < 0.01$), pathological injury and apoptosis were significantly reduced ($p < 0.01$). Compared with CI/RI group, the expression of P-JNK/JNK, Cleaved caspase-3/caspase-3, Bax, and Bad proteins in CI/RI + GA group, CI/RI + SP and CI/RI + SP + GA groups were significantly decreased ($p < 0.01$). The expression of B-cell lymphoma 2 (Bcl-2) was significantly increased ($p < 0.01$, $p < 0.05$).

Conclusions: GA can restore neurological dysfunction and inhibit hippocampal neuronal apoptosis in CI/RI mice, at least partially through inhibition of the JNK/Caspase-3 signaling pathway and regulation of apoptosis signals.

1. Introduction

Across the globe, stroke is one of the main causes of death. Every year, strokes claim the lives of around 6 million individuals. Eighty-five percent of strokes are ischemic strokes [1]. It is particularly noteworthy that cerebral ischemia/reperfusion injury (CI/RI) is the main cause of ischemic stroke injury [2,3].

A lot of research has been done in the field of cerebral ischemia-reperfusion. Pathological changes resulting from ischemia/reperfusion injury can be caused by a variety of mechanisms, including anomalies in energy metabolism, oxidative stress, glutamate

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toxicity, Ca2+ overload, excessive nitric oxide (NO) generation, and apoptosis [3–5]. Among them, apoptosis is one of the important factors affecting ischemic encephalopathy. It is one of the main pathogenic mechanisms that cause neurological dysfunction [6]. Therefore, neuronal apoptosis has become one of the research hotspots in the field of cerebral ischemia-reperfusion [7].

c-Jun N-terminal kinase (JNK) is the main signaling pathway that mediates apoptosis of nerve cells. Studies have demonstrated that there are two primary mechanisms via which JNK triggers cellular apoptosis: a transcription-dependent pathway and an independent pathway. Key factors in the JNK transcription-dependent pathway include JNK, P-JNK, p53, activator protein (AP)-1, etc. [8–10]. The key factors of JNK non-transcription-dependent pathway include JNK, P-JNK, Bax, Bcl-2, etc. [11–13].

A lot of work has been done to reduce stroke morbidity and mortality. Of the more than 1000 animal models of stroke that have been evaluated, more than 400 drugs have been shown to be effective [14,15]. Regretfully, the majority of these therapies are not helpful during the acute stage of a stroke, and bleeding and reperfusion injury raise safety issues. Finding safe and efficient interventions is still a pressing issue.

Tibetan golden acupuncture treatment (GA) is one of the external treatment methods in Tibetan medicine. For more than 1000 years, people in Tibet have used it to treat central nervous system injuries, hypertension, epilepsy, and other diseases. GV20 (Baihui) is the most commonly used acupoint in the treatment of stroke with golden acupuncture. Our previous research found that acupuncture at GV20 (Baihui) can reduce the apoptosis of neurons in animal models of cerebral ischemia/reperfusion injury [16–18].

However, uncertainty concerning GA's mode of action is a major barrier to its clinical utilization. The purpose of this work was to determine whether GA suppresses JNK/caspase-3 pathway, which in turn prevents nerve cell apoptosis in cerebral ischemia-reperfusion rats. We expect that this study will help reveal the mechanism of action of GA in the treatment of ischemic brain injury.

2. Materials and methods

2.1. Experimental animals

60 adult healthy male C57BL/6 mice, weighing 25–30 g and aged 8 weeks, were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

Adaptive breeding environment: SPF level animal room.

Adaptive feeding duration: 7 days.

Pre operative preparation: Mice were deprived of water for 12 h before surgery.

Ethics review body: Ethics Committee for Animal Experience and Use Committee of Beijing University of Chinese Medicine (No. BUCM-2023070605-3061)

2.2. Experimental protocols

Experiment I. To observe whether GA has beneficial effects on CI/RI mice, we used the Morris water maze (MWM) and Neurological Severity Score (NSS) to examine the effects of EA on neurological function. TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining and flow cytometry (FCM) were performed to determine the neuronal damage in the hippocampus. 36 mice were randomly divided into 3 groups with 12 mice in each group. Of these, the brain tissue of six was used for FCM detection, and the other six were used for pathological detection.

Sham group: locate the common carotid artery without blocking blood flow.

CI/RI group: BCCAo surgery performed.

CI/RI + GA group: BCCAo + GA.

Experiment II. To determine whether GA-induced anti-apoptotic effects were associated with the JNK/Caspase-3 signaling pathway, Western blotting was performed to measure the changes in the expression of JNK, phosphorylated JNK (P-JNK), JNK, cleaved caspase-3, caspase-3, Bcl-2, Bax and Bad in the hippocampal tissue samples of mice in each group. Accordingly, in Experiment II, 30 mice were randomly divided into five groups (6 mice/group):

Sham group: locate the common carotid artery without blocking blood flow.

CI/RI group: BCCAo surgery performed.

CI/RI + GA group: BCCAo + GA.

CI/RI + SP group: BCCAo + SP600125 (Intraperitoneal injection)

CI/RI + SP + GA group: BCCAo + GA + SP600125 (Intraperitoneal injection)

2.3. BCCAo

Previous research [16] served as the foundation for the BCCAo surgical method. In brief, mice were sedated with pentobarbital (1 %, 50 mg/kg) administered intraperitoneally (i.p.). Locate both common carotid arteries. Bilateral carotid artery blood flow blockage process: block for 20 min, restore blood flow for 10 min, and block again for 20 min. At the same time, 3 ml of blood was taken from the tail vein.

In Sham group, surgical intervention consisted just of Bilateral carotid arteries exposure, with no blood flow restriction or tail venepuncture.

2.4. GA treatment

GA was done (in the CI/RI + GA and CI/RI + SP + GA groups) at GV20. 75 % ethanol was used to sterilize the skin at the acupoint and the golden needle. At GV20, a golden needle (0.35 × 40 mm; ZHONGYANTAIHE, Suzhou, China) was inserted. A 0.5 cm diameter and 0.5 cm height cylindrical moxa roll was put into the needle handle and lighted. A 0.5 cm diameter and 0.5 cm height cylindrical moxa roll was put into the needle handle and lighted. Each roll took 10 min to burn out. GV20 is positioned around the midline of the skull, roughly halfway down an imaginary line connecting the auricle apices. GA treatment began 2 h after surgery, once a day for 14 days.

CI/RI + SP and CI/RI + SP + GA mice were intraperitoneally injected with 30 mg/kg SP600125 60 min before GA treatment (Selleckchem, Houston, TX, USA).

The mice in Sham, CI/RI, and CI/RI + SP groups were kept stationary for 10 min instead of getting GA. There was no animal death during the 14-day GA treatment.

2.5. Neurological Severity Score (NSS)

The neurological deficit score was derived from the behavioral symptoms [19]. After each mouse was assessed separately, the total mouse brain stroke index—a single-blind method—was utilized to calculate the overall scores for each mouse in the experimental group. There are 25 total points available (mild ischemia $\leq$10, severe ischemia >10).

2.6. Morris water maze (MWM) tests

Test time points: before surgery, 24 h after surgery, after 7 treatments, after 14 treatments.

Track tracking device: TECHMAN WMT-100S Water Maze Video Analysis Device, China.

The testing process is based on previous research [16]. The mice swam for an unlimited 60 s in a pool. We used the tracking device to record the travel trajectory and analyze the data.

2.7. Sample collection

After the experiment, the mice were euthanized following an i.p. pentobarbital overdose (1 %, 150 mg/kg). The brains were removed and the hippocampi were dissociated for protein extraction. The remaining mice were perfused with 4 % paraformaldehyde via the heart. The brains were removed and immersed in 4 % paraformaldehyde for histopathological evaluation.

2.8. HE staining

We dewaxed and rehydrated the paraffin slices. The slices underwent two 5-min incubations with xylene and 100 % ethanol, followed by 3-min incubations with 95 %, 90 %, and 80 % ethanol. Subsequently, the slices were stained for ten to 15 min with hematoxylin and then rinsed with water for 10 min. Then, for 1 s, 1 % hydrochloric acid alcohol was applied to visualize blue. Following a 10-min water wash, the slices were stained for 5 min with eosin and twice-dehydrated for 1 min each with 80 %, 95 %, and 100 % ethanol. After being cleaned twice with xylene for 5 min each time, the resultant samples were mounted with neutral gum and captured on camera under a light microscope.

2.9. TUNEL staining

TUNEL detection Kit: Promega, Madison, WI, USA

The specific testing process is based on previous research [16]. The sections were inspected using a light microscope at 400× magnification. Six random locations of sections in the hippocampus CA1 layer were counted in order to determine the cell counts of positive neurons and total neurons. Quantify neuronal damage using the percentage of both.

2.10. Flow cytometry (FCM)

The hippocampal tissues were placed into a 325 standard sieve (opening 0.0018 inches) nylon bag and gently squeezed. The squeezed cells were collected in a flow tube and washed once with PBS at 500 g for 3 min. A total of 1 × 106 cells were harvested and then washed twice with PBS. Cells were re-suspended with a 500 μL 1 × Annexin V binding buffer at a concentration of 1 × 106/mL, followed by the addition of 5 μL Annexin V fluorescein isothiocyanate (FITC) dye and 5 μL propidium iodide (PI) dye. After vortexing, the samples were incubated at RT for 15 min. Then, FACSCanto plus (BD Biosciences, Newark, NJ, USA) was used to determine the apoptosis. Annexin V−/PI− cells represented living cells, while Annexin V+/PI− and Annexin V+/PI + cells represented apoptotic and dead cells, respectively.

2.11. Western blotting

Extract hippocampal protein samples for detecting the expression levels of JNK, P-JNK, caspase-3, cleaved caspase-3, Bcl-2, Bax

and Bad. The specific testing process is based on previous research [16].

The Primary antibodies included the following: anti-JNK3 (1:1000, sc-81469, Santa Cruz), anti-P-JNK3 (1:1000, sc-293136, Santa Cruz), anti-caspase-3 (1:1000, 66470-2-Ig, Proteintech), anti-cleaved caspase-3 (1:1000, 25546-1-A, Proteintech), anti-Bax (1:1000, 23931-1-AP, Proteintech), anti-Bcl-2 (1:1000, 60178-1-Ig, Proteintech), and anti-Bad (1:1000, 10435-1-AP, Proteintech). Anti-GAPDH (1:5000, KGAA002, KeyGEN BioTECH). The secondary antibodies included the following: goat anti-rabbit IgG-HRP (1:5000, KGAA35, KeyGEN BioTECH). Scanning instrument: Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA)

2.12. Statistical analysis

Software: IBM SPSS Statistics 26.0 (SPSS Inc., Chicago, IL, USA)

One-way analysis of variance (ANOVA) was used to assess the data, and for pairwise comparisons, post hoc least significant difference (LSD) tests were used.

Data expression form: mean ± standard deviation (SD)

statistically significant: $p < 0.05$, and $p < 0.01$ was used to conduct the statistical study.

3. Results

3.1. GA improves neurological deficits

There was a significant difference in NSS scores between the groups of mice undergoing BCCAo surgery and the Sham group ($P < 0.01$). Moreover, there was no difference in NSS scores between the groups of mice undergoing BCCAo surgery ($P > 0.05$). The CI/RI model was successfully copied (see Fig. 1).

After 7 and 14 treatments, the NSS scores of the CI/RI + GA group were significantly lower than that of the CI/RI group ($P < 0.01$). The NSS score continued to decrease after 14 treatments compared to 7 treatments ($P < 0.01$) (Fig. 2 (A)).

Before surgery, all mice underwent a 5-day directional cruise experiment (4 times per day per mouse), and there was no statistically significant difference in the escape latency (EL) between the groups of mice ($P > 0.05$). 24 h after surgery, there was a significant increase in EL between the groups of mice receiving BCCAo surgery and the Sham group ($P < 0.01$), and there was no difference between the groups of mice receiving BCCAo surgery ($P > 0.05$). After 7 and 14 treatments, the EL of the CI/RI + GA group was lower than that of the CI/RI group ($P < 0.05$, $P < 0.01$). After 14 treatments, compared to 7 treatments, the EL continued to decrease ($P < 0.05$, $P < 0.01$) (Fig. 2(B–D)).

There was no difference in platform crossings (PC) and time spend in the target square (TSTQ) among the groups of mice before surgery ($P > 0.05$). After 24 h of surgery, the PC and TSTQ of mice in each group receiving BCCAo surgery significantly decreased compared to the Sham group ($P < 0.01$), and there was no difference between the groups of mice receiving BCCAo surgery ($P > 0.05$). After 7 and 14 treatments, the PC and TSTQ of the CI/RI + GA group was higher than that of the CI/RI group ($P < 0.05$, $P < 0.01$). Fig. 2 (E) shows the representative strategies for searching for the platform of each group after 14 treatments.

These results suggest that GA could improve neurological deficits in CI/RI mice.

3.2. GA lowers apoptosis of hippocampal neurons

The HE staining results are as follows: In the Sham group, the cells in the hippocampal CA1 region are arranged in an orderly manner, with normal morphology, intact skeleton, and clear nucleoli. The cells in the CI/RI group are arranged in a disordered and sparse manner, with incomplete skeletons and unclear nucleoli. Compared with the CI/RI group, the CI/RI + GA group had more regular cell arrangement, clearer nucleoli, and more complete structure (Fig. 3 (A)).

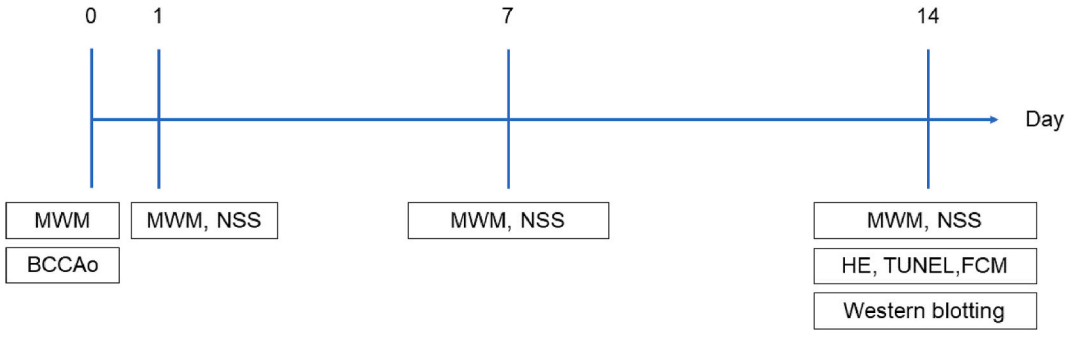

Fig. 1. In Experiment I, 3 groups were analyzed: Sham group, CI/RI group, CI/RI + GA group. We used MWM and NSS to examine the effects of GA on neurological function. TUNEL staining and FCM were performed to determine the neuronal damage in the hippocampus. In Experiment II, 5 groups were analyzed: Sham group, CI/RI group, CI/RI + SP group, CI/RI + GA group, and CI/RI + SP + GA group. Western blotting was performed to measure the changes in the expression of JNK, P-JNK, cleaved caspase-3, caspase-3, Bcl-2, Bax and Bad in the hippocampal tissue samples.

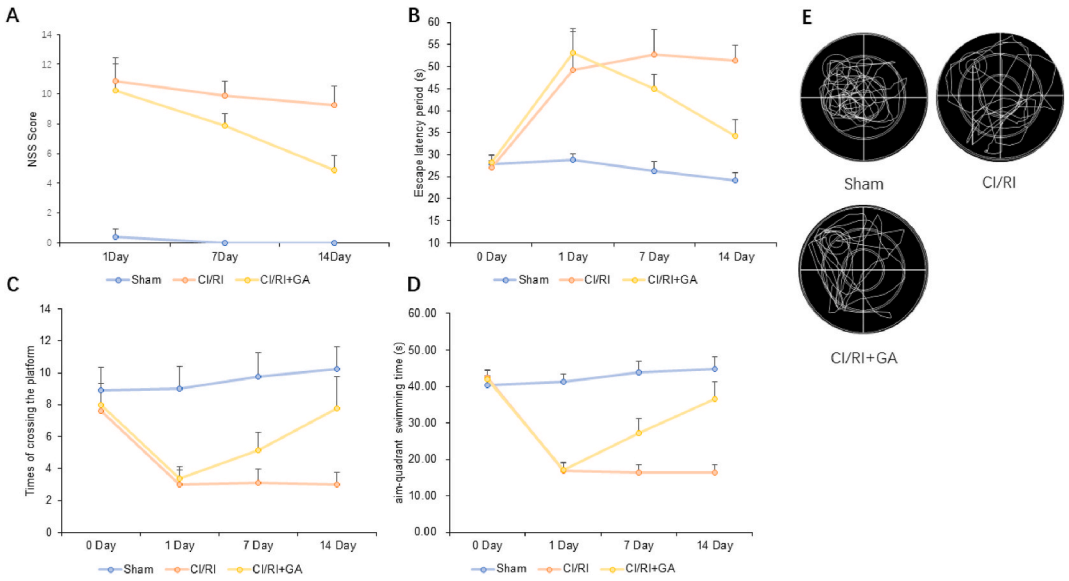

Fig. 2. Results of the NSS test and Morris water maze test before and after intervention (n = 6, mean ± SD). Day 0 is before the modeling procedure, day 1 is 24 h after the procedure, day 7 is after 7 treatments, and day 14 is after 14 treatments. (A) Results of the NSS test of each group. (B) Escape latency of mice in each group. (C) The number of times mice in each group crossed the platform. (D) Duration of stay in quadrant III of each group. (E) Representative strategies for searching for the platform of each group after 14 treatments.
(A) Results of the NSS test of each group. 1 Day: CI/RI, CI/RI + GA vs Sham ($P < 0.01$). 7 Day, 14 Day: CI/RI + GA vs CI/RI ($P < 0.01$).
(B) Escape latency of mice in each group. 0 Day: No significant difference was observed ($P > 0.05$). 1 Day: CI/RI, CI/RI + GA vs Sham ($P < 0.01$). 7 Day: CI/RI + GA vs CI/RI ($P < 0.05$). 14 Day: CI/RI + GA vs CI/RI ($P < 0.01$).
(C) The number of times mice in each group crossed the platform. 0 Day: No significant difference was observed ($P > 0.05$). 1 Day: CI/RI, CI/RI + GA vs Sham ($P < 0.01$). 7 Day, 14 Day: CI/RI + GA vs CI/RI ($P < 0.01$).
(D) Duration of stay in quadrant III of each group. 0 Day: No significant difference was observed ($P > 0.05$). 1 Day: CI/RI, CI/RI + GA vs Sham ($P < 0.01$). 7 Day, 14 Day: CI/RI + GA vs CI/RI ($P < 0.01$).

The TUNEL results are as follows: In the Sham group, the cells in the hippocampal CA1 area are arranged neatly, stained blue, and the nucleoli are clear. The number of positive cells, swollen cells, and incomplete cells increased in the CI/RI group. Compared with the CI/RI group, the positive cells and swollen cells in the CI/RI + GA group (Fig. 3 (B)). Quantify neuronal damage using the percentage of positive neurons to the total number of neurons. The apoptosis rate in the CI/RI group was significantly higher than that in the Sham group ($p < 0.01$). The apoptosis rate of the CI/RI + GA group was significantly lower than that of the CI/RI group ($p < 0.01$) (Fig. 3 (D)).

FCM was used to detect the apoptosis rate of neurons in the hippocampus, which was significantly higher in the CI/RI compared to that of the Sham group ($p < 0.01$). Compared with the CI/RI, the apoptosis rates of the CI/RI + GA group was significantly decreased ($p < 0.05$ and $p < 0.01$) (Fig. 3(C–E)).

3.3. GA inhibits JNK signaling pathway activation

We hypothesized that the inhibitory effect of GA on neuronal apoptosis is related to the JNK signal pathway. To test this possibility, we first detected the expression of p-JNK, JNK, cleared caspase-3 and caspase-3 by Western blotting (Fig. 4 (A)).

Compared to the CI/RI group, significant reductions in p-JNK/JNK and cleaved caspase-3/caspase-3 ratios were observed in the CI/RI + SP, CI/RI + GA and CI/RI + SP + GA groups ($p < 0.01$). The results suggest that GA mainly inhibited activation of JNK in CI/RI mice (Fig. 4(B and C)).

To further confirm the link between the GA intervention and JNK-induced apoptosis, we measured the expression of Bcl-2, Bax and Bad in the mouse hippocampus by Western blotting.

The Western blotting results showed notably higher expression of Bax and Bad in the CI/RI group compared to the Sham group ($p < 0.01$) (Fig. 5(C and D)). The expression of Bcl-2 was significantly lower than that of the Sham group ($p < 0.01$) (Fig. 5(B)). Compared to the expression in the CI/RI group, the expression of Bax and Bad in the CI/RI + SP, CI/RI + GA and CI/RI + SP + GA groups was significantly lower ($p < 0.01$) (Fig. 5(C and D)). The CI/RI + SP, CI/RI + GA, CI/RI + SP + GA groups showed significantly higher expression of Bcl-2 than the CI/RI group ($p < 0.01$, $p < 0.05$) (Fig. 5 (B)).

Furthermore, compared to the expression in the CI/RI + SP group, the expression of Bad in the CI/RI + GA and CI/RI + SP + GA groups was significantly lower ($p < 0.05$) (Fig. 5 (D)).

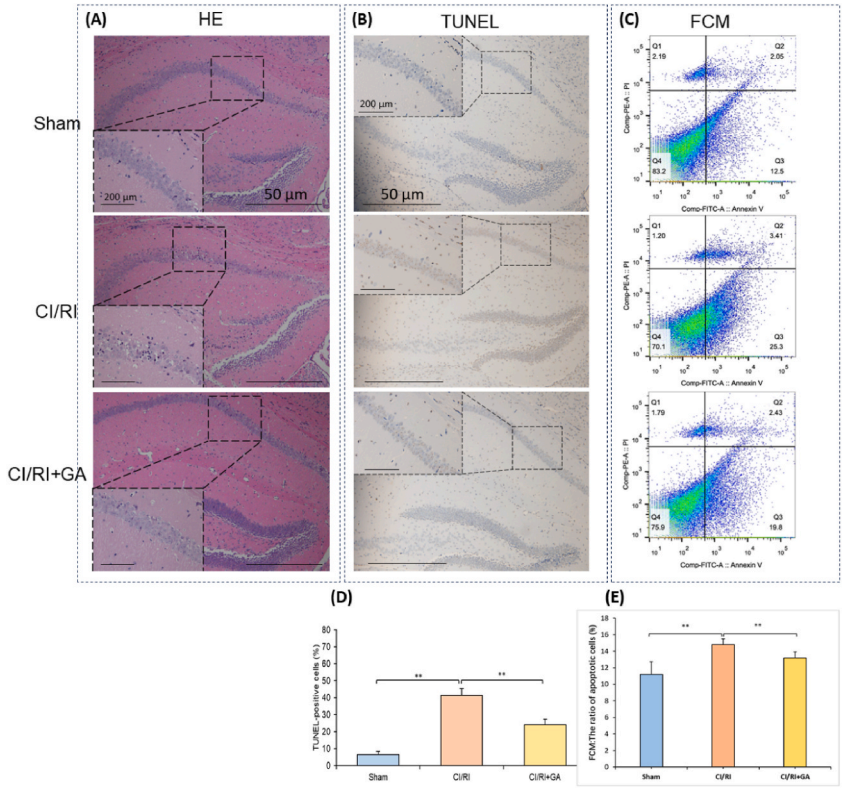

Fig. 3. GA inhibited neuronal injury in the hippocampal. (A) Representative photographs of HE staining. (B) Representative photographs of TUNEL staining. (C) Representative photographs of FCM. (D) Comparison of apoptosis of hippocampal CA1 cells in each group detected by TUNEL. (E) Comparison of hippocampal apoptosis detected by FCM in each group. (n = 6, mean ± SD). *p < 0.05; **p < 0.01.

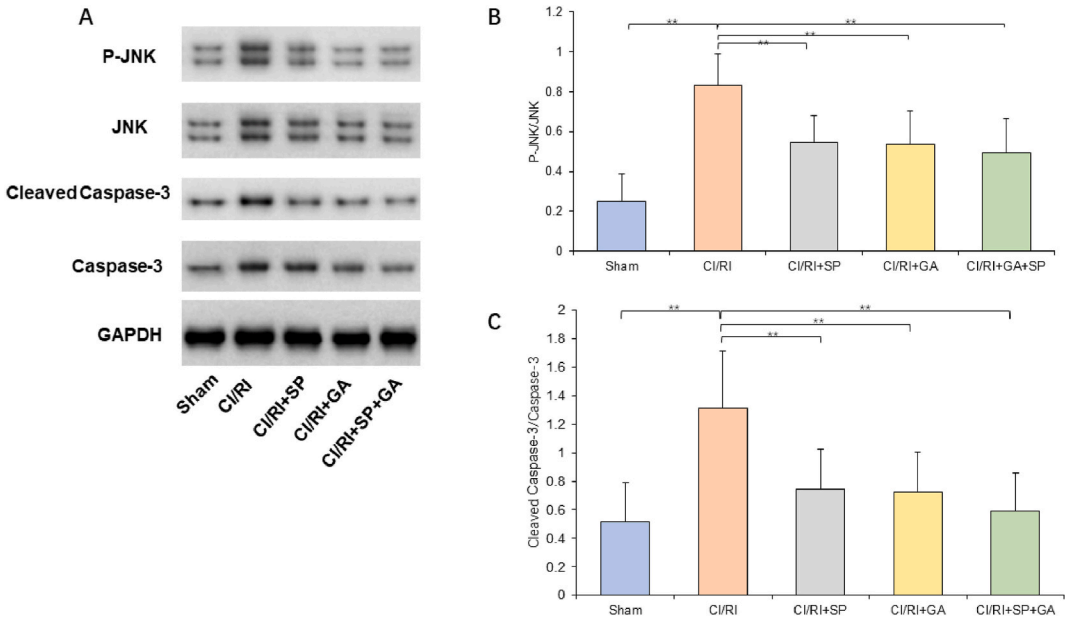

Fig. 4. Comparison of the expression of JNK and caspase-3 in the hippocampus of mice, measured by Western blotting. (A) Relative expression of hippocampal P-JNK, total JNK, cleaved caspase-3 and caspase-3. (B) Ratio of P-JNK to total JNK expression. (C) Ratio of cleaved caspase-3 to caspase-3 expression. Data are mean ± SD (n = 6 per group). *p < 0.05; **p < 0.01.

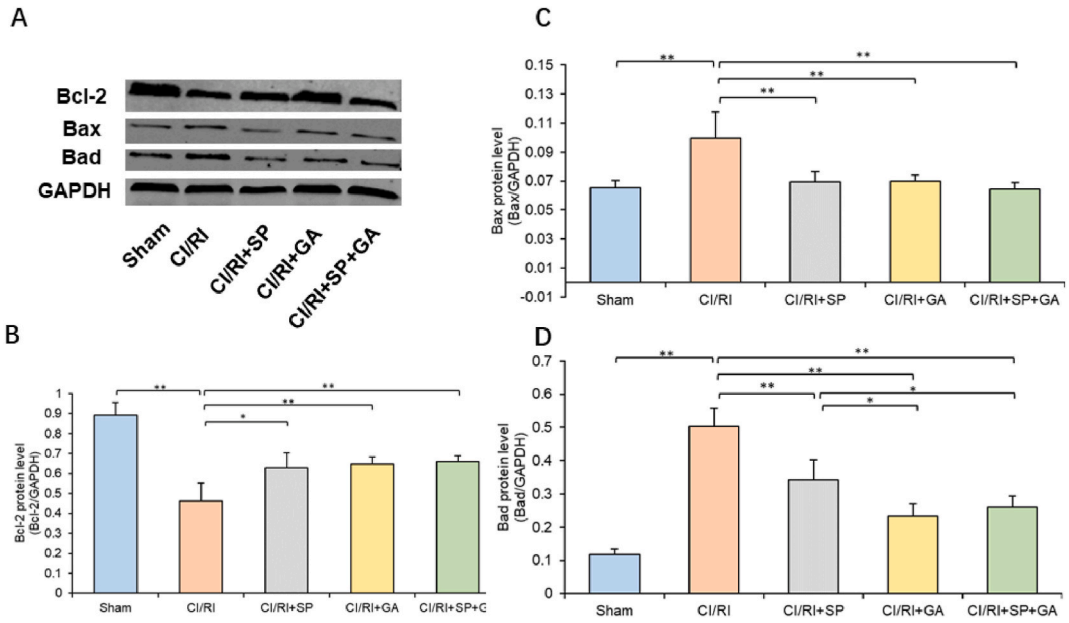

Fig. 5. Comparison of the expression of activator protein Bcl-2, Bax and Bad in the hippocampus of mice, measured by Western blotting. (A) Relative expression of hippocampal Bcl-2, Bax and Bad. (B–D) Densitometry analysis of Bcl-2, Bax and Bad. Data are mean ± SD (n = 6 per group). *p < 0.05; **p < 0.01.

4. Discussion

In this study, GA treatment improved the neural function of CI/RI mice, especially the learning and memory ability, and inhibited the activation of JNK/Caspase-3 pathway in CI/RI mice. The data suggest that GA treatment may be effective in ameliorating neurological impairment, particularly cognitive impairment in CI/RI, at least partially by inhibiting the JNK/Caspase-3 signaling pathway.

The animal model used in this work for the BCCAo-induced CI/RI caused significant neurological impairments, decreased motor and cognitive performance, and frequent histological changes. These results are consistent with earlier research showing that CI/RI mice acquire significant neurological and motor dysfunctions as a result of brain injury [16–18]. On the other hand, GA treatment for one or two weeks improved the neurological score, cognitive dysfunction and enhanced histopathological examination. This finding signifies the neuroprotective ability of GA.

Many studies have found that the death of nerve cells caused by whole brain or local cerebral ischemia occurs mainly through apoptosis [20,21]. Apoptosis is a type of programmed death that is different from cellular necrosis and is precisely regulated by genes. It is an important homeostatic regulation mechanism of the body. TUNEL and FCM results showed that the apoptosis of nerve cells in CI/RI + GA group was significantly decreased compared with that in CI/RI group.

As an important link in the mitogen-activated protein kinase (MAPK) signaling cascade, JNK plays an important regulatory role in the process of cerebral ischemiareperfusion (CIRI)-induced neuronal apoptosis [22]. The JNKs, known as stress-activated protein kinases (SAPKs), belong to the family of MAPKs. JNKs are a family of multifunctional signaling molecules that are activated in response to a wide range of cellular stresses and are involved in the regulation of cellular proliferation, differentiation and apoptosis [23].

JNK signaling pathway can be activated by cytokines, growth factors, stress and other factors. The activation of JNK signals in the brain of mice can be triggered during the modeling process, so there is no special JNK activator group.

SP600125 is a JNK-specific reversible small molecule inhibitor, discovered in 2001, which is insoluble in water and soluble in dimethyl sulfoxide. It exerts its inhibitory effect by competitively binding to the adenosine triphosphate (ATP)-binding sites of JNK1, JNK2 and JNK3. Therefore, SP600125 may potentially reduce JNK activity to prevent neuronal degeneration. In this study, both GA and SP600125 were able to improve neural dysfunction by inhibiting apoptosis of nerve cells. Interestingly, GA has a better inhibitory effect on Bad protein than SP600125. More importantly, the effects of acupucnture may not be mediated exclusively through the JNK signaling pathway, and acupuncture affects multiple CIRI-related pathways [24,25]. In this study, P-JNK levels were decreased to varying degrees after SP600125 or the GA intervention. JNK is a specific transcription factor that activates caspase cascade reactions to initiate cellular apoptosis. Caspase is a key downstream factor of the JNK signaling pathway [26], and caspases act as the effectors of apoptosis downstream of JNK [27].

On the other hand, caspases can induce JNK phosphorylation by activating upstream protein kinases in the MAPK pathway, leading to apoptosis [28]. Cysteinyl aspartate–specific proteases or caspases are protease proteins that are activated at the end of the process of apoptosis to lyse the membrane and degrade the DNA in the damaged cell [29]. Caspase-3 activation is a sensitive assay used to determine cell death via the apoptotic pathway [30,31].

The JNK signaling pathway also can rapidly exert corresponding biological effects by directly regulating the structure and function of cytoplasmic substrates. A part of activated JNK stays in the cytoplasm and directly regulates the activity of Bcl-2 family members (Bim, Bax, Bcl-2) through phosphorylation, thereby mediating apoptosis in the mitochondrial pathway [32]. This process does not rely on the expression of new genes. The Bcl-2 family is the main regulator of the JNK transcription-independent route. It is divided into three categories: (1) pro-apoptotic proteins, such as Bad and Bax; (2) anti apoptotic proteins, such as Bcl-2 and Bcl-xL and (3) BH3 only proteins, such as Bim and Bid [33]. Among them, Bax is the main mediator of the mitochondrial pathway. Activated Bax translocates to the outer mitochondrial membrane to increase its permeability and release pro apoptotic mitochondrial proteins into the cytoplasm, which initiate the caspase-dependent mitochondrial pathway of apoptosis by forming apoptotic bodies.50 Our current study found that the expression of Bcl-2 decreased in the hippocampus of BCCAo model mice, while the expression of Bax and Bad increased. Our results also showed that GA could increase the expression of Bcl-2 and reduce the expression of Bax and Bad. We speculate that GA can also play a regulatory role in the downstream proteins of the JNK pathway.

5. Conclusion

In conclusion, this work offers crucial preclinical evidence that GA, at least in part, inhibits the JNK/caspase-3 signaling pathway and controls apoptotic signals to cure neurological dysfunction, including cognitive abnormalities, in CI/RI mice. In CI/RI mice, our results similarly showed a moderate synergy between the GA and SP600125 treatments. All things considered, these findings imply that GA might be useful in addition to or instead of CI/RI, and more clinical research is necessary.

CRediT authorship contribution statement

Yaru Liu: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation. **Lamu Yixi:** Writing – review & editing, Methodology, Formal analysis. **Guilin Jin:** Writing – review & editing, Methodology. **Mingke Feng:** Methodology. **Chun hua:** Methodology. **Da wa:** Methodology.

Ethics statement

The experimental protocols used in this study were reviewed and approved by the Ethics Committee for Animal Experimentation and Use Committee of Beijing University of Chinese Medicine (No. BUCM-2023070605-3061) before performing experiments. The work described has been carried out in accordance with either the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive 2010/63/EU or the National Institutes of Health – Office of Laboratory Animal Welfare policies and laws. All animal studies comply with the ARRIVE guidelines.

Data availability statement

The data associated with this study has not been uploaded into a publicly available repository. However, data will be made available on reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

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