Compatibility of ingredients of Danshen (Radix *Salviae Miltiorrhizae*) and Honghua (*Flos Carthami*) and their protective effects on cerebral ischemia-reperfusion injury in rats

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Abstract. Danshen (Radix *Salviae Miltiorrhizae*) and Honghua (*Flos Carthami*) (Danhong) are two drugs commonly prescribed together, which are often used in the treatment of cerebrovascular diseases in China. Due to the complexity of the ingredients of Danhong, the present study focused on performing the orthogonal compatibility method on the primary effective molecules of this drug: Tanshinol, salvianolic acid A, salvianolic acid B and hydroxysafflor yellow A. These four molecules were studied to determine their protective effects and to screen for the most compatible ingredients to improve cerebral ischemia-reperfusion injury (IR) in rats. Focal middle cerebral artery occlusion was performed to establish the cerebral IR model in rats. Male Sprague-Dawley rats were randomly divided into sham operation group, IR group and nine orthogonal administration groups with different ratios of Danhong effective ingredients and Danhong injection group. Neurological deficit score and cerebral infarction volume were measured postoperatively. Morphological pathological alterations were observed via H&E staining. Bcl-2 and Bax were quantified using ELISA. Immunohistochemistry was conducted to analyze the expression of caspase-3 in the hippocampus. The expression levels of cytochrome *c*, apoptotic peptidase activating factor 1 (apaf-1), caspase-9, caspase-3 and p53 mRNA in the hippocampus were assessed via reverse transcription-quantitative PCR. The results demonstrated that different compatibility groups significantly reduced the neurological function score and decreased the volume of cerebral infarct compared with the IR group. These groups were also indicated to improve the pathological damage to the brain tissue. In addition, certain compatibility groups significantly decreased the number of caspase-3 positive cells in the hippocampus and the expression levels of cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53 mRNA in the brain tissue. Orthogonal group 4 (30 mg/kg tanshinol; 2.5 mg/kg salvianolic acid A; 16 mg/kg salvianolic acid B; 8 mg/kg hydroxysafflor yellow A) was indicated to be the most effective. The four effective ingredients of Danhong exhibited a protective effect on rats with cerebral IR injury, potentially through the inhibition of apoptosis via the downregulation of key targets upstream of the caspase-3 pathway. In addition, the present study provided novel insights for the continued study of the drug compatibility rules of TCM.

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Introduction

Stroke has become the main clinical type of cerebrovascular disease, which is a type of disorder of blood circulation in brain tissues (1). Pathologically, stroke can be divided into ischemic stroke and hemorrhagic stroke (2). More than 80% of the global burden of stroke is attributed to ischemic stroke (3). Ischemic strokes often present with high rates of incidence, recurrence, disability and mortality for patients (4). In 2008, an epidemiological survey indicated that strokes, with an incidence of 136.64 per 100,000 individuals, had replaced cancer as the leading cause of mortality in China (5). At present, intravenous thrombolytic therapy is the main clinical treatment for ischemic stroke (1,6), and there is still a lack of effective drugs to protect neurons from death. Therefore, there is a need for multi-target and improved therapeutic drugs, which is why the beneficial effects of Traditional Chinese Medicine (TCM) is worth investigating (7).

Danshen and Honghua (Danhong) are classic blood-activating drugs often used for promoting blood circulation and believed to remove blood stasis in TCM. They have a long

history in the treatment of cardiovascular and cerebrovascular diseases in traditional clinical trials (8-11). With the progress of modern research and separation technology, it has been revealed that the primary effective ingredients in Danshen are tanshinol, salvianolic acid A and salvianolic acid B. These water-soluble molecules were indicated to exhibit a variety of favorable effects, including neuroprotective activity, antioxidation, regenerative effects and responses similar to those of an antidepressant (12-14). Hydroxysafflor yellow A is the main bioactive component in Honghua, which could protect against ischemic stroke by promoting the dilation of cerebral vessels to improve cerebrovascular permeability (15-17). In addition, these four molecules displayed protective and regulatory effects on disturbed metabolism and the regulation of neuroinflammatory responses (17-22).

Collectively, the four effective ingredients of Danhong were indicated to attenuate cerebral ischemic injury *in vitro* (23). In the present study, the orthogonal compatibility of the four effective ingredients of Danhong (tanshinol, salvianolic acid A, salvianolic acid B and hydroxysafflor yellow A) were examined to explore the protective effect of Danhong on cerebral ischemia-reperfusion (IR) injury in rats. The current study aimed to provide novel insights and guidance for the clinical and experimental treatment of ischemic cerebrovascular disease.

Materials and methods

Animals. Healthy adult male Sprague-Dawley rats (total, 216; weighing 260-300 g) with clean grade were purchased from Zhejiang Laboratory Animal Center. Animal license number was SCXK (Zhejiang) 2014-0001. The temperature of the animal room was controlled at 25±1˚C, and air humidity was 60-65%. The rats were placed in a 12:12 h light/dark cycle with access to food and water *ad libitum*. The rats were euthanized via cervical dislocation under pentobarbital sodium anesthesia [1% in normal saline (NS); 35 mg/kg; intraperitoneally administered].

Chemicals and reagents. Danhong injection was supplied by Shandong Buchang Pharmaceuticals Co., Ltd. 2,3,5-triphenyltetrazolium chloride (TTC) and H&E were obtained from ShangHai SSS Reagent Co., Ltd. Xylene was purchased from Huadong Medicine Co., Ltd. Rat Bcl-2 (cat. no. MB-7297B) and Bax ELISA kits (cat. no. MB-6629A) were obtained from Shanghai YuanYe Biotechnology Co., Ltd. DAB chromogenic kit (cat. no. ZLI-9018) was obtained from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. TRIzol® reagent (cat. no. 15596-026) and caspase-3 antibody (1:100 dilution; cat. no. 43-7800) were purchased from Thermo Fisher Scientific, Inc. Tanshinol (purity >98%; cat. no. 76822-21-4; batch. no. SZ201707038), salvianolic acid A (purity >98%; cat. no. 96574-01-5; batch. no. SZ201706001), salvianolic acid B (purity >98%; cat. no. 121521-90-2; batch. no. SZ201706003) and hydroxysafflor yellow A (purity >98%; cat. no. 78281-02-4; batch. no. Z201702005) were obtained from Nanjing Shizhou Biotechnology Co., Ltd.

Instruments. The instruments used in the present study were as follows: OHAUS AR153CN electronic balance (OHAUS Instruments Shanghai Co., Ltd.), analytical balance (Mettler Toledo), Pall Cascada Bio Mk2 Water Filtration system (Pall Life Sciences), fluorescence quantitative PCR instrument (Bio-Rad Laboratories, Inc.), ZH-003 stainless steel brain matrices (Anhui Zhenghua Biological Equipment Co., Ltd.), Rotary Microtome Microm HM 340E (Thermo Fisher Scientific, Inc.) and Leica DM LB2 microscope camera (Leica Microsystems GmbH).

Transient focal cerebral ischemia model. The experimental procedure was developed and performed after certain adjustments to the method by Longa *et al* (24). The rats were anesthetized intraperitoneally with 1% pentobarbital sodium (35 mg/kg). Their body temperature was kept constant at 37˚C. The rats were immobilized in the supine position and sanitized with alcohol before the skin was removed. A median longitudinal incision was made on the neck. The superficial fascia was cut from the bilateral submandibular glands to expose one side of the mastoid muscle. The muscle gap was bluntly separated between the right sternocleidomastoid muscle and the sternohyoid muscle to expose the right side. This allowed for visualization of three major blood vessels: The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). The root of the ECA and the proximal end of the CCA were ligated and the ICA was clamped with an arterial clip. Subsequently, a nylon wire with a smooth rounded tip (diameter, 0.28-mm; Beijing Cinontech Co., Ltd.) was inserted from CCA into ICA gently, and the arterial clip was removed. The insertion depth was stopped at the origin of the middle cerebral artery (18-20 mm), and the ischemic time was recorded. After ischemia for 1 h, the wire was gently withdrawn for reperfusion, and rats were euthanized 3 days after. The incision was sutured layer by layer and disinfected. The rats were returned to their cages and kept in the lateral position after the operation, and their body temperature was maintained at 37˚C.

Groups and treatment. Sprague-Dawley rats were randomly divided in one of 12 groups: Sham operation (sham), IR untreated model (IRU), Danhong injection group (DHI) and orthogonal groups [L_9 (3^4)]. The nine different combinations of the four key ingredients of Danhong were prepared according to orthogonal experimental design (25,26), which is a design method to study multi-factors and multi-levels. The orthogonal design is presented in Table I. For example, Group 1 is made of four components at dose 1 (15 mg/kg tanshinol, 2.5 mg/kg salvianolic acid A, 8 mg/kg salvianolic acid B and 2 mg/kg hydroxysafflor yellow A). The doses of the four individual components were all within the safe range according to previous pharmacological research and related literature (16-22).

Each group contained 18 rats (six rats were used for TTC staining, H&E staining and immunohistochemistry and PCR, respectively). Firstly, the drug was dissolved in physiological saline. Subsequently, the orthogonal group dose was administered to the tail vein of the L_9 (3^4) groups directly at 0 h after reperfusion. Sham and IRU groups were administered an equal amount of physiological saline. The positive control group was administered Danhong injection (2 ml/kg) (27-29).

Table I. Doses of nine compatibility groups of four effective ingredients according to L_9 (3^4).

	Dose (mg/kg)			
Group	A	B	C	D
1	15	2.5	8	2
2	15	5	16	4
3	15	10	24	8
4	30	2.5	16	8
5	30	5	24	2
6	30	10	8	4
7	60	2.5	24	4
8	60	5	8	8
9	60	10	16	2

A, tanshinol; B, salvianolic acid A; C, salvianolic acid B; D, hydroxysafflor yellow A.

Neurological assessments. Assessments of neurological function were performed following reperfusion in accordance with previously described methods (24). Neurological function was assessed using the modified five-point scale scoring system ranging from 0 to 4, with higher scores being indicative of a more severe neurological impairment. Rats with scores of 1-4 following MCAO were used for analysis.

Measurement of infarct volume. Rats were euthanized under anesthesia on the 3rd day after surgery for TTC staining. The rat brains were cut into small sections (2.0 mm), immersed in 2% TTC at 37˚C for 30 min. Areas of red staining indicated normal brain tissue, and pale gray areas represented infarcted tissue. Image-Pro Plus v6.0 software (Media Cybernetics, Inc.) was used to calculate the infarct volumes. The following formula was used to calculate cerebral infarction rate: Infarct Rate=Infarct Volume/Whole Brain Volume x100%.

H&E staining. A total of 3 days after cerebral IR, the rats were anesthetized with 35 mg/kg pentobarbital sodium and then fixed with 200 ml 4% paraformaldehyde via perfusion of the heart until the right atrial appendage produced clear liquid. The rats were decapitated, and the brains were fixed in 4% paraformaldehyde (Ph 7.4) for 24 h at 4˚C. After gradient elution (100 and 95% ethanol for 5 min, respectively), brain tissues were embedded in paraffin and serially sliced (3-4 μm). Subsequently, the slices were immersed in hematoxylin for 5 min and eosin for 2 min at room temperature. The results of H&E staining were observed under a light microscope (magnification, x100).

Measurement of Bcl-2 and Bax levels in serum. At day 3 after MCAO, the rats were deeply anesthetized with 35 mg/kg pentobarbital sodium. A total of ~6 ml blood was drawn from the abdominal aorta and subsequently centrifuged at 1500 x g for 15 min at 4˚C. The levels of Bcl-2 and Bax in the serum were measured via ELISA using commercially available kits according to the manufacturer's instructions.

Immunohistochemistry. After fixation, embedding and routine paraffin sectioning of 3-4-μm as aforementioned, the experiment followed the procedure of DAKO En Vision™ two-step immunohistochemistry kit (cat. no. K5007; Hangzhou Xincheng Biotech Co., Ltd.) (30). Under a light microscope (magnification, x200), the positive cell status of immunohistochemistry was shown as yellow or yellow brown in the cytoplasm. The staining result was determined based on immunoreactivity score (31) by multiplying the intensity of staining (0=not stained; 1=low intensity; 2=moderate intensity; 3=high intensity) and the percentage of immune positive cells (0=not stained; 1=1-10%; 2=11-50%; 3=51-80%; 4≥80%).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Frozen brain tissue was placed in a centrifuge tube and the RNA from the right hippocampus of each group of rats was extracted with TRIzol® reagent. RNA concentration and purity were determined using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Inc.). The extracted RNA was then reverse transcribed into cDNA using a ThermoScript RT-PCR system (cat. no. 11146016; Toyobo Life Science) according to the manufacturer's instructions. The target genes and GAPDH internal reference gene (Sangon Biotech Co., Ltd.) were amplified by an Applied Biosystems 7500 Fast RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 94˚C for 3 min, followed by 95˚C for 10 sec, 58˚C for 30 sec and 72˚C for 15 sec for a total of 40 cycles. After the reaction was completed, melting curve analysis was performed to identify the specificity of the PCR reaction product. The relative expression of each target gene normalized to GAPDH was analyzed using the $2^{-\Delta\Delta Cq}$ method (32). The primer sequences are listed in Table II.

Statistical data analysis. All statistical analyses were performed using SPSS v25.0 software (IBM Corp.), and one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test was used. Data are presented as the mean ± standard deviation or the median (interquartile range) for normally or nonnormally distributed parameters, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of compatibility groups of four effective ingredients on neurological deficits in rats with cerebral IR injury. The neurological deficit of the IRU group was more severe ($P<0.01$) than that of the sham group. Compared with the IRU group, the DHI group indicated a significant improvement in the symptoms of neurological deficit ($P<0.05$). In addition, all orthogonal compatibility groups were indicated to exhibit an improvement in the symptoms of neurological deficit to different degrees compared with IRU group. Specifically, the symptoms of neurological deficit in the orthogonal groups 4 and 6 were more similar to those in DHI group, and had an improved neurological score compared with the other orthogonal groups. These results are presented in Table III.

Table II. Primer sequences of selected genes designed for reverse transcription-quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Apaf-1	TGGATGAAGCCATGTCCATA	TCCCAGAGAACACACAGCAC
Cytochrome *c*	AAGACTGGACCAAACCTCCA	CTCCATCAGGGTATCCTCTCC
Caspase-9	GCCTCATCATCAACAACGTG	CTTCACCTCCACCATGAAGC
Caspase-3	CTGGACTGCGGTATTGAG	GGGTGCGGTAGAGTAAGC
p53	GCTGAGTATCTGGACGACA	CAGGCACAAACACGAACC
GAPDH	GGAAATCGTGCGTGACATTA	AGGAAGGAAGGCTGGAAGAG

Apaf1, apoptotic peptidase activating factor 1.

Table III. Effects of compatibility groups of four effective ingredients on neurological deficit in rats with cerebral IR injury.

Group	Neurological score
1	2 (2-2.25)
2	2 (1.75-2)
3	2 (2-2)
4	2 (1-2)
5	2 (1.75-2.25)
6	2 (1-2)
7	2 (2-2.25)
8	2 (2-2.25)
9	2 (1.75-2)
Sham	0
IRU	3 (2.75-3)[a]
DHI	1.5 (1-2)[b]

Values are expressed as the median (interquartile range) (n=6). [a]P<0.01 vs. sham group; [b]P<0.05 vs. IRU group. IR, ischemia-reperfusion; IRU, IR untreated; DHI, Danhong injection.

Effects of compatibility groups of four effective ingredients on cerebral infarct volume in rats with cerebral IR injury. Following TTC staining, the brain sections of the sham operation group appeared red. The cerebral infarct area of the IRU group was more pronounced (P<0.01) than that of the sham group. Compared with the IRU group, the infarct volume of the DHI group was observed to be significantly reduced (P<0.01). In addition, the cerebral infarct volume of each drug group decreased to different degrees. The cerebral infarct volume in the orthogonal compatibility groups 2, 4, 6 and 7 was significantly decreased compared with the sham group (P<0.01 or P<0.05). These results are presented in Fig. 1.

Effects of compatibility groups of four effective ingredients on pathological alterations of brain tissue in rats with cerebral IR injury. There was no evident pathological damage in the brain tissue of the sham group (Fig. 2A). The structure was normal and clear: The arrangement of cells were tight and uniform, the nucleus was intact and the intercellular space was normal without edema. Typical necrotic foci were observed in the brain tissue of the IRU group (Fig. 2B). Cell edema was visible, the number of cells was reduced, and the arrangement of cells was sparse and disordered. In addition, the boundaries between cells were blurred, the nuclei were atrophied, and a triangular dense nucleus was visible. Compared with the IRU group, brain tissue damage was markedly improved in the DHI group (Fig. 2C). The DHI group presented an increased number of normal neurons and only partial edema degeneration. The orthogonal compatibility of Danshen and Honghua was observed to be most effective in the reduction of pathological tissue damage in groups 2 and 4. These results are presented in Fig. 2.

Effects of compatibility groups of four effective ingredients on the expression levels of Bcl-2 and Bax in the serum of rats with cerebral IR injury. The serum ratio of Bcl-2/Bax in the IRU group was significantly lower (P<0.01) than the serum ratio of the sham group. When compared with the IRU group, the DHI group and the orthogonal administration groups (groups 2, 3, 4, 5, 6 and 8) indicated a significant increase in the Bcl-2/Bax ratio (P<0.01 or P<0.05). In addition, there was no significant difference observed among orthogonal groups (groups 2, 3, 4, 5, 6 and 8) and the DHI group (P>0.05). However, orthogonal groups 1, 7 and 9 showed statistical difference compared with DHI group (P<0.01 or P<0.05). The result of group 4 was the closest to that of DHI group, which indicates that group 4 and the DHI group exhibited similar efficacy. These results are presented in Table IV.

Effect of DHI and compatibility groups of four effective ingredients on caspase-3 expression in the CA1 area of the hippocampus as detected by immunohistochemistry. Rats in the sham-operated group (Fig. 3A) exhibited low numbers of yellow brown caspase-3 positive cells in the CA1 area of the hippocampus. When compared with the sham group, the IRU group (Fig. 3B) presented increased cytoplasmic staining of caspase-3 in the hippocampal CA1 region (P<0.01). Orthogonal group 4 was observed to exhibit significantly reduced expression of caspase-3 protein (P<0.05) when compared with the IRU group. The results are reflected in Fig. 3 and Table V.

Expression levels of cytochrome c, apoptotic peptidase activating factor 1 (apaf-1), caspase-9, caspase-3 and p53 mRNA. RT-qPCR results indicated that the expression level of cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53 mRNA in the

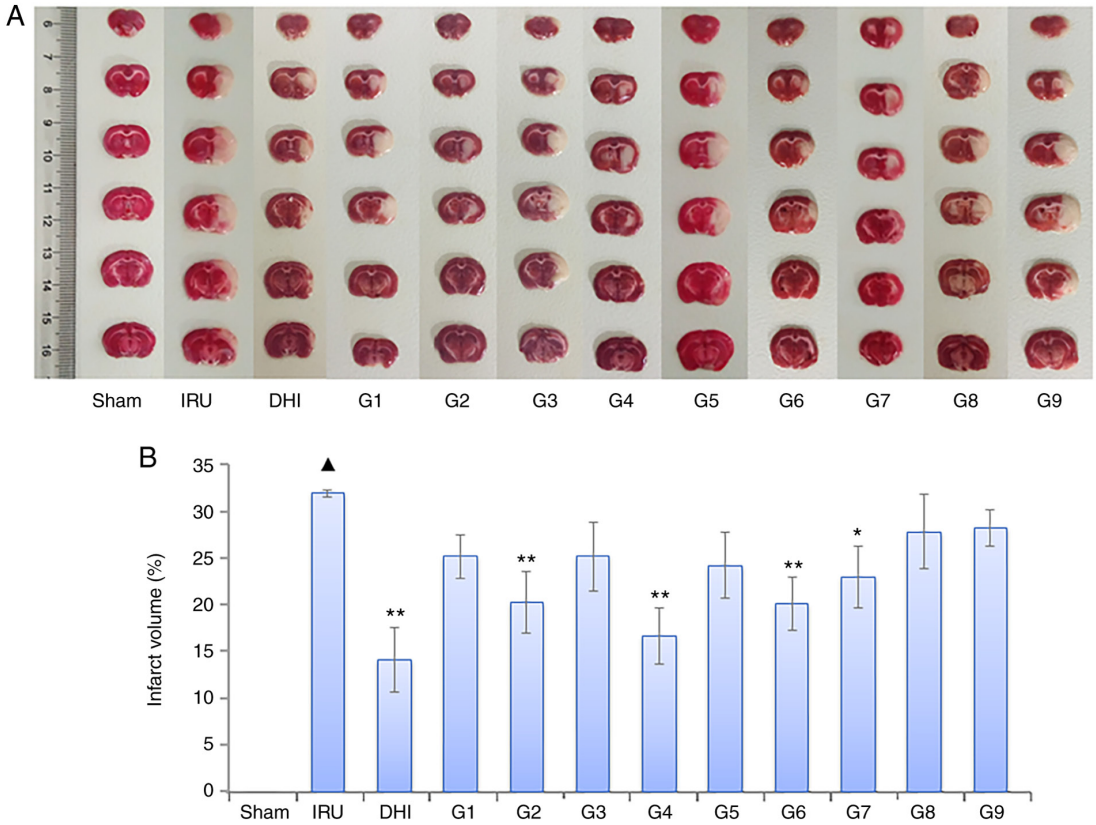

Figure 1. Effect of DHI and orthogonal groups on cerebral infarct volume in rats after focal cerebral IR. Infarct volume was assessed by TTC staining at day 3 after middle cerebral artery occlusion. (A) Representative TTC staining of the cerebral infarct in coronal sections of rat brain. (B) Infarct volumes assessed by TTC staining. The data are presented as the mean ± standard deviation (n=6). ▲P<0.01 vs. sham; *P<0.05, **P<0.01 vs. IRU group. IR, ischemia-reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride; IRU, IR untreated; DHI, Danhong injection; G, orthogonal group.

Table IV. Effects of compatibility groups of four effective ingredients on the secretion of Bcl-2 and Bax in the serum of rats with cerebral IR injury.

Group	Bcl-2/ng·ml^{-1}	Bax/ng·ml^{-1}	Bcl-2/Bax
1	97.58±9.30	6.52±1.02^{a}	14.97±1.43^{b}
2	109.08±11.86	5.04±0.79^{c}	21.64±2.35^{c}
3	102.15±11.25	5.64±0.72^{c}	18.11±1.99^{d}
4	117.33±16.11^{c}	4.53±0.52^{c}	25.90±3.56^{c}
5	101.08±11.88	5.84±0.93^{c}	17.31±2.03^{d}
6	107.88±11.06	5.09±0.60^{c}	21.19±2.17^{c}
7	98.12±11.32	6.18±0.84^{b}	15.88±1.83^{b}
8	102.56±11.70	5.69±0.66^{c}	18.02±2.06^{d}
9	99.76±12.82	7.51±1.05^{a}	13.28±1.71^{a}
Sham	149.38±26.59	3.48±0.18	42.93±7.64
IRU	82.17±18.34^{e}	7.67±0.75^{e}	10.71±2.39^{e}
DHI	121. 21±17.79^{c}	4.49±0.39^{c}	27.00±3.96^{c}

Values are expressed as the mean ± SD (n=6). ^{a}P<0.01 and ^{b}P<0.05 vs. DHI group; ^{c}P<0.01 and ^{d}P<0.05 vs. IRU group; ^{e}P<0.01 vs. sham group. IR, ischemia-reperfusion; IRU, IR untreated; DHI, Danhong injection.

Table V. Effect of compatibility groups of four effective ingredients on caspase-3 protein expression in rats after cerebral IR injury.

Group	Caspase-3
1	6 (4.75-6.25)
2	4 (2.75-5)
3	4 (3.75-5)
4	3.5 (2.75-4)^{a}
5	4.5 (3.5-6)
6	4.5 (3.75-5.25)
7	5 (4.75-6)
8	5 (3.75-6)
9	5.5 (3.75-7)
Sham	2 (1.75-2)
IRU	7 (5.5-8)^{b}
DHI	4 (2.75-5)

Values are expressed as the median (interquartile range) (n=6). ^{a}P<0.05 vs. IRU group; ^{b}P<0.01 vs. sham group. IR, ischemia-reperfusion; IRU, IR untreated; DHI, Danhong injection.

IRU group was significantly higher than that of the sham group (P<0.01). Compared with the IRU group, the orthogonal compatibility groups were indicated to exhibit decreased expression level of cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53 mRNA genes. In groups 2, 4 and 6, the expression level of cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53 mRNA was

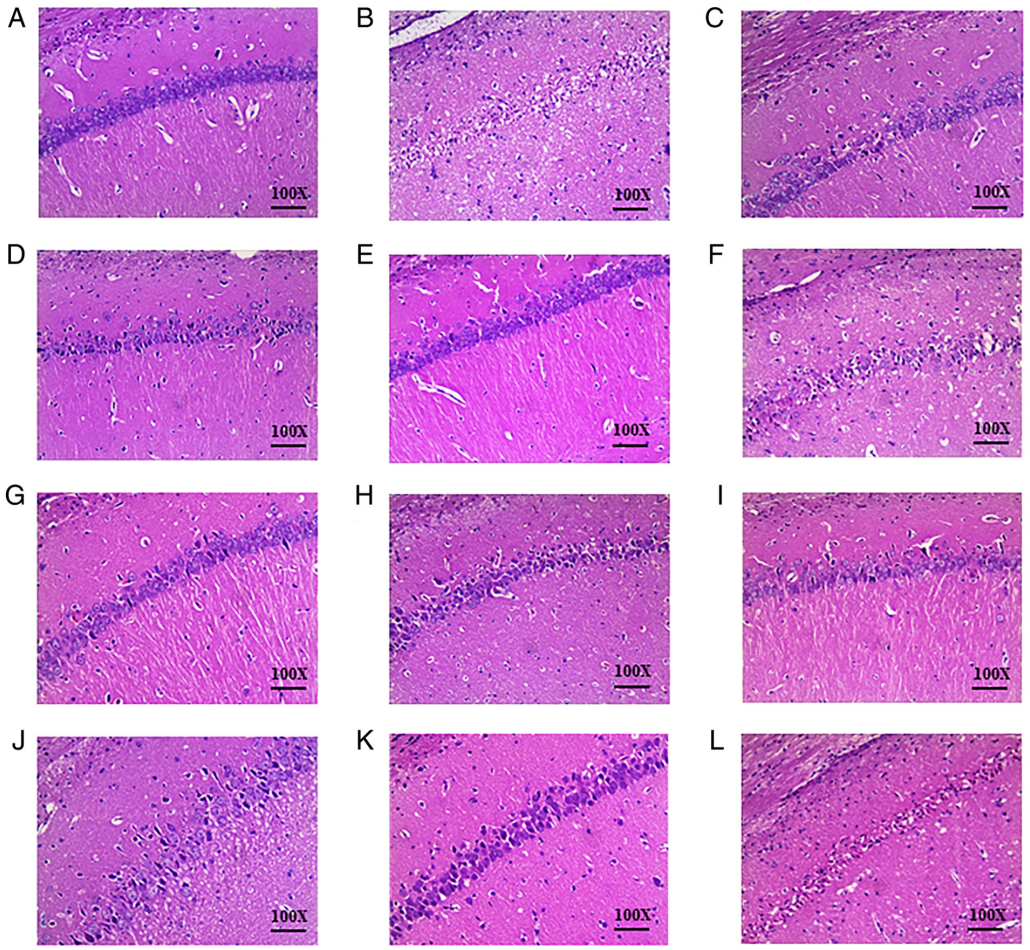

Figure 2. Effect of DHI and compatibility groups of four effective ingredients on brain histopathological alterations in the ischemic penumbra at day 3 of reperfusion after middle cerebral artery occlusion (magnification, x100). (A-L) Sham, ischemia-reperfusion untreated, DHI and orthogonal compatibility 1-9 groups, respectively (n=6). DHI, Danhong injection.

significantly decreased (P<0.01 or P<0.05). When compared with the DHI group, the expression levels of cytochrome *c* in groups 1, 7 and 8, apaf-1 in groups 7 and 9, caspase-9 in groups 7 and 8, caspase-3 in groups 5, 7 and 9 exhibited significant differences (P<0.01 or P<0.05). This indicated that the efficacy of the orthogonal compatibility groups 2, 4 and 6 and the DHI group was similar. These results are presented in Table VI.

Discussion

Known as one of the top four life-threatening diseases (33), strokes are frequent in clinic patients (34). Ischemic strokes are common, accounting for ~87% of all strokes worldwide (2,35). The incidence of ischemic strokes is higher than that of other types of stroke, which could pose a serious threat to human health (1). Consequently, the prevention and treatment of ischemic stroke and cerebrovascular disease has become a priority throughout the world (36). It is also of great clinical significance and social value to explore the effective treatment methods for patients with ischemic stroke (37).

The compatibility law is one of the core issues in the study of prescription science. It requires a higher level of understanding and generalization of prescription compatibility methods (36,38). This law was a helpful and significant guide for writing clinical prescriptions and further developing the theory of prescription science (37). The study of the compatibility of prescription drugs has been considered important by ancient and modern doctors (38).

Drug pairs are a commonly used compatibility form of TCM clinical prescriptions (38). Drug pairs follow the theory of TCM, including four odors and five flavors, ascents and descents, channel tropism, toxicity and side effects and the principle of complementary or opposite combination (39). A drug pair has the characteristics of a simple structure and clear compatibility effect (8). This theory is the culmination of accumulated clinical medication experience by physicians of past dynasties (40). The present study on the main effective ingredients of Danshen and Honghua as effective prescriptions will help clarify the mechanism of action of these drugs and reveal their useful characteristics (41).

The pathophysiological process of cerebral IR injury is a complex cascade reaction (2,42). The pathogenesis involves a variety of dysregulations, including excitatory amino acid toxicity, intracellular calcium overload, excessive formation of oxygen free radicals, cascade free radical chain reactions, inflammatory reactions, mitochondrial dysfunction and apoptosis (42-45). These events can ultimately cause irreversible brain injury (46). A notable cause of IR injury is the increased apoptosis of local neurons after the initial cerebral ischemia (44).

Table VI. Effect of compatibility groups of four effective ingredients on the expression levels of cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53 in rats after cerebral IR injury.

Group	Cytochrome *c*	Apaf-1	Caspase-9	Caspase-3	p53
1	3.64±0.86[a]	2.69±0.60	2.43±0.67	2.69±0.68	2.61±0.54
2	2.32±0.57[b]	2.25±0.51[c]	1.98±0.54[b]	1.73±0.36[b]	2.14±0.40[b]
3	3.18±0.61	2.76±0.72	2.64±0.76	2.51±0.57	2.55±1.05
4	2.02±0.56[b]	1.86±0.57[b]	2.01±0.48[b]	1.90±0.53[b]	2.32±0.71[c]
5	2.31±0.59[b]	2.65±0.65	2.61±0.72	2.84±0.39[d]	2.85±1.05
6	2.46±0.63[b]	1.95±0.49[b]	1.91±0.56[b]	1.92±0.70[b]	2.41±1.14[c]
7	3.31±0.74[d]	3.08±0.66[d]	2.88±0.40[d]	2.84±0.44[d]	2.26±1.26[c]
8	3.70±0.67[a]	2.94±0.91	2.96±0.66[d]	2.78±0.79	2.65±1.02
9	2.74±0.61[b]	3.09±0.78[d]	2.69±0.73	2.96±0.70[d]	2.96±0.99
Sham	1.01±0.18	1.02±0.21	1.01±0.18	1.01±0.18	1.02±0.26
IRU	4.45±0.86[e]	3.65±0.79[e]	3.43±0.85[e]	3.43±0.85[e]	4.22±0.78[e]
DHI	1.75±0.51[b]	1.75±0.51[b]	1.69±0.27[b]	1.69±0.27[b]	2.11±0.73[b]

Values are expressed as the mean ± SD (n=6). [a]P<0.01 and [d]P<0.05 vs. DHI group; [b]P<0.01 and [c]P<0.05 vs. IRU group; [e]P<0.01 vs. sham group. IR, ischemia-reperfusion; IRU, IR untreated; DHI, Danhong injection; Apaf-1, apoptotic peptidase activating factor 1.

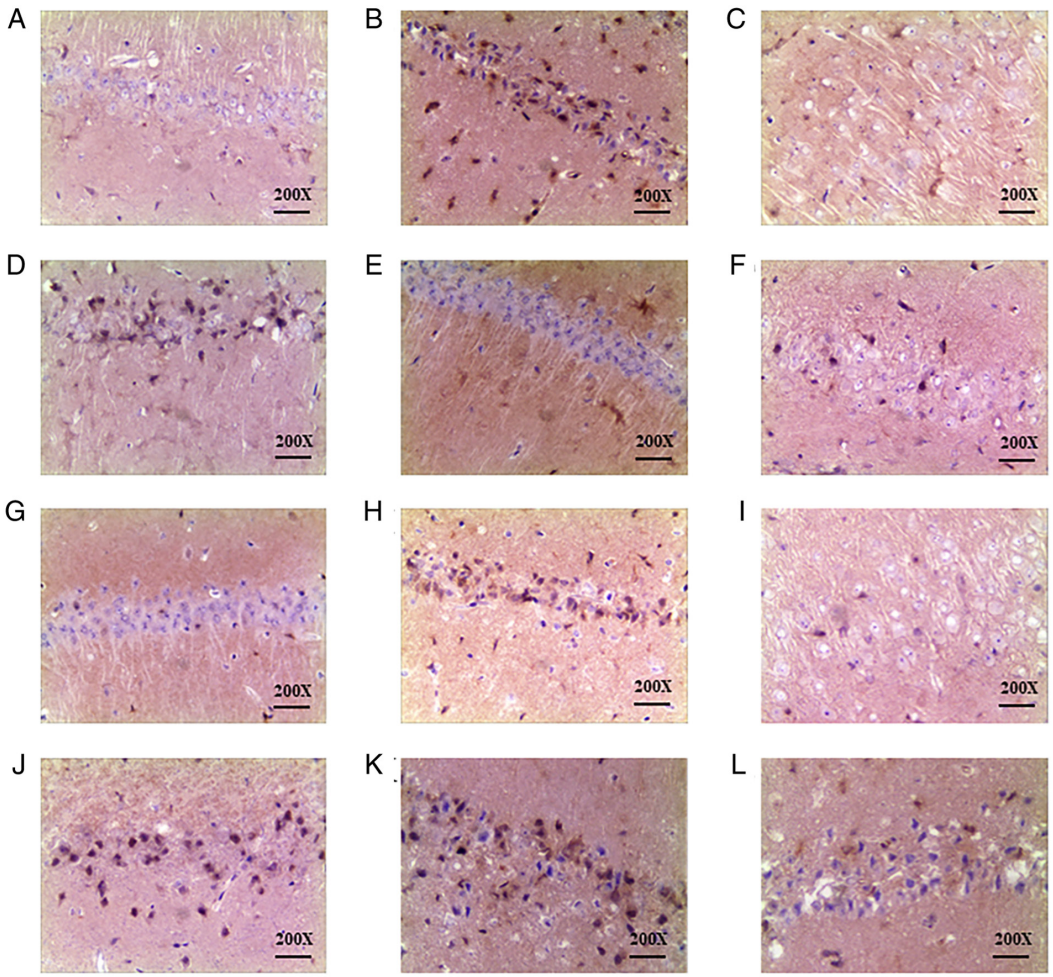

Figure 3. Effect of DHI and compatibility groups of four effective ingredients on caspase-3 protein expression in rats after cerebral IR injury (magnification, x200). (A-L) Sham, IR untreated, DHI and orthogonal compatibility 1-9 groups, respectively (n=6). IR, ischemia-reperfusion; DHI, Danhong injection.

The regulation of factors and signal transduction pathways involved in neuronal apoptosis reduces the degree of brain injury during ischemia and prevents further development of apoptosis (44,45). This encourages the possibility of a breakthrough

in the treatment of cerebrovascular diseases. The caspase family serves an important role in the apoptotic process of neurons (47,48). This family of proteins represents the common pathway for the final implementation of apoptosis (49).

Cerebral ischemia and hypoxia can initiate a series of pathological changes within cells (42). One important response is the opening of a permeability transition pore that activates the endogenous apoptotic pathway (50). The precursors of caspase-9, procaspase-9 and cytochrome *c* are then released from the mitochondria to form apoptotic bodies with apaf-1 (47,51). These apoptotic bodies activate caspase-9 and downstream caspase-3, which causes apoptosis (50,51).

The damage of the mitochondrial membrane is also closely associated with Bcl-2 family members, including Bcl-2, Bax and Bad (52). These proteins are involved in the regulation of apoptosis (53). Bcl-2 and Bax are a group of channel proteins, which can affect the state of cells by regulating the permeability of the mitochondrial membrane. Specifically, Bax can regulate the permeability of the mitochondrial extracorporeal membrane, causing increased release of cytochrome *c* from the mitochondria and promotion of apoptosis (54,55). Bcl-2 inhibits the activation of the caspase family and halts apoptosis by preventing the formation of the Bax channel (56-58). The p53 protein is a key molecule in promoting neuronal apoptosis (59), which can upregulate Bax and downregulate Bcl-2 (51,54). It can also cause a caspase family cascade reaction and promote cell apoptosis (47,60).

The present study has several limitations. Firstly, the experimental period in the present research was 3 days as a result of the small treatment time. Therefore, the efficacy of drug treatment for 1, 5 and 7 days was not examined. Secondly, as oxidative stress and mitochondrial dysfunction are upstream factors leading to apoptosis, a further study could evaluate the comprehensive and in-depth effect of these pathways regulated by the combination of Danshen and Honghua after cerebral IR injury.

The present study indicated that the expression levels of apoptosis-related factors, such as cytochrome *c*, apaf-1, caspase-9, caspase-3 and p53, were significantly increased after cerebral IR injury. In addition, the damage of hippocampal cells was improved to varying degrees after drug treatment. These findings suggested that the combination of Danshen and Honghua exhibited a protective effect on rats after cerebral IR injury. In addition, orthogonal group 4 (30 mg/kg tanshinol; 2.5 mg/kg salvianolic acid A; 16 mg/kg salvianolic acid B; and 8 mg/kg hydroxysafflor yellow A) exhibited a significant inhibition of apoptosis. These drugs may function by inhibiting key targets upstream of caspase-3 to prevent apoptosis. Ultimately, the effective and compatible ingredients of Danshen and Honghua were revealed to exhibit a significant protective effect on cerebral IR injury in rats.

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Availability of data and materials

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

Authors' contributions

JY and HZ conceived the idea and designed the study. HW and LC performed the experiments. ZD and ZL established the cerebral IR model in rats. ZL wrote the manuscript. YY and HW participated in the data acquisition and statistical analysis, and YY revised the manuscript. All authors have read and approved the final manuscript. JY and HZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Animal welfare and experiments were strictly in accordance with the Regulation for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission, 1988) and approved by the Institutional Animal Care and Use Committee of Zhejiang Laboratory Animal Center (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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