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Mechanism underlying the protective effect of *Kaixin Jieyu Fang* on vascular depression following cerebral white matter damage

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Abstract

The Chinese compound *Kaixin Jieyu Fang* can be used to treat vascular depression; however, the underlying mechanism remains unclear. This study established a rat model of chronic cerebral ischemia-caused white matter damage by ligation of the bilateral common carotid arteries. Rats received daily intragastric administration of a suspension of *Kaixin Jieyu Fang* powder. After 3, 7 and 21 days of treatment, the degree of white matter damage in the cerebral ischemia rat model was alleviated, Bcl-2 protein and mRNA expression in brain tissue increased, and Bax protein and mRNA expression decreased. These results indicate that *Kaixin Jieyu Fang* can alleviate cerebral white matter damage, and the underlying mechanism is associated with regulation of Bcl-2/Bax protein and mRNA expression, which is one of possible mechanism behind the protective effect of *Kaixin Jieyu Fang* against vascular depression.

Key Words: nerve regeneration; vascular depression; ligation of the bilateral common carotid arteries; chronic cerebral ischemia; white matter damage; Kaixin Jieyu Fang powder; Bcl-2; Bax; NSFC grant; neural regeneration

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Introduction

Vascular depression, first proposed by Alexopoulos et al.[1] and Krishnan et al.[2], is a depressive syndrome closely related to cerebrovascular disease or vascular risk factors and mostly occurs in the elderly, in particular individuals aged over 65 years, where it is the main type of depression. In addition to cognitive disorders and neuropsychological impairments, white matter changes are often observed, with extremely high white matter signals observed in MRI images of patients with vascular depression[3]. Most patients with white matter damage present with depressive mood and functional deficits in motor, attention and judgment, although not achieving the diagnostic criteria of depression. Shibata et al.[4] found that cerebral ischemia-caused white matter damage is often accompanied by depressive symptoms and white matter damage is possible between cerebrovascular disease and late-onset depression. Therefore, it has been accepted that white matter damage can cause vascular depression and is the main pathological change contributing to the occurrence and development of vascular depression[5-7].

The pathological mechanism underlying vascular depression remains unclear. Chronic cerebrovascular diseases, including hypertension, arteriosclerosis and cerebral ischemia, are considered factors that can cause ischemic white matter damage[8], abnormal neurotransmitter (norepinephrine, 5-hydroxytryptamine) transmission in the frontal lobe, hippocampal gyrus and temporal lobe, decreased levels of 5-hydroxytryptamine and norepinephrine in the brain, damaged striatum-pallidum-thalamus-cortex, and emotional, learning and memory information transfer disorders, leading to depression. White matter damage is an independent obvious predictor of the occurrence and development of vascular depression[9].

There have been no ideal western medicines for vascular depression: simple anti-depressive Western medicines, such as fluoxetine, show poor sensitivity and have toxic and adverse events[10]. In the clinic, several anti-depressive Western medicines or Chinese medicines that can activate blood circulation to dissipate blood stasis combined with anti-depressive Western medicines are used to treat vascular depression[11-12]. *Kaixin Jieyu Fang* was made from two prescriptions of *Kaixin San* and *Sini San* supplemented with Radix Morindae Officinalis, consisting of eight Chinese herbs including *Radix Ginseng*, *Radix Bupleuri*, *Fructus Aurantii Immaturus*, *Radix Morindae Officinalis*, *Poria*, *Radix Polygalae*, *Radix Paeoniae Rubra* and *Radix Glycytthizae*. *Sini San* is a common prescription used for the treatment of depression. Pharmacological findings showed that *Sini San* can hypno-

tize, regulate 5-hydroxytryptamine, promote the production of nitric oxide and strengthen the immunity of organisms[13]. *Kaixin San* supplemented with *Radix Morindae Officinalis* exhibits anti-depressive effects[14].

The Chinese compound of these two prescriptions, *Kaixin Jieyu Fang*, can relieve deficiency syndrome, supplement *qi* and remove stasis, thereby exhibiting significant clinical therapeutic effects on vascular depression. Our preliminary pharmacological findings have shown that *Kaixin Jieyu Fang* can improve cognitive disorder in rats with vascular depression[15-17].

There is evidence that apoptotic genes are involved in the occurrence and development of cerebral ischemia, and that Bcl-2/Bax protein and mRNA expression is associated with cerebral histomorphological damage and dysfunction[18]. However, whether *Kaixin Jieyu Fang* can improve these depressive symptoms by reducing white matter lesions remains unknown. This study established a rat model of chronic cerebral ischemia by ligation of the bilateral common carotid arteries[19], and intragastrically administered a suspension of *Kaixin Jieyu Fang* powder. We observed the morphological changes in rat white matter to investigate the interventional effects of *Kaixin Jieyu Fang* on white matter damage.

Results

Quantitative analysis of experimental animals

A total of 180 rats were included in this study. The cerebral ischemia model was established in 135 rats by ligation of the bilateral common carotid arteries, and 45 rats died during this procedure. The remaining rats were randomly and equally divided into a model group and a *Kaixin Jieyu Fang* group. The 45 rats not subjected to cerebral ischemia induction only received the procedure of suture insertion without ligation (sham operation group). Rats in the *Kaixin Jieyu Fang* group were intragastrically administered a suspension of *Kaixin Jieyu Fang* powder (1.8 g/kg per day). Rats in the sham operation and model groups received equal amounts of sterile water. At 3, 7 and 21 days of treatment, 15 rats per group were selected for laboratory examination (5 for pathomorphological observation of the white matter and 10 for analysis of Bcl-2 and Bax mRNA and protein expression).

Kaixin Jieyu Fang improved white matter damage in cerebral ischemic rats

Hematoxylin-eosin staining results under the optical microscope are shown in Figure 1. In the sham operation group, white matter nerve fibers in the optic chiasma were densely and orderly arranged, no tissue swelling was observed, glial cell morphology was normal, and cytoplasm staining was even. Compared with the sham operation group, after 3, 7 and 21 days of treatment, arrangement of nerve fibers was disordered, matrix was sparse and swollen, interstitial edema was severe, and these pathological changes aggravated with prolongation of ischemic time in the model group. Compared with the model group, pathomorphological changes were alleviated at 3 days, the arrangement of nerve fibers was slightly ordered and interstitial edema was significantly alleviated at 7 and 21 days in the *Kaixin Jieyu Fang* group, similar to those in the sham operation group.

Kaixin Jieyu Fang improved the ultrastructure of white matter in cerebral ischemic rats

Using transmission electron microscopy, nerve fibers in the left frontal lobe white matter of rats in the sham operation group contained axons in the center enveloped by myelin sheath, which presented as concentric arrangements of alternately dark and bright lamellae with indistinct microfilaments and microtubules. Compared with the sham operation group, at 3 days after cerebral ischemia, the nerve fiber myelin sheath was occasionally thickened and delaminated; at 7 days, myelin sheath thickening and delamination were more obvious and demyelination occurred more frequently; at 21 days, demyelination was more obvious, and microfilaments and microtubules in the myelin sheath were indistinct in the model group. Compared with the model group, at 3 days after cerebral ischemia, damage to myelin sheath structure was alleviated, and at 7 and 21 days, myelin sheath thickening and delamination was reduced and the number of myelin sheaths was increased in the *Kaixin Jieyu Fang* group, which were similar to the sham operation group (Figure 2).

Effects of *Kaixin Jieyu Fang* on Bcl-2/Bax mRNA expression in the brain tissue of cerebral ischemic rats

RT-PCR results showed that at 3, 7 and 21 days after cerebral ischemia, Bcl-2 mRNA expression in rat brain tissue was significantly decreased ($P < 0.01$), while Bax mRNA expression was significantly increased ($P < 0.05$ or $P < 0.01$) in the model group when compared with the sham operation group; however, Bcl-2 mRNA expression in rat brain tissue was significantly increased ($P < 0.05$ or $P < 0.01$), while Bax mRNA expression was significantly decreased ($P < 0.05$), in the *Kaixin Jieyu Fang* group when compared with the model group (Figure 3).

Effects of *Kaixin Jieyu Fang* on Bcl-2/Bax protein expression in the brain tissue of cerebral ischemic rats

Western blot analysis showed that at 3, 7 and 21 days after cerebral ischemia, Bcl-2 expression in rat brain tissue was significantly decreased ($P < 0.01$), while Bax expression was significantly increased ($P < 0.01$) in the model group when compared with the sham operation group; however, at 3, 7 and 21 days, Bcl-2 expression in rat brain tissue was significantly increased ($P < 0.05$ or $P < 0.01$), while Bax expression was significantly decreased ($P < 0.05$) in the *Kaixin Jieyu Fang* group when compared with the model group (Figure 4).

Discussion

Previous studies have demonstrated that occlusion of the middle cerebral artery leads to cerebral tissue ischemia/hypoxia, neuronal injury and white matter lesions[20-21]. These pathological changes are similar to those of vascular disease-caused leukoencephalopathy. At present, permanent occlusion of the bilateral common carotid arteries (2-VO) has been widely used in the study of ischemic cerebrovascular disease and is considered an ideal model of chronic cerebral

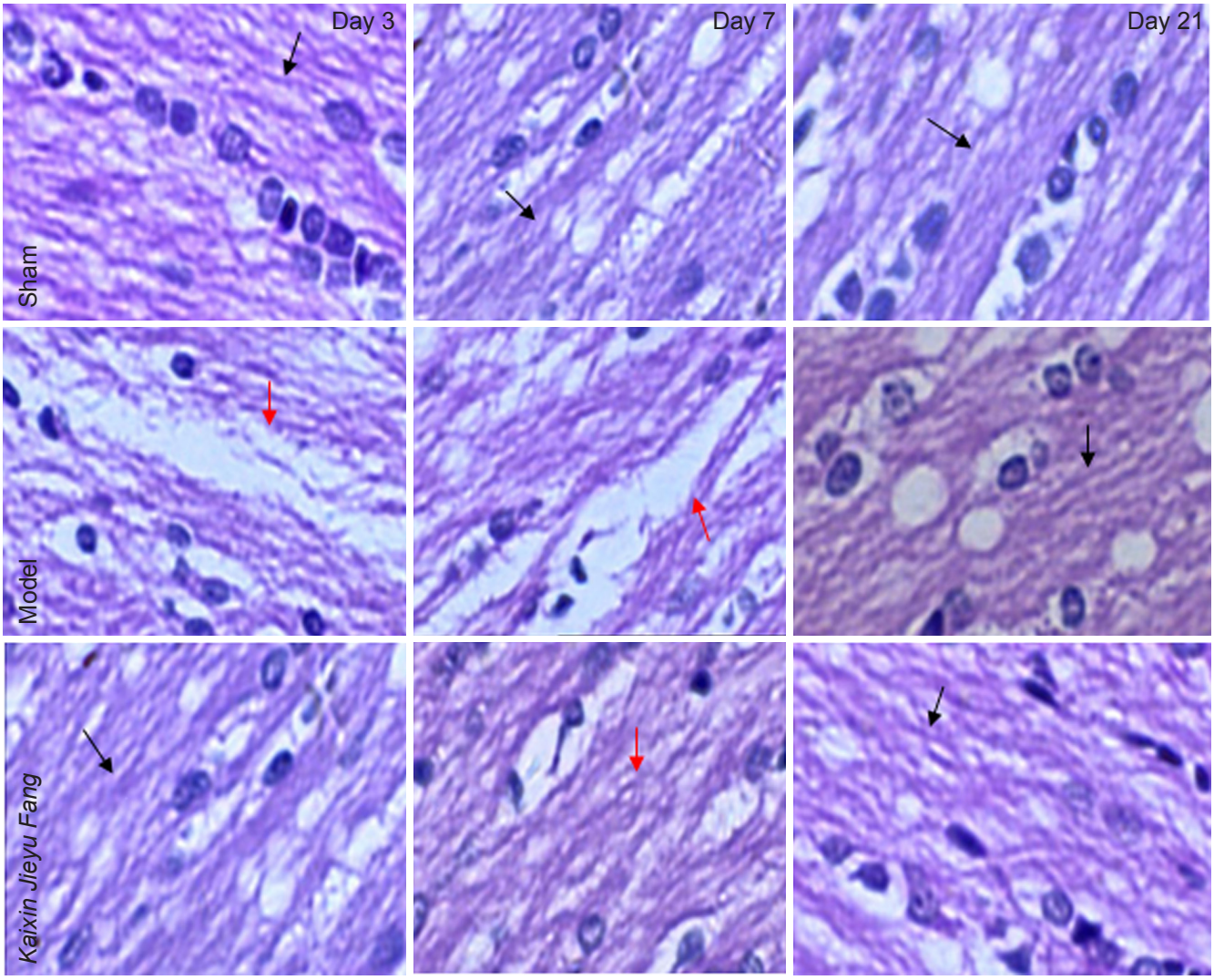

Figure 1 Effect of *Kaixin Jieyu Fang* on the pathological morphology of white matter in cerebral ischemic rats (hematoxylin-eosin staining, × 200).

Compared with the sham operation (sham) group, the arrangement of nerve fibers in the optic chiasma was disordered (black arrows), the matrix was sparse and swollen (red arrows) and pathological changes gradually worsened with the prolongation of ischemia time in the model group. Compared with the model group, the pathological changes were alleviated at 3 days, and the arrangement of nerve fibers was slightly ordered (black arrows), and tissue swelling was markedly alleviated at 7 and 21 days in the *Kaixin Jieyu Fang* group, which were similar to the sham group.

ischemia[22-23]. This study established a rat model of chronic ischemic white matter damage by permanent occlusion of the bilateral common carotid arteries, observed the ultrastructure of white matter, and validated white matter lesions after cerebral ischemia/hypoxia. Results from this study showed that cerebral ischemia/hypoxia by the 2-VO method led to thickening and delamination of the cerebral myelin sheath, indicative of successful induction of white matter lesions. There is evidence that white matter damage leads to decreased nerve fiber conduction and disordered information transfer, thereby causing decreased cognitive function (such as depression)[24-25]. Our preliminary experiments have confirmed that after cerebral ischemia/hypoxia, there is increased oligodendrocyte cell death in rat brain white matter[26]. Oligodendrocyte apoptosis is also a main contributor to white matter damage[27], leading to myelin sheath degeneration even loss and finally resulting in white matter damage[28]. The toxic substances produced during ischemia/hypoxia, such as free radicals and excitatory amino acids, can promote the activation of pro-apoptotic genes, leading to cell death. Bcl-2 family members are involved in apoptosis regulation[29], and the regulation mainly presents at the gene transcription and protein modification level. Bcl-2 and Bax are the key anti-apoptotic and pro-apoptotic genes, respectively[30-34], and exhibit a critical role in post-ischemia cell apoptosis[35]. After cerebral ischemia, increased Bcl-2 expression can reduce cerebral infarction area, exhibiting neuroprotective effects on brain tissue[36].

Bax protein, a component of mitochondrial ion channels, has a similar structure to Bcl-2, and can form heterodimers by binding to Bcl-2 and promote cell apoptosis. Simultaneously, Bax itself forms homodimers and accelerates cell apoptosis. This study detected Bcl-2/Bax mRNA and protein expression and investigated their role in white matter dam-

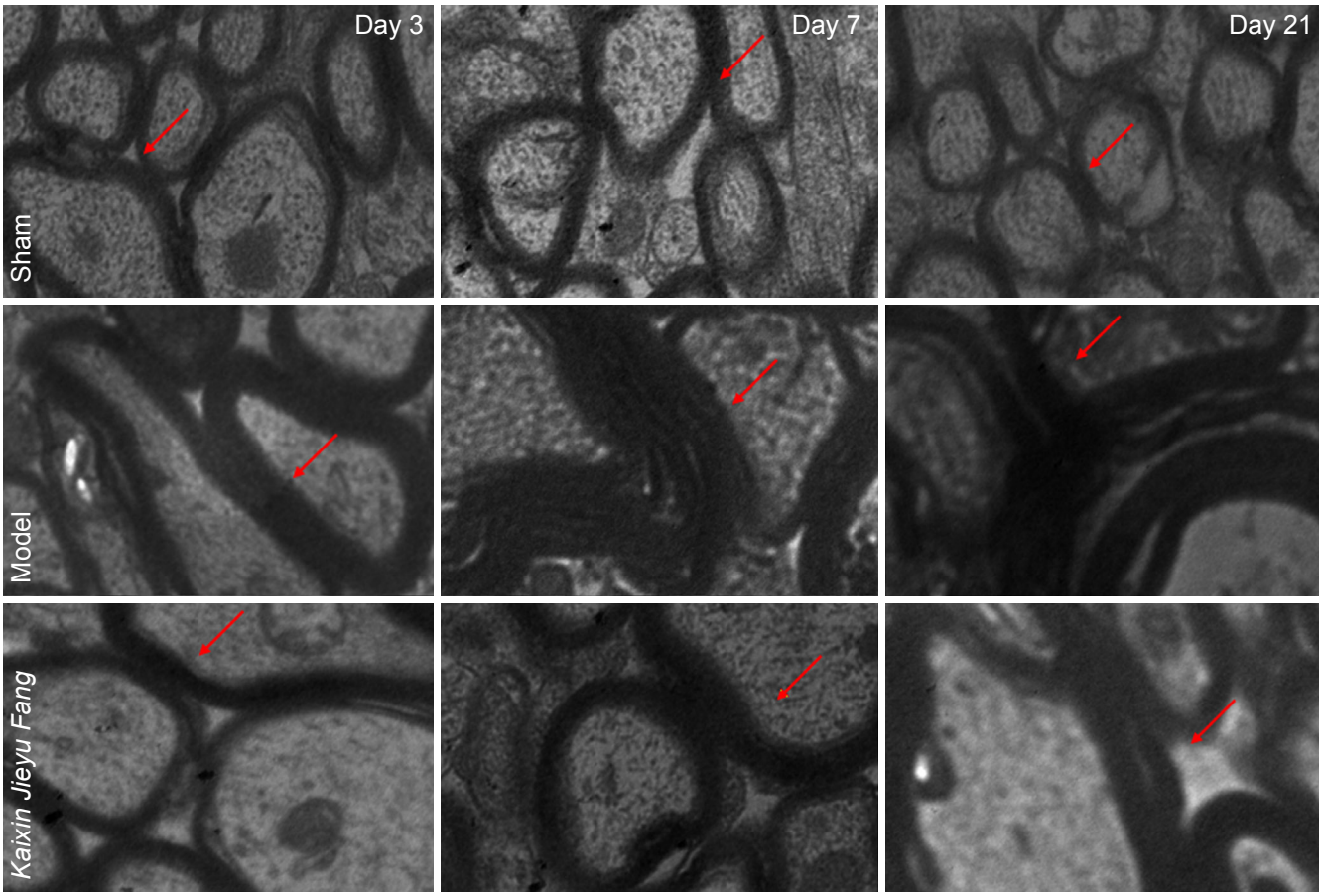

Figure 2 Effect of *Kaixin Jieyu Fang* on the ultrastructure of white matter in cerebral ischemic rats (× 20,000).
Transmission electron microscopy revealed that the myelin sheaths surrounding the nerve fibers in the left frontal lobe white matter were occasionally thickened and delaminated (arrow) at 3 days and myelination was more obvious at 7 and 21 days in the model group when compared with the sham operation (sham) group. Compared with the model group, damage to myelin sheath structure was slightly alleviated at 3 days, and myelin sheath thickening and delamination was obviously reduced, leading to an increased number of myelin sheaths (arrows) in the *Kaixin Jieyu Fang* group, which was similar to the sham group.

age.

Results from this study showed that at 3–21 days after cerebral ischemia, the mRNA and protein expression of the pro-apoptotic gene Bax increased and that mRNA and protein expression of Bcl-2 decreased. Based on our preliminary findings[37], increased levels of toxic substances, such as free radicals, nitric oxide, up-regulation of Bax mRNA and protein expression, and down-regulation of Bcl-2 mRNA and protein expression, lead to oligodendrocyte apoptosis and finally white matter damage, which is consistent with previous findings[38-39]. After *Kaixin Jieyue Fang* treatment, Bcl-2 mRNA and protein expression increased and Bax mRNA and protein expression decreased, suggesting that *Kaixin Jieyue Fang* influences the Bcl-2 family of apoptosis-related genes and regulates the abnormal expression of Bcl-2/Bax expression after cerebral ischemia.

Taken together, *Kaxin Jieyu Fang* can improve white matter damage by down-regulating Bax mRNA and protein expression and up-regulating Bcl-2 mRNA and protein expression, suggesting that *Kaixin Jieyu Fang* significantly regulates Bcl-2/Bax expression. The neuroprotective effect of *Kaixin Jieyu Fang* on white matter damage after cerebral ischemia is closely related to regulation of Bcl-2/Bax expression, which is likely to be one of the mechanisms underlying *Kaixin Jieyu Fang* treatment of vascular depression.

Materials and methods

Design

A randomized controlled animal experiment.

Time and setting

This study was performed in the Laboratory of Pharmacology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing, China from May 2010 to May 2011.

Materials

Animals

A total of 135 healthy male Wistar rats, aged 12 weeks, weighing 280 ± 20 g, were provided by Academy of Military Medical Sciences, Beijing, China (license No. SCXK (Army) 2007-004). These rats were housed at 24°C with a relative humidity of 40–60% and good ventilation. They were fed with common feed and had free access to water. All experimental protocols were performed according to the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China[40].

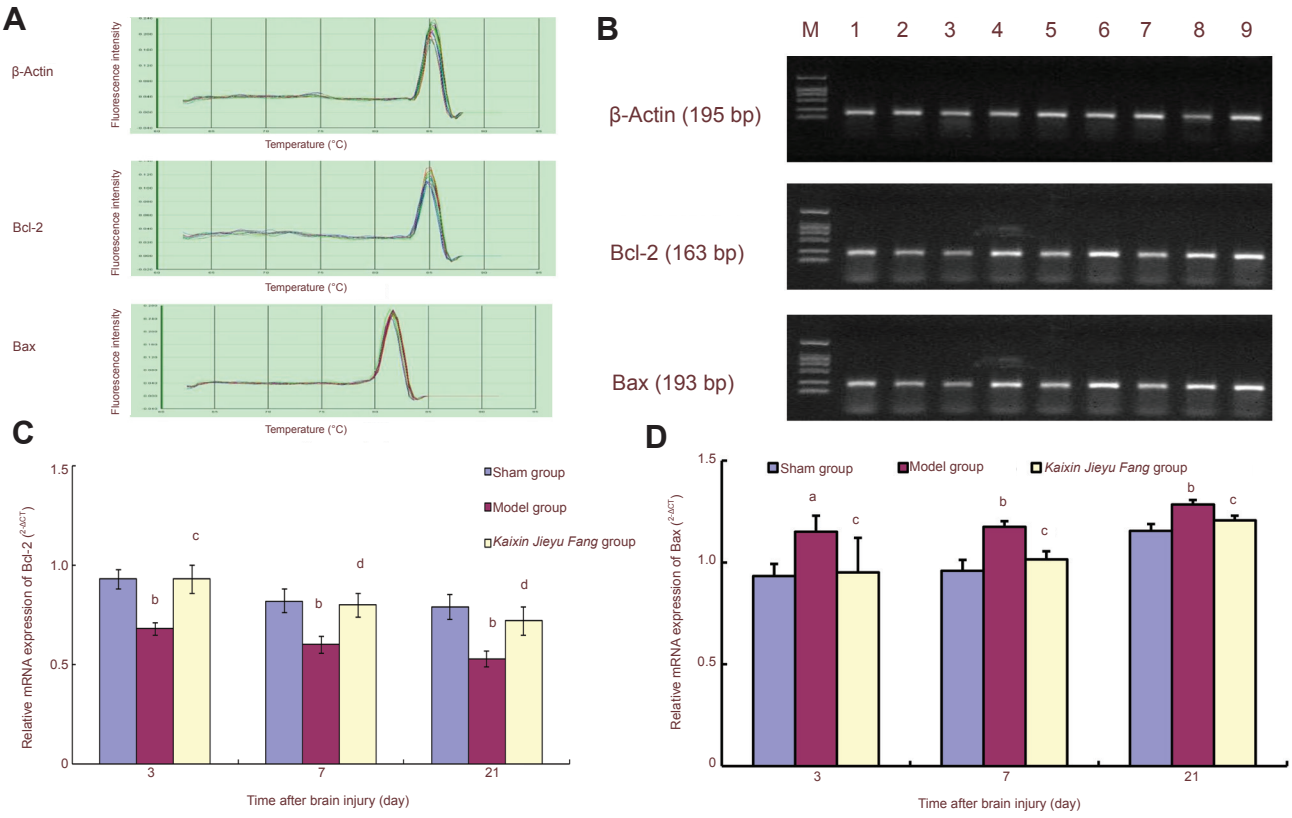

Figure 3 Detection of Bcl-2 and Bax mRNA expression in brain tissue of cerebral ischemic rats after *Kaixin Jieyu Fang* treatment by RT-PCR.
(A) Bcl-2, Bax and β-actin dissociation curves for PCR reactions.
(B) Agarose electrophoresis analysis. M: DNA marker; 1–9 refers to relative mRNA expression of Bcl-2 and Bax in the rat brain tissue at 3, 7, 21 days after brain injury, precisely 1, 4, 7: sham operation (sham) group; 2, 5, 8: model group; 3, 6, 9: *Kaixin Jieyue Fang* group.
(C, D) The relative mRNA expression of Bcl-2 and Bax. The relative mRNA expression of Bcl-2 and Bax was expressed as the copy number of Bcl-2 and Bax to β-actin ($2^{-\Delta CT}$). Experimental data were expressed as mean ± SD, $n = 10$ per group per time point. Data were analyzed by one-way analysis of variance and the least significance difference test for pairwise comparison. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs.* sham group; $^{c}P < 0.05$, $^{d}P < 0.01$, *vs.* model group.

Drugs

Kaixin Jieyu Fang was composed of *Radix Ginseng*, *Radix Bupleuri*, *Fructus Aurantii Immaturus*, *Radix Morindae Officinalis*, *Poria*, *Radix Polygalae*, *Radix Paeoniae Rubra* and *Radix Glycytthizae* at a mass ratio of 3:3:2:3:3:2:3:2. All Chinese herbs (Room of Pharmacology, Guang'anmen Hospital, China) were dried at 50°C for 6 hours in the temperature-constant electrothermal drying oven (Tianjin Taisite Instrument Co., Ltd., Tianjin, China). Each Chinese herb was broken into small pieces, then ground into power, screened using a powder sieve machine, and mixed together to form *Kaixin Jieyu Fang* powder.

Methods

Cerebral ischemia model

After 7 day environmental adaptation, cerebral ischemia models were established in Wistar rats by ligation of the bilateral common carotid arteries[19]. Within 12 hours before surgery, rats were fasted but had free access to water. Following anesthesia by intraperitoneal injection of chloral hydrate (350 mg/kg; batch No. 20100304, Sinopharm Chemical Reagent Co., Ltd., Beijing, China), hair shaving and ethanol/povidone iodine sterilization, a median incision was made in front of the neck, and the bilateral common carotid arteries were bluntly dissociated. The common carotid artery on each side was ligated at the distal and proximal ends separately using No. 5 silk suture and then cut in the middle. To reduce surgery-caused errors, only suture insertion was performed without ligation and bilateral common carotid arteries were not cut off. After surgery, the wounds were treated with benzylpenicillin sodium solution (4×10^5 U/kg) and sutured with No. 0 silk suture. Body temperature was maintained using a heat lamp. Benzylpenicillin sodium solution was intraperitoneally injected for 5 successive days to prevent infection.

Kaixin Jieyu Fang administration

According to our preliminary findings[17], 1.8 g/kg per day was the optimal effective dose of *Kaixin Jieyu Fang* for rats, and the resulting therapeutic effect equaled to fluoxetine hydrochloride. Rats in the *Kaixin Jieyu Fang* group were intragastrically administered *Kaixin Jieyu Fang* suspension (1.8 g/kg per day) harmonized with purified water on day 2 after cerebral ischemia induction, once a day. Rats in the sham operation and model groups received equal amounts

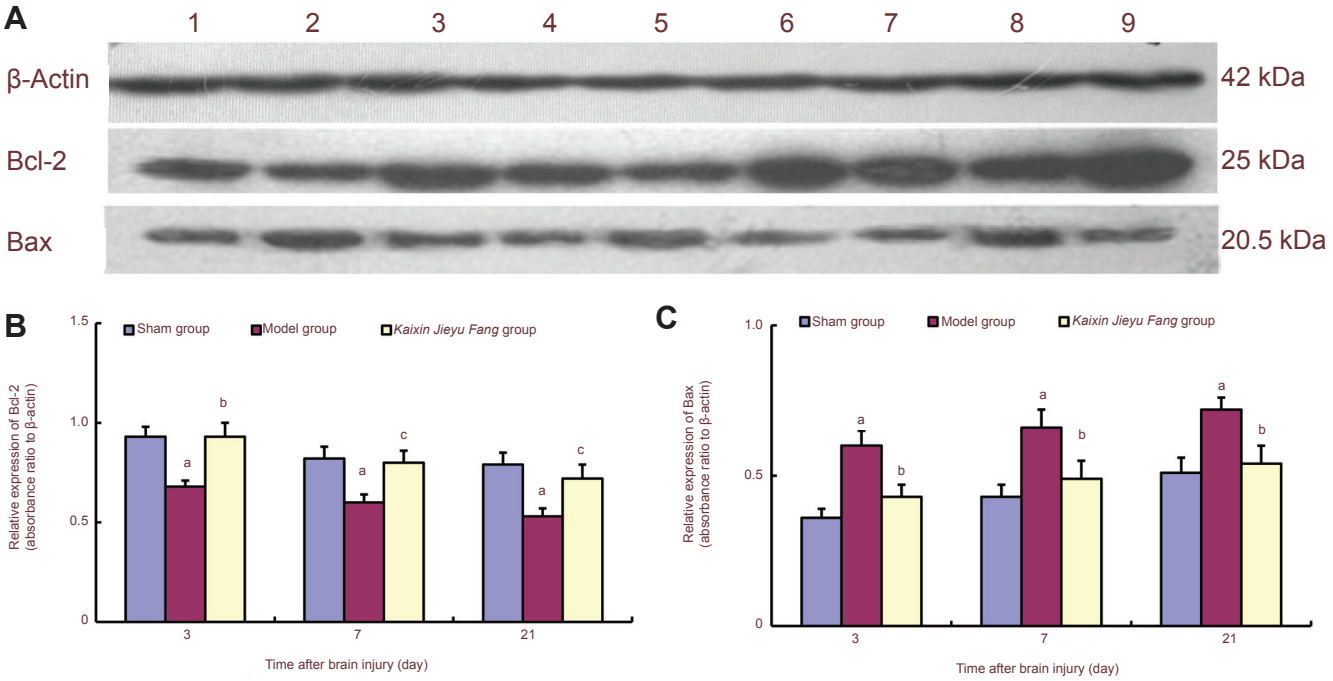

Figure 4 Bcl-2/Bax protein expression change in the brain tissue of rats with cerebral ischemia after *Kaixin Jieyu Fang* treatment (western blot analysis).
(A) Detection of Bcl-2 and Bax protein expression in rat brain tissue by western blot analysis. 1–9 refers to relative protein expression of Bcl-2 and Bax in rat brain tissue at 3, 7, 21 days after brain injury. 1, 4, 7: Sham operation (sham) group; 2, 5, 8: model group; 3, 6, 9: *Kaixin Jieyue Fang* group.
(B, C) Relative expression of Bcl-2 and Bax. Relative protein expression of Bcl-2/Bax is the absorbance value of Bcl-2/Bax gene to β-actin. Experimental data were expressed as mean ± SD, $n = 10$ per group per time point. Data were analyzed by one-way analysis of variance and the least significance difference test for pairwise comparison. $^{a}P < 0.01$, *vs.* sham group; $^{b}P < 0.05$, $^{c}P < 0.01$, *vs.* model group.

of sterile water (0.5 mL/kg per day). After 3, 7 and 21 days of intervention, 15 rats per group were selected for later laboratory examinations.

Pathological changes in rat white matter by hematoxylin-eosin staining

After sacrifice by decapitation, a 5-mm-thick tissue block spanning the optic chiasma was dissected, then fixed in 4% (w/v) paraformaldehyde, dehydrated through a series of ethanol washes (80%, 95%, 100% for 1 hour each), cleared with xylene, soaked in paraffin, embedded and sliced into 4-μm-thick sections, dewaxed, rehydrated, stained with hematoxylin-eosin, dehydrated by ethanol gradients again, cleared with xylene and mounted with gum. Finally, the morphology of the left white matter was observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Ultrastructure of rat white matter under the transmission electron microscope

After sacrifice by decapitation, the corpus callosum below the left frontal lobe was fixed with 2 L of 2.5% (v/v) glutaraldehyde (pH 7.2), chopped into 1 mm × 1 mm × 1 mm blocks, rinsed with 0.1 mol/L PBS at 4°C over 4 hours, fixed in 1% (v/v) osmic acid, rinsed with 0.1 mol/L PBS three times, hydrated through a series of acetone washes (50%, 70%, 90% and 100% for 15 minutes each), soaked in a mixture of epoxy resin and epoxy resin (1:1) for 2 hours, then in epoxy resin, embedded with Epon812, sliced with an LKB11800 ultramicrotome (Sunrise Technology Co., Ltd., Yantai, Shandong Province, China), and stained with uranyl acetate and lead nitrate. Finally, pathological changes in the ultrastructure of the corpus callosum below the left frontal lobe were observed using a transmission electron microscope (Olympus).

Detection of Bcl-2 and Bax mRNA expression in the left brain tissue by real-time fluorescence quantitative PCR

According to the kit instructions, rat left brain tissue was fixed in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. The absorbance at 260 and 280 nm (A_{260} and A_{280}) of total RNA was measured using the DU640 ultraviolet spectrophotometer (Beckman, Pasadena, CA, USA) and the concentration was determined. Total RNA was identified by 1.2% (w/v) agarose gel electrophoresis and then reverse transcribed into cDNA using MMLV First-Strand cDNA Synthesis Kit, *i.e.*, K1622RT reverse transcription kit (MBI Fermentas, Burlington, Canada). The real-time fluorescence quantitative PCR system consisted of cDNA 1μL, 0.05 μg/μL upstream primer 1 μL, 0.05 μg/μL downstream primer 1 μL, 2 × SYBR green PCR Master mix (Applied Biosystems, Foster, CA, USA) 10 μL, and diethylpyrocarbonate water was added to a final volume of 20 μL. The primer sequences were synthesized by SBS Genotech Co., Ltd., Beijing, China.

The primer sequences are as follows.

Gene	Primer sequence	Length of product (bp)
β-Actin	Forward: 5'-cca tgg aga agg ctg gg-3'	195
	Reverse: 5'-caa agt tgt cat gga tga cc-3'	
Bcl-2	Forward: 5'-ggg atg cct ttg tgg aac ta-3'	163
	Reverse: 5'-att tgt ttg ggg cag gtc t-3'	
Bax	Forward: 5'-aga cac ctg agc tga cct tg-3'	193
	Reverse: 5'-aag ttg cca tca gca aac at-3'	

The PCR conditions: predenaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds, and a final extension at 72°C for 8 minutes. PCR amplification was observed through 1.2% (w/v) agarose gel electrophoresis. Simultaneously, β-actin was designated and quantitatively detected. The specificity of PCR products was monitored through the melting curve, and the standard curves of the target and reference gene β-actin were prepared. The ratio of Bcl-2 and Bax gene to β-actin was used as the relative expression of target gene. The formula used was $2^{-\Delta CT}=2^{-(\text{target gene } \Delta CT - \text{reference gene } \Delta CT)}$.

Detection of Bcl-2 and Bax expression in rat left brain tissue by western blot analysis

Rat left brain tissue was lysed and then homogenized on ice. Brain tissue (10 mg) was lysed in 200 μL protein lysis buffer and then Bcl-2 and Bax protein expression was detected using the BCA protein kit (KeyGen Biotech, Nanjing, Jiangsu Province, China). Protein samples were separated on a 12% (w/v) separating gel and 4% (w/v) stacking gel, transferred to membrane for 90 minutes, washed with Tris-buffered saline containing 0.1% (v/v) Tween 20 for 5 minutes, and blocked with 5% (w/v) skim milk for 1 hour. Membranes were then incubated at 4°C overnight after addition of rabbit anti-rat Bcl-2 antibody (1:500; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) and rabbit anti-rat Bax antibody (1:500; Zhongshan Golden Bridge Biotechnology Co., Ltd.), followed by incubation at room temperature for 2–3 hours with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1: 500; Zhongshan Golden Bridge Biotechnology Co., Ltd.). Membranes were then developed with 3,3′-diaminobenzidine (DAB), and intensified by enhanced chemiluminescence technique. Images were analyzed by Lab Works software (SunBio Biomedical Technology (Beijing) Co., Ltd., Beijing, China).

Statistical analysis

All data were statistically processed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. One-way analysis of variance and least significance difference test were used. A level of $P < 0.05$ was accepted as statistically significant.

Author contributions: *Zhang Y was responsible for experimental design and evaluation, data processing, integration and analysis, paper writing; and provided technique and material support. Huang SJ was in charge of funds, guided the study and proof read the paper. Wang YY and Pan JH guided the study and provided technical and material support.*

Conflicts of interest: *None declared.*

Peer review: *This study investigated and revealed the mechanism underlying vascular depression, which is of great clinical significance for the treatment of depression in the elderly. Based on the self-developed Kaixin Jieyu Fang, this study used modern cell and molecular biology techniques to search for a possible therapeutic target.*

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