Zhongfeng capsule protects against cerebral ischemia-reperfusion injury via mediating PI3K/Akt and TLR4/NF-κB signal pathway regulated neuronal apoptosis and inflammatory reaction

Chunlu Yan (yanchl1979@126.com)
Gansu University of Chinese Medicine
Fangyu An
Gansu University of Chinese Medicine
Jiayu Wang
Gansu University of Chinese Medicine
Yao Shi
Gansu University of Chinese Medicine
Lingqing Yuan
Gansu University of Chinese Medicine
Donghui Lv
Gansu University of Chinese Medicine
Yanzhen Zhao
Gansu University of Chinese Medicine
Yongqi Liu
Gansu University of Chinese Medicine
Yongfeng Wang
Gansu University of Chinese Medicine

Research Article

Keywords: zhongfeng capsules, cerebral ischemia-reperfusion injury, apoptosis, inflammatory reactions, neuroprotective effect

Posted Date: March 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1464064/v1

Abstract

Inflammation reactions and neuronal apoptosis are the major pathophysiological mechanism of cerebral ischemia/reperfusion injury (CI/RI). Zhongfeng capsules (ZFCs), composed of panax notoginseng, hirudo, red ginseng, eupolyphaga, pangolin scales, rhubarb, and radix salviae miltiorrhizae. It has been reported that composition of ZFCs has a definite curative effect on CI/RI. Thus far, the specific molecular mechanisms of ZFCs remain unclear. This article would set rats model of middle cerebral artery occlusion(MCAO) to research the probable mechanisms of ZFCs underlying inflammation reactions and neuronal apoptosis signaling pathways. Our analyses revealed that in the ZFCs treatment group, there was an obvious decrease in neurologic impairment, as well as alleviation of the area of cerebral infarction, cerebral edema, histopathologyrats and neuronal apoptosis were also alleviated. The IL-1β, IL-6, and TNF-α were found to be down-regulated expression in the ZFC treatment group as well as the expression of Bcl-2, p-PI3K, p-Akt, and I-κBα was increased and the expression level of TLR4, NF-κB, Bax, caspase-3 was tended to decrease. The results indicated that ZFCs was effective in protecting the brain against CI/RI. One of their neuro-protective mechanisms may be associated with TLR4/NF-κB signalling, and they may negatively regulate the transcriptional activity of NF-κB and the secretion of downstream inflammatory factors. Another of their neuro-protective mechanisms may occur by regulating the expression of Bcl-2-Bax proteins of PI3K/Akt pathway. Our findings could facilitate that the suppressing PI3K/Akt signal pathway-mediated neuronal apoptosis, suppressing TLR4/NF-κB signal pathway-mediated inflammatory reaction were the potential mechanisms for the regulations, activation of PI3K/Akt pathway can inhibit the secretion of proinflammatory cytokines, which may be another functional mechanism of ZFCs against CI/RI.

1. Introduction

An ischaemic stroke can result in death or permanent disability, and occurs in patients worldwide[1]. The main pathogenesis of ischaemic stroke includes inflammatory reactions, oxidative stress, excitatory amino acid toxicity, and apoptosis[2]. There are reports that the activation of apoptosis, necrosis and necroptosis may be one of the chief pathogenic mechanisms underpinning cerebral ischaemia-reperfusion damage[3-4]. A lot of attention has been attracted about the molecular mechanisms of stroke-associated apoptosis in order to improve treatment and recovery. Apoptosis is also closely linked to the pathology of cerebral ischaemia-reperfusion damage[5]. Maintaining a balance among the Bcl-2 family proteins (such as Bax and Bcl-2) has an important role in regulating apoptosis [6]. Caspase-3 of caspase family protein participates in apoptosis cascade reaction, which is a mutual downstream effector of various apoptosis ways[7]. The mechanism of apoptosis in CI/RI is complex, and involves multiple signals. One pathway involved in CI/RI is PI3K/Akt signalling, which can further phosphorylate mTOR and other target proteins by activating Akt, thus mediating different biological effects involving inflammation, autophagy, apoptosis[8-9].

It is believed that CI/RI induces an inflammatory response that elicits tissue damage in several organs[10]. According to reports, some of the key inflammatory factors (IL-1β, IL-6, TNF-α) play key

regulatory roles in inflammatory responses, which was enhanced leukocyte adherence, cerebral impregnation, as well as endothelium dysfunction, resulting in neuronal ischaemic injury[11].Neuroinflammatory responses induced by a massive release of chemokines, proinflammatory cytokines further exacerbate cerebral injury in CI/RI[12-16]. TLR4 expressed in nerve cells was in response to the inflammatory reaction[17]. The activation of TLRs (TLR2, TLR4, and TLR6) promotes the secretion of downstream chemokines, adhesion factors, type I interferons, costimulatory molecules, and proinflammatory factors by indirectly activating the NF-κB pathway[18-20]. Therefore, a key reason for the neuro- inflammation observed in CI/RI is the activation of the TLR4/NF-κB channel[21], thus the inhibition of TLR4/NF-κB activity may well be a new target for CI/RI therapy.

In recent years, research has focused on cerebral ischaemic diseases in order to reduce CI/RI. The treatment of CI/RI typically includes chemicals such as calcium antagonists, free radical scavengers, and neuronal protective drugs. However, a number of adverse reactions, such as gastrointestinal problems and cerebral haemorrhage, have seriously affected the therapeutic outcomes of long-term clinical treatment programs[22]. Studies have confirmed that traditional Chinese medicinal ingredients have substantial advantages in treating CI/RI[23-25]. Traditional Chinese medicine (TCM) is garnered considerable attention for its multi-efficacy, multi-target, and multi-linked effects.

Zhongfeng capsules (ZFCs) are composed of *Panax notoginseng*, *Hirudo*, *Red ginseng*, *Eupolyphaga sinesis*, *pangolin scales*, *Rhubarb*, and *Radix salvia* miltiorrhizae. It activates and promotes blood circulation by warming yang. It has been reported that the individual components in ZFCs have a definite curative effect in the treatment of CI/RI. For example, *P.notoginseng* in ZFCs can protect the neurovascular unit in rats with CI/RI by inhibiting apoptosis[26]. Shuizhi extract has significant protective activity against cerebral cell apoptosis in rat with middle cerebral artery occlusion[27-28]. The neuroprotective effect of *E. sinesis* water on CI/RI in rats may occur via anti-peroxidation[29]. Salvianolic acid, an active component of R. salvia miltiorrhizae, alleviates CI/RI in rats by promoting SIRT1 expression, reducing the release of inflammatory factors (such as P-53) in downstream pathways, and inhibiting neuronal apoptosis[30]. However, the specific molecular mechanisms associated with ZFCs is unknown. This study reveals the protective effects of ZFCs in treating a CI/RI rat model and improves our understanding of the mechanisms involved.

2. Materials And Methods

2.1 Drug

ZFCs were bought from the Teaching Hospital of TCM Research of Henan(Lot No. 20180901, Henan, China). Zhongfeng capsules (ZFCs) are composed of *Panax Notoginseng*, *Hirudo*, *Red Ginseng*, *Eupolyphaga*, *Pangolin Scales*, *Rhubarb*, and *Radix Salvia Miltiorrhizae*. It was identified by Institute of Traditional Chinese Medicine, and was produced by Affiliated Hospital of Henan Academy of Chinese Medicine. It weighs 0.5g per capsule, and contains 1.4245 g of the raw powders per capsule. dl-3-

butylphthalide was purchased from CSPC NBP PHARMACE UTICAL CO, LTD(Lot No. 118190343, Hebei, China).

2.2 Experimental animals

SD male rats were weighed 200+20 g, and purchased from Gansu University of Chinese Medicine (Lanzhou, China, Certificate No.: SCXK (gan) 2015-0002). All animals were controlled by humidity and temperature, specific pathogen free grade animal room kept in 12 h cycle light or dark; animals ate freely, drunk freely. Animals were fasted 12 h before surgery, drinking freely.

2.3 Grouping and drug intervention

Sixty healthy SD male rats were andomized into sham control group (SHAM), ischemia reperfusion group(I/R), I/R+dl-3- butylphthalide(I/R+DBT) group(0.05g/kg); and I/R+ Zhongfeng capsule at high-dosage(I/R+ZFCH), middle-dosage(I/R+ZFCM), and low-dosage(I/R+ZFCL) groups (1.08g, 0.54g, and 0.27g/kg, respectively)six groups with 10 rats in each group: Dosages were calculated using the body surface area exchange algorithm[31]. The dosage of ZFCs for adult is 6 g/d, which can be converted into the dosage of rats with standard body weight: 6×0.018×5=0.54 g/d, this dose was used as the medium dose of intervention in rats. Therefore, the high-dosage, middle-dosage and low-dosage of ZFCs were 1.08, 0.54, and
0.27g/d. Rats in each group were given equal volume of saline and gavage administration, repectively for 10 consecutive days after operation.

2.4 Middle cerebral artery occlusion rat model

We performed modified a Zea-Longa method to induce CI/RI[32-33]. Briefly, all anesthetized rats were ligated the right carotid common artery (CCA), external carotid artery (ECA). Following this, at the CCA incision, we inserted nylon wire(0.285mm diameter) into the internal carotid artery(ICA), block the middle cerebral artery (MCA) for 2hour by moving abut 18mm through pulling forward. Reperfusion was established by pulling out the nylonwire. The SHAM rats were not blocked the MCA. The successfully establishment of MCAO rat was evaluated by zeal longa method 24 hours after operation. A score of 1-3 grades indicated that MCAO model rats are successfully established[15]. 24h after the MCAO surgery, I/R+ZFC treatment group dosages were determined to be 1.08g, 0.54g, and 0.27g/kg for the high, middle, and low groups, respectively. Rats in the interfered group were administered intragastrically by dl-3-butylphthalide or ZFC for 10 consecutive days after surgery[26,34], however, rats were infused normal saline in SHAM group and I/R group. The rats were executed after neurological evaluation, serum, brain tissue samples were collected. The sera was used for the ELISA, the brain tissue was used for the immunohistochemical stains, HE stains, TTC stains, TUNEL stains, RT-PCR, and WB assays.

2.5 Neurobehavioral deficit assessement

Neurological impairment[35] was assessed as previously described. The specific scoring criteria are as follows[15]; rats with unconscious, unable to stand and walk were scored 4 grades; rats with the flexion of the front paw, resistance to lateral pressure and a left turning were scored 3 grades; the flexion of the front paw resistant to lateral pressure without the turn were scored 2 grades; the opposite front paw not able to be fully extended were scored 1 grades; and no symptoms of nervous system injury were recorded as 0 grades.

2.6 Infract size in brain tissues measurement

The rats were killed with the injection of sodium pentobarbital (100mg/kg) in abdominal cavity, the brain tissues were collected and frozen. Then, each brain tissue was sectioned coronally and cut into 5 pieces. All sections were soaked with TTC solution (Sangon Biotech, Shanghai, China), and they were photographed for 10-15 min at 37 ℃. The infract size expressed as infarct area percentage(%) was calculated using Adobe Photoshop CS6 (Adobe Systems Inc., USA)[36].

2.7 Haematoxylin-eosin staining for brain histopathology

Rats were administered general anesthesia and the extracted brain tissue fixed by 4% paraformaldehyde. Next, it was dehydrated, embedded, sliced, stained for 5 min and 5-10 min respectively using hematoxylin and 1% eosin. Finally, All sections were photographed by inverted microscope(BX53, Olympus, Tokyo, Japan). The cell-damaging was observed in 5 high power fields of ischemic cortex(×20), its evaluation criteria are as follows, the cell arrangement was disorderly and irregular and hyperchromic, pyknosis or eosinophilic degeneration appeared, intercellular space was enlarged, interstitial edema was obvious, and capillary lumen was collapsed[37].

2.8 Cerebral water content

The cerebral tissue was removed and dried to a constant weight (100 ℃ for 72h). The cerebral water content were measured with the wet-dry method[38].

2.9 ELISA for inflammatory cytokines

Serum was isolated from the blood by centrifuging the blood and stored at -80 ℃. ELISA with the Multiskan Sky Microplate Spectrometer (Beijing Haonuos Technology Co. Ltd., Beijing, China) was checked the IL-1β, IL-6, TNF-α levels.

2.10 TUNEL Staining for neuronal apoptosis

After behavioral evaluation, the rats were anesthetized and sacrificed by femoral artery blood collection, the cerebral tissue was quickly dissected and fixed by 4% paraformaldehyde, paraffin embedding. sections of brain tissue were randomly selected. TUNEL method was applied to detect cells of apoptosis according to the instructions of TUNEL kit(Yeasen Biotech Co. Ltd., Shanghai, China). After TUNEL dyeing, anti fluorescence quenching agent was added dropwise for sealing, neuronal apoptosis was

observed by fluorescence microscope (IX73-A12FL/PH, Olympus, Japan). Two sections of each sample were taken to acquire data from different five fields of view in ischemic area at high power magnification(20×), the numbers of TUNEL positive staining cells (green represents the positive cells) were analyzed by pathological image analysis system, and calculate the neuronal apoptosis rate[39].

2.11 RT-PCR analysis for gene expression

The brains of the anesthetized rats were removed and were quickly frozen at -80 ℃ for analyzing in the next step. PCR primers pairs were as follows: for GAPDH (sense, 5'AGTGCCAGCCTCGTCTCATA3', anti-sense, 5'TTGTCACAAGAGAAGGCAG C3'), IL-1β (sense, 5'TGACCCATGTGAGCTGAAAG3', anti-sense, 5'CGTTGCT TGTCTCTCCTTGTA3'), IL-6 (sense, 5'CTTCACAAGTCGGAGGCTTAAT', anti- sense, 5'GCATCATCGCTGTTCATACAATC3'), TNF-α (sense, 5'ACCTTATCTA CTCCCAGGTTCT3', anti-sense, 5'GGCTGACTTTCTCCTGGTATG'), TLR4 (sense, 5'CCGCTCTGGCATCATCTTCA3', anti-sense, 5'CTCCCACTCGAGGTAGGTG T3'), NF-κB p65 (sense, 5'TGCCGAGTAAACCGGAACTC3', anti-sense, 5'CAGC CAGGTCCCGTGAAATA3'), Bcl-2 (sense, 5'AGCATGCGACCTCTGTTTG A3', anti-sense, 5'TCACTTGTGGCCCAGGTATG3'), Bax(sense, 5'GGGTTTCATCCA GGATCGAGCA3', anti-sense, 5'ACACTCGCTCAGCTTCTTGGT3'), Caspase-3 (sense, 5'GAGCTTGGAACGCGAAGAAA3', anti-sense, 5'AGTCCATCGACTTGC TTCCA3'). RNA in brain was extracted by Trizol method(Yeasen Biotech, Shanghai, China), RNA concentration was determined using an ultramicro ultraviolet-visible spectrophotometer (Q5000, Quawell Technology Inc., San Jose, CA, USA). Reverse- transcription kit (Yeasen Biotech, Shanghai, China) was used for cDNA synthesis, then the target gene was amplified by PCR(7500, fluorescence quantitative PCR, Foster City, CA, USA). The condition of RT-PCR amplified reaction was 95℃ preheat for 2minutes, 95℃ degeneration for 40seconds, 58℃ annealing for 50 seconds, 60℃ extension for 2minutes, which were cycled for 42 times. The GAPDH gene served as the control gene, and the relative quantity of objective gene was detected by $2^{-\triangle\triangle Ct}$ formula[40].

2.12 Immunohistochemical staining measure for inflammatory cytokines

The cerebral ischemia tissue of animals were embedded in paraffin and dried. The slices were inactivated with 3% hydrogen peroxide solution. After antigen repair and 5% serum blocking for 1h, the slices were cultured with antibodies IL-1β (1:400, Bioss, Beijing, China), IL-6 (1:500, Genetex, San Antonio, USA), TNF-α (1:300, Genetex, San Antonio, USA) throughout the night, washed with PBS for 3 times, then dropped into the secondary antibody, then the sections were washed by PBS and colored by DAB. Hematoxylin counterstaining was used. After sealing, three visual fields were randomly selected for observation. The cell was investigated, photographed with a microscope(BX43+sc50, Olympus, Japan). The cell was positive cells, which they were stained brown. Using image Plus 6.0 software(Media Cybernetics, Rockville, MD, USA) to measure the positive cells of each visual field, and the IHC scores was calculated[41].

2.13 Western blot analysis

50 mg ischemic brain tissue was plused 500 μl lysis buffer (Solarbio, Beijing, China), and the total protein of ischemic brain tissue was prepared. Using an ultramicro ultraviolet-visible spectrophotometer to select for protein content(50 μg/μl). Then, total protein(10 μl/sample) was loaded and ran on 10–15% polyacrylamide gels, PAGE gel area was selected to conduct trarsmembrane according to relative molecular weight of target protein. Five percent skimmed milk was applied for the closure steps. Western blotting analyses were performed with antibodies against PI3K (1:1000, Affinity, Shanghai, China), p-PI3K (1:1000, Immunoway, Plano, USA), Akt (1:1000, Immunoway, Plano, USA), p-Akt (1:1000, Genetex, San Antonio, USA), NF-κB p65 and I-κBα(1:1000, Immunoway, Plano, USA), Bcl-2 (1:1000, Genetex, San Antonio, USA), Bax (1:1000, Genetex, San Antonio, USA), Caspase-3 (1:1000, Immunoway, Plano, USA), and GAPDH (1:2000, Immunoway, Plano, USA). These antibodies were applied in cold storage all night. Next day, the membrane was incubated by secondary antibody IgG(1:10000, Immunoway, Plano, USA) for 1 h. Objective protein were expressed after a reaction with ECL reagent (Yeasen Biotech Co. Ltd., Shanghai, China) and exposured in darkroom. Using image J software to analyze the target protein's gray value of mixed protein band[39].

2.14 Data analysis

Data analysis was presented as the mean ± STDEV, and using one-way ANOVA of SPSS 20.0 software to tanalyzed data. The LSD method was used for homogeneous variance and tamhane's method was used for heterogeneous variance in group comparison. The criterion of significant difference was $p < 0.05$.

3. Results

3.1Zhongfeng capsules decreases neurological deficit scoresand alleviate cerebral swellingsin rats model of ischemic/reperfusion

To estimate the neuroprotective effects of ZFCs on rats model of ischemic/ reperfusion, neurologic impairment was scored by the Zea–Longa method. Results reflects an higher tend on the neurological deficit scores of I/R rats($^{\#\#}p < 0.01$, Figure1A). Whereas, the neurological deficit scores decreased obviously in ZFCs (1.08 g/kg) group than the I/R group($^{*}p < 0.05$, Figure 1A). Additionally, DBT(0.05 g/kg) had similar decreases in the neurological deficit scores ($^{*}p < 0.01$, Figure 1A).

Moreover, to evaluate alleviating effect of cerebral swellings of ZFCs on rats model of ischemic/reperfusion, we measured the brain water content. Results showed that the content of brain water significantly raised in I/R rats($^{\#\#}p < 0.01$, Figure1B), yet with the ZFC(1.08, 0.54, 0.27 g/kg) treatments, this was remarkably reduced ($^{**}p< 0.01$, Figure 1B). Additionally, DBT(0.05 g/kg) had similar decreases in the brain water contents ($^{**}p < 0.01$, Figure 1B).

3.2 Zhongfeng capsules reduces infarct volume in rats model of ischemic/ reperfusion

To measure the infarct volume in ischemic hemisphere, we performed TTC staining. The area of cerebral infarction showed a significant increase in I/R group($^{\#\#}p$<0.01, Figures 2A-B), yet with the ZFC(1.08 g/kg) treatments, this was remarkably reduced. ($^{**}p$ < 0.01, Figure 2A-B). Additionally, DBT(0.05 g/kg) had similar decreases in the the infarct volume ($^{**}p$ < 0.01, Figure 2B). Therefore, we suggest that ZFCs may induce expansion of the infarct size.

3.3 Zhongfeng capsules alleviates inflammatory cytokines in rats model ofischemic/reperfusion

It was previously reported that the inflammatory response was caused by inflammatory destruction in I/R rats[8]. To study whether ZFCs treatment has anti-inflammatory efficiency, the inflammatory cytokines expression in sera was determined by ELISA, RT-PCR, immunohistochemical staining. Results reflects a higher tend on the IL-1β, IL-6, and TNF-α concentrations of I/R rats($^{\#\#}p$<0.01, Figures 3A-C), meanwhile, the IL-1β, IL-6, and TNF-α expressions also increased obviously in I/R group($^{\#\#}p$<0.01, Figures 3D-L) . Incontrast, ZFCs administration (1.08 g/kg) treatment caused the IL-1β, IL-6, TNF-α concentrations of sera and the IL-6 expression of cerebral tissue dropped obviously($^{*}p$ < 0.05 or $^{**}p$ < 0.01, Figure 3A-C, E, H, K), ZFCs (1.08, 0.54 g/kg) treatment doses markedly decreased the TNF-α expression of cerebral tissue ($^{**}p$ < 0.01, Figure 3 F,I, L), ZFCs (1.08, 0.54, 0.27 g/kg) groups markedly lowered the IL-1β expression of cerebral tissue ($^{**}p$ < 0.01, Figure 3 D,G, J). Additionally, DBT(0.05 g/kg) showed similar results between the concentrations of inflammatory cytokines and the expression of inflammatory cytokines($^{*}p$ < 0.05 or $^{**}p$ < 0.01, Figure 3A-L).

3.4Zhongfeng capsules decreases neural cell apoptosis and ischemic cerebral injury

We explored the pathologic morphologic change of ischemic cerebral cortex by HE staining. HE results showed that the cell arrangement was disorderly and irregular and hyperchromic; moreover, pyknosis or eosinophilic degeneration appeared, intercellular space was enlarged, interstitial edema was obvious, and capillary lumen was collapsed in I/R group(Figure 4A). Incontrast, ZFCs administration(1.08 g/kg) treatment caused the cells to swell slightly, decrease nuclear pyknosis and eosinophilic degeneration, and increase the intercellular space (Figure 4A). Whereas, TUNEL observation hints that showed that neuronal apoptosis was appearant(Figure 4B). ZFC (1.08, 0.54, 0.27 g/kg) treatment doses had the lower concentration of apoptosis index (Figure 4C, $^{**}p$<0.01). Therefore, we suggest that ZFCs can accelerate the neurofunctional recovery in rats model of ischemic/reperfusion.

3.5 Zhongfeng capsules inhibits the NF-κB channel

The activation of the NF-κB channel, as everyone knows, closely related with inflammation, we ultimately studied whether ZFCs can inhibit the inflammation of the NF-κB channel, thus, potentially helping to relieve the CI/RI damage. Consequently, using RT-qPCR to analyze the NF-κB p65 mRNA expression as well as TLR mRNA expression, its upstream gene, in the brain tissues. The NF-κB p65 and I-κBα expression of cerebral tissue was semi-quantitated by Western-blot.
The expression of the total NF-κB and TLR genes was higher in I/R group than SHAM group($^{\#}p$ <

0.05 or $^{\#\#}p < 0.01$, Figures 5A-B), meawhile, it also increased the level of the total NF-κB p65 protein and decreased the level of the I-κBα protein in cerebral tissue ($^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, Figure 5C-E). ZFCs(1.08, 0.54, and 0.27 g/kg) treatment notably down-regulated the levels of the NF-κB p65 gene ($^{*}p<0.05$ or $^{**}p<0.01$, Figure 4B); The ZFCs(1.08 g/kg) treatment also notably inhibitedthe gene expressions of the TLR($^{*}p<0.05$, Figure 5A) . Moreover, the ZFCs(1.08 g/kg) treatment notably decreased the protein levels of the NF-κB pathway ($^{**}p<0.01$, Figure 5C-D), however, ZFCs(1.08, 0.54, and 0.27 g/kg) treatment notably increased the protein levels of the I-κBα, in brain tissues($^{**}p<0.01$, Figure 5C,E).

3.6 Zhongfeng capsulesRegulate the PI3K/Akt channel

The apoptosis of I/R inducted might be related with regulation of PI3K/Akt channel. T-qPCR results reflected a sharp decrease trend on the levels of bcl-2 gene in I/R model, however, the level of bax and caspase-3 genes reflected a sharp up-regulate trend ($^{\#\#}p < 0.01$, Figures 6A-C). According to western-blot findings, the level of p-PI3K, p-Akt, Bcl-2 proteins obviously down-regulated, whereas the level of Bax, Caspase-3 proteins obviously raised in I/R model ($^{\#\#}p < 0.01$, respectively, Figures 6D-I). Comparatively, ZFCs (1.08, 0.54, 0.27 g/kg) treatment markedly increased the specific value between pPI3K/PI3K and pAkt/Akt, as well as the overall levels of Bcl-2 ($^{**}p < 0.01$, Figures 6 D-G). Furthermore, ZFCs (1.08, 0.54, 0.27 g/kg) treatment markedly lowered the Bax and Caspase-3 protein level ($^{**}p < 0.01$, Figures 6 D, H, and I). Our data indicates a possible mechanism of ZFCs may upregulate the phosphorylation level of PI3K/ Akt pathway, so as to reduce neuro-apoptosis of ischemia through regulating apoptosis proteins.

4. Discussion

CI/RI belongs to Traditional Chinese Medicines apoplexy and was first recorded in Neijing (China). CI/RI, as per western medicine, is an acute brain dysfunction disease, where during a certain period of cerebral ischemia, the symptoms and signs are more serious than those of occlusion after the reperfusion of infarct related vessels. More than 15 million patients suffer from ischemic cerebrovascular disease every year globally and about 2 million patients of these are in China every year; The disease rate is still increasing each year and is profoundly affecting the health of the people[42-43]. Currently, thrombolysis and neuroprotective agents are the main treatment methods for CI/RI. These treatments, which aims to recover the defective neurological function; however, the effectiveness of these medicines remains unsatisfactory. As the TCM advantages of being a multi-component, multi-target treatment with low toxicity and high safety, study of TCM on CI/RI has been received much attention[44]. Additionally, as a unique advantage to TCM, the two-way regulation of TCM has gradually attracted the attention of the public. Components of ZFC is mainly *panax notoginseng*, *hirudo*, *red ginseng*, *eupolyphaga*, *pangolin scales*, *rhubarb* and *radix salvia miltiorrhizae*. ZFC and their properties are as follows; *panax notoginseng* and *pangolin scales* disperseing blood stasis to stop bleeding; *hirudo eupolyphaga*, and *rhubarb removeing* blood stasis and dredging meridians; *red ginseng* shengjin anshen; *radix salvia miltiorrhizae* blood-activating and stasis-eliminating. This whole prescription has the effect of dispelling

wind, strengthening the body, warming meridians, and dredging collaterals. According to the formula analysis, ZFCs may be an effective treatment for CI/RI; however, the specific molecular mechanism is unclear.Blood-activating and Stasis-eliminating

The pathogenesis of CI/RI so far remains unclear, and the inflammatory reaction plays a critical roles during the process of brain injury caused by CI/R. NF-κB is a protein involved in several types of immune stress responses, including cell apoptosis, cell proliferation and cell differentiation. Moreover, it is also an important inflammatory factors in regulating cellular inflammatory responses can cause neuronal death in the process of ischemic brain injury. Previous findings have confirmed the importance of NF-κB in the pathogenesis of acute ischemic damage and neurodegenerative diseases[45-46]. Furthermore, it has discovered that the TLR4/NF-κB channel can be regulated to modulate the inflammatory response[47]. Therefore, it is crucial to prevent the abnormal activation of the TLR4/NF-κB channel. Without stimulation, the trimer formed from the binding of NF-κB and I-κB can inhibit its own specific binding to target the DNA regulatory region and ensure inactive NF-κB exists in the cytoplasm. After cerebral ischemia, I-κB phosphorylates and degrades under the action of the I-κB kinase (IKK) complex, and it dissociates from the NF-κB dimer. Moreover, NF-κB activation was resulted in the NF-κB release and its exposure to the p65 subunit, which then migrates into the nucleus, initiating the transcription of a series of cytokines, chemokines, adhesion molecules, and other genes, thus promoting the expression of inflammatory factors[48-49]. Furthermore, a large number of neutrophils are chemotactic and release oxygen free radicals, proteolytic enzymes, and peroxidase, which aggravate brain tissue damage.

In addition, TLR4 belongs to the toll-like receptor family which is associated with the generation and development of several different pathemas closely. When an organism is stimulated by various physiological or pathological stimuli, TLR activates the NF-κB channel through the dimerization of its ligand binding[50], which up-regulates the expression of various inflammatory cytokines(IL-1β, IL-6, TNF-α, etc); this is done by promoting NF-κB p65 to translocate into the nucleus and connecting with the target sequences[51-52]. Evidence has shown that several inflammatory signals are released after CI/RI; it makes platelet, brain vessel endothelial cells and glial cells release inflammatory cytokines, like TNF-α and IL-1 in this instance[53]. IL-1β is an important pro-inflammatory factor, which has a close link with immune and inflammatory response, the TNF-α can mediate the activation of neutrophils and lymphocytes, promote the expression of adhesion factors, induce the synthesis of inflammatory mediators, which aggravate brain injury especially in reperfusion[54]. Other studies have confirmed that the overexpression of IL-1 β, IL-6, TNF-α from CI/RI can lead to a variety of pathological changes[15]. These are key inducers in the triggering of the inflammatory cascade reaction of ischemic brain injury[55]. Hence, inflammation inactivation may be a valuable target for anti-ischemic cerebral injuries[56]. It has been demonstrated that the activating of TLR4/NF-κB channel may cause unwarranted inflammation, the interplay between inflammatory factors and NF-κB is one of the main reasons to cause CI/RI inflammation reactions. In the current study, ZFCs were shown to sharply decrease the IL-1 β, IL-6, TNF-α, TLR4, NF-κB levels of CI/RI rat, and it significantly inhibited the reductions in I-κBα. These findings

suggest that ZFCs might inhibit inflammatory response to ameliorate CI/RI injury via the TLR4/NF-κB channel, so as to relieve the pathomorphological damage of brain tissue and play a neuroprotective role.

Apoptosis maybe another important pathological step in CI/RI[57]. The PI3K/Akt pathway regulation is very important for the CI/RI apoptosis , among the several other signaling pathways that are involved[7-9]. When CI/RI occurs, the mitochondrial structure is destroyed, and cytochrome c (Cytc) is released. This promotes the homologous oligomerization of apoptotic protein activating factor 1, which then (Aparf 1), Aparf 1 promotes the binding of Caspase-9 to apoptotic bodies, which activates Caspase-9 and further activates other Caspases, such as Caspase-3, thus inducing apoptosis[58]. Bax and Bcl-2 are also participated in apoptosis regulatory. Bcl-2 can prevent the release of Cytc, promote the glutathione (GSH) accumulation of nucleus, change the redox balance of nucleus, reduce the reactivity of Caspases, and then inhibit apoptosis[59]. Focal cerebral ischemia promotes irreversible nerve injury at the ischemic center. However, the ischemic penumbra can keep the cells, which is dependent on its residual blood flow and the energy provided by the new blood vessels. Furthermore, the blood-brain barrier is still relatively intact, thus, apoptosis is reversible to an extent. Therefore, PI3K/Akt signaling pathway can raise Bcl-2 level and reduce Bax level, which is an important anti apoptotic channel[60]. In our study, the PI3K/Akt signaling pathway was highly activated which was intervened by ZFCs in R/I rats. It can further promote the level of Bcl-2 expression , inhibit the level of Bax and Caspase-3 expression, and play the role of anti neuronal apoptosis. Taken together, our data showed that ZFCs can inhibit the neuron apoptosis in rats with CI/RI injury by raising the antiapoptosis protein level and lowering the proapoptosis protein level in PI3K/Akt signal pathway, so as to exert the neural protective effect.

Our study presented first evidence approaching the mechanism on the protective role of Zhongfeng capsule (ZFC) that is composed of *panax notoginseng*, *hirudo*, *red ginseng*, etc for ischmic stroke. And a comparatively comprehensive set of results suggest that the suppressing PI3K/Akt signal pathway-mediated neuronal apoptosis, suppressing TLR4/NF-κB signal pathway-mediated inflammatory reaction were the potential mechanisms for the regulations (Figure. 7). On the other side, activation of PI3K/Akt channel can inhibit the release of proinflammatory media, including NF-κB, IL-1β, IL-6, TNF-α, which may be another functional mechanism of Zhongfeng Capsule against CI/RI (Figure. 7). But, in PIK/Akt channel and TLR4/NF-κB channel, the primary target channel of ZFCs may be PIK/Akt signaling pathway mediated apoptosis. However, which is the most effective and direct target molecule in PI3K/Akt channel-mediated neuronal apoptosis and TLR4/NF-κB channel- mediated inflammatory response? Are there other miRNAs involved in the regulation of PI3K/Akt channel and TLR4/NF-κB channel? It has not been studied in this study.

5. Conclusion

In our finding, ZFCs were related to the decrease expression of IL-1β, IL-6, TNF-α, TLR4, NF-κB in CI/RI rat, and it significantly inhibited the reductions in I-κBα, it can also further raise the Bcl-2 expression level, reduce the Bax and Caspase-3 expression levels, and exert effects through anti-neuronal apoptosis. Given all of that, our findings reveal that ZFCs can alleviate CI/RI damage by decreasing inflammatory

factor secretion and neuronal apoptosis via the TLR4/NF-κB, and PI3K/Akt channel. Moreover, ZFC treatment delayed the pathological damage of brain tissue, which was dependent on the inflammatory reaction and apoptosis during cerebral CI/RI injury. The molecular mechanism of ZFC on CI/RI is shown in Figure. 7; however, due to the complexity of the ZFC components, the factor playing the lead protective role remains to be further simulated and analyzed using bioinformatics technology, such as molecular docking and network pharmacology. Following this, the specific molecular mechanism can be further studied through the experiments of CI/RI in vivo.

Declarations

Ethics statements

The animal study was reviewed and approved by Institutional Animal Ethical Committee of Gansu University of Chinese Medicine.

Conflict of Interest

All authors claim that there is no commercial or financial relationships in our research.

Author Contributions

Yongfeng Wang, Yonqi Liu designed the research; Jiayu Wang, Yao Shi performed the experimental work; Chunlu Yan, Fangyu An wrote the manuscript; Donghui Lv, Yanzhen Zhao analyzed the data; Yongfeng Wang, Yonqi Liu helped make the charts and check the manuscript. All authors participated in the discussion, compile and examination of the final version. All authors also participated in the view and approval of the manuscript.

Funding

This work was supported by Natural fund of Gansu Province (1610RJZA061), the Improvement Project of College Students' Employment and Entrepreneurship Ability of Gansu Province (6-1) and the Open Project of Gansu Research Center of Traditional Chinese Medicine (zyzx-2020-07).

Data availability statement

The data analyzed during the current study are available from the corresponding author upon reasonable request. Requests to access the datasets should be directed to yanchl1979@126.com.

Acknowledgments

We would like to thank Editage (www.editage.cn) for English language editing.

References

1. Wang P, Zhang MX, Zhuang PW, Lu ZQ, Han J, Zhang YJ. Progress on inflammatory reaction mechanism of ischemia/reperfusion injury. *Journal of Tianjin University of Traditional Chinese Medicine* 33(2014) 317-320.
2. WANG X, AN F, WANG S, et al. Orientin attenuates cerebral ischemia/ reperfusion injury in rat model through the AQP-4 and TLR4/NF-κB/TNF-α signaling pathway[J].J Stroke Cerebrovasc Dis,2017,26(10): 2199 -2214.
3. Vallabhapurapu S, Karin M. Regulation and Function of NF-κB Transcription Factors in the Immune System. *Trends Annu Rev Immunol* 29(2009) 693-733.doi: 10.1146/annurev.immunol.021908.132641.
4. Denk A, Wirth T, Baumann B. NF-κB transcription factors: critical regulators of hematopoiesis and neuronal survival. *Cytokine Growth Factor Rev* 11(2000): 303-320.doi: 10.1016/s1359-6101(00)00009-5.
5. Flood PM, Qian L, Peterson LJ, Zhang F, Shi JS, Gao HM, Hong JS, Transcriptional Factor NF-κB as a Targetfor Therapy in Parkinson's Disease. Parkinson's Disease,2011,2011: 216298. doi: 10.4061/2011/216298.
6. Li F, Chang YQ, Ding J. Clinical research progress of nuclear transcription factor NF-κB in hypothalamus. *Med Pharm J Chin PLA* 28(2016) 113-116.
7. Deng BX, Lu JD. Evaluate the Expression of NF-κB and the Intervene Mechanismof the Traditional Chinese Medicine (TCM) in Cerebral Ischemia Reperfusion. *Journal of Hunan Univ of CM* 34(2014) 54-58.
8. Zhou JJ, Wu CT, Li G, Zhang QP, He YH. Research progress of autophagy protection on traditional Chinese medicine intervention in cerebral ischemia- reperfusion injury. *Hunan Journal of Traditional Chines Medicine* 37(2021) 191-194.
9. Min DY, Li Y, Guan L, Chang J, Zhang HN, Cui XY, Wang P, Cao YG. Protective mechanism of Naoxinqing Capsule in rat models of cerebral ischemia/reperfusion injury. *Chinese Journal of Tissue Engineering Research* 24 (2020) 215-222.
10. Mou SM, Sui XL, Gao L, Gao N, Yang F, Zhang XZ, Li Protective mechanism of mild hypothermia on cerebral ischemia-reperfusion injury. *Chin J Arterioscler* 28(2020) 118-122,146.
11. Jia BB, Cai QY, Zhang L, Shen AL, Chu JF, Peng J. Protective effect of Qingda Granule on inflammatory injury in rats with cerebral ischemia-reperfusion. *Chinese Journal of integrative Medicine on Cardio-Cerebrovascular Diseases* 18(2020) 2597-2602.
12. Liao XB, Liu ZB, Lv ZC, Kang CX. Effect of miR-122 on cerebral infarction volume and insulin like growth factor-1 receptor pathway in mice with cerebral ischemia-reperfusion injury. *J Anatomy* 44(2021) 507-512.
13. Huang H, Qi SY, Zhang K, Yang F, Wang Q. Inhibition of NLRP3 inflamma- some activation by neferine alleviates brain tissue damage and immune disorders in rats with cerebral ischemia-reperfusion. *Immunological Journal* 37(2021) 704-709.

14. Jiang FF, Chen ZZ, Xu YW, Wan HF, Lin HY. Study on Mechanism of hyperosi- de Protecting Cerebral Ischemia Reperfusion Injury in Rats. *Chin J Mod Appl Pharm* 38 (2021) 1448-1453.
15. Ouyang B, Liu XD, Yang XQ, Ding H, Huang XP, Deng CQ. Neuroprotective effect of borneol combined with astragaloside IV and Panax notoginseng saponins in cerebral ischemia reperfusion injury rat model through Notch signaling pathway. *Chinese Traditional and Herbal Drugs* 51(2020) 5990-5997
16. Han YD, Li DF, Bai B, Wang MQ. Effect of DA3-CH on oxidative stress injury in diabetic and cerebral ischemia/reperfusion rats. *Chin J Geriatr Heart Brain Vessel Dis* 23(2021) 1205-1208.
17. Wang Y, He Y, Yang JH, Wan HT. Effects of Yangyin Tongnao Granules on the expression of Caspase-3, Bax, Bcl-2 mRNA in rats after cerebral ischemia/ reperfusion injury. *CJTCMP* 33(2018) 3875-3878.
18. Zhao YL, Xue Y, Liu ZH, Ren S, Guan XC, Li M, Zhao X, Song Y, Ren XP. Role of the Janus kinase 2/signal transducers and activators of transcription 3 pathway in the protective effect of remote ischemia preconditioning against cerebral ischemia-reperfusion injury in rats. *NeuroRepor* 30(2019) 664-670.
19. Liu B, Ding LJ, Li F, Gong QH. Effect of Gastrodin on related genes mRNA expressions in cerebral ischemia-reperfusion rats. *Journal of Zunyi Medical University* 39(2016) 139-142.
20. Longa EZ, Weinstein PR, Calson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20(1989) 84-91. doi: 10.1161/01.str.20.1.84.
21. Huang JH, Huang XH, Chen ZY, Zheng QS, Sun RY. Dose conversion among different animals and healthy volunteers in pharmacological study. *Chin J Clin Pharmacol Ther* 9(2004) 1069-1072.
22. Cao ZW, Zhang R, Li M, Guan YM, Wang QS. The mechanism of *salvianolic acids*(SA) on improving cerebral ischemic damage through silencing information regulator protein 1 (SIRT1) /high-mobility group box 1 (HMGB1) signal pathway in rat. *J Apoplexy and Nervous Diseases* 38(2021) 4-8.
23. He WH, Zeng R, Cheng XH. The protective effect of Eupolyphaga sinesis water against cerebral ischemia-reperfusion injury. *Pharmacology and Clinics of Chinese Materia Medica* 24(2008) 43-44.
24. Lin MB, Huang X, Zhang J. “Effects of the antagonism of leech extract on the apoptosis of primary cultured neonate rat cerebral cortical neurons caused by hypoxia.” *West China Journal of Pharmaceutical Science* 23(2008) 543-545.
25. Cao CL, Ma CS, Ma WL, Yang TZ. Anti-apoptotic effect of traditional Chinese medicine hirudo on cerebral cells after occlusion/reperfusion. *Journal of Hebei Medical University* 21(2000) 193-195.
26. Liang P, Hung Q, Nong LY, Lin J, Liu L, Meng LQ. The overall protective effects of Panax Notoginseng on neurovascular units in rats with acute cerebral ischemia reperfusion injury. *Chinese Pharmacological Bulletin* 36(2020) 1756- 1764.
27. Zhao J, Yu S, Zheng W, Feng G, Luo G, Zhao WY, Curcumin improves out-comes and attenuates Focal cerebral ischemic injury via antiapoptotic mechanisms in rats. *Neurochem Res* 35(2010) 374-379.
28. Jia Q, Zhen YH, Hong X. Neuroprotective effects of leonurine on ischemia /reperfusion-induced mitochondrial dysfunctions in rat cerebral cortex. *Biol Pharm Bull* 33(2010) 1958-1964.

29. Wan JY, Wan HF, Yang RB, Wang HT, Yang JH, He Y, Zhou HF. Protective effect of Danhong Injection combined with Naoxintong Capsule on cerebral ischemia-reperfusion injury in rats. *J Ethnopharmaco*l 144(2018) 348-357.
30. Chen L, Zhao Y, Zhang T, Dong X, Xie R, Li Z, Li Y, Zhao W, Son H. Protective effect of Sheng-Nao-Kang decoction on focal cerebral ischemia- reperfusion injury in rats. *J Ethnopharmacol* 151(2014) 228-236.
31. Kong YN, Zhan SH, Gong ZG, Yang SH, Lu F, Zhuang LH, Liu MX. Study on the mechanism of electroacupuncture in alleviating inflammatory reaction of cerebral ischemia reperfusion injury mice through TLR4 signaling pathway. *Chinese Imaging Journal of Integrated Traditional and Western Medicine* 18(2020) 568-571,581.
32. Kong Y, Le Y. Toll-like receptors in inflammation of the central nervous system. *Int Immunopharmacol* 11(2011) 1407-1414. doi: 10.1016/j.intimp.2011.04.025.
33. West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. *Annu Rev Cell Dev Biol* 22(2006) 409-437.
34. Kenny EF, O'neill LA. Signalling adaptors used by Toll-like receptors an update. *Cytokine* 43(2008) 342-349. doi: 10.1016/j.cyto.2008.07.010.
35. Picinini M, Midwood KS. DAMP ening inflammation by modulating TLR signalling. *Mediators Inflamm* 2010(2010) 672395. doi: 10.1155/2010/672395.
36. Chen WM, Qian MZ. Influence of gypenosides on the injury induced by ischemia-reperfusion and NF-κB p65 signaling pathway in rat brain. *Journal of Zunyi Medical University* 40(2017) 273-277.
37. Jin XF, Zhang XN, Zhou XH, Gao WJ. Effects of astragaloside IV on inflammatory factors and ultrastructure in rats with cerebral ischemia reperfusion. *Chin J Comp Med* 30(2020) 1-6.
38. Fang XY, Zheng Y, Jia JJ, Miao MS. The effects of cerebral ischemic preconditioning on antioxidant enzymes in cerebral ischemia-reperfusion rats. *Pharmacology and Clinics of Chinese Materia Medica* 32(2016) 193-196.
39. Chang HE, Zheng N, An HW, Dun LL, Liu GC, Zhou ZY. Effects of Tongqiao-Huoxue Decoction on Levels of TNF-α and IL-6 in Rats with Cerebral Ischemia Reperfusion Injury. *Journal of Chengdu University of TCM* 41(2018) 18-20.
40. Kim BH, Lee YG, Park TY, Kim HB, Rhee MH, Cho JY. Ginsenoside Rp1, a ginsenoside derivative, blocks lipopolysaccharide-induced interleukin-1 beta production via suppression of the NF-kappa B pathway. *Planta Med* 75(2009) 321–326. doi: 10.1055/s-0028-1112218.
41. Licata G, Tuttolomondo A, Corrao S, Di Raimondo D, Fernandez P, Caruso C, Avellone G, Pinto A. Immunoinflammatory activation during the acute phase of lacunar and non-lacunar ischemic stroke: Association with time of onset and diabetic state. *Int J Immunopath Pharmacol* 19(2006) 639-646. doi: 10.1177/ 039463200601900320.
42. Dai Z, Zhu Y, Wang ZY. Effect of Angelica Polysaccharide on the expression of IL-1 β, TNF-α and NF-κ B in rats with cerebral ischemia-reperfusion injury. *Chinese Patent Medicine* 42(2020) 2176-2178.

43. Tian Z, Tang C, Wang Z. Neuroprotective effect of ginkgetin in experimental ischemia/reperfusion via apoptosis inhibition and PI3K/Akt/mTOR signaling pathwayivation. *J Cell Biochem* 120(2019) 18487-18495.
44. Li YZ, Guo SQ, Liu WC, Jin T, Li XF, He XY, Zhang X, Su HX, Zhang N, Duan CZ. Silencing of SNHG12 enhanced the effectiveness of MSCs in alleviating ischemia/reperfusion injury via PI3K/Akt/mTOR signaling pathway. *Front Neurosci* 13(2019) 645. doi: 10.3389/fnins.2019.00645.
45. Jia ZZ, Yuan Q, Chen HY, Zhang T, Zhao L, Wang SX, Chai LJ, Guo H, Hu LM. Effects of salvia polyphenolic acids and panax notoginseng saponins on neuronal apoptosis via PI3K/Akt signaling pathway in cerebral ischemia/reperfusion injury rats. *Chinese Pharmacological Bulletin* 36(2020) 1214-1220.
46. Love S. Apoptosis and brain ischaemia. *Prog Neuropsycho Pharmacol Biol Psychiatry* 27(2003) 267-282. doi: 10.1016/S0278-5846(03)00022-8.
47. Radak D, Katsiki N, Resanovic I, Jovanovic A, Sudar-Milovanovic E, Zafirovic S, Mousad SA, Isenovic ER. Apoptosis and acute brain ischaemia in ischemic stroke. *Curr Vasc Pharmacol* 15(2016) 115-122. doi: 10.2174/15701611156661611040 9
48. Huang JL, Li Y, Zhao BL, Li JS, Zhang N, Ye ZH, Sun XJ, Liu WW. Necroptosis signaling pathways in stroke: from mechanisms to therapies. *Curr Neuropharma- col* 16((2018) 1327–1339. doi: 10.2174/1570159X16666180416152243.
49. Amani H, Mostafavi E, Alebouyeh MR, Arzaghi H, Akbarzadeh A, Pazoki- Toroudi H, Webster TJ. Would colloidal gold nanocarriers present an effective diagnosis or treatment for ischemic stroke? *Int J Nanomed* 14(2019) 8013–8031.
50. Zhang YH, Yao MJ, Liu JX. Advances in modern research on ischemic cerebr- ovascular diseases. *Chinese Pharmacological Bulletin* 35(2019) 1185-1188.
51. Ahsan A, Zheng YR, Wu XL, Tang WD, Liu MR, Ma SJ, Jiang L, Hu WW, Zhang XN, Chen Z. Urolithin A-activated autophagy but not mitophagy protects against ischemic neuronal injury by inhibiting ER stress in vitro and in vivo. *CNS Neurosci Ther* 25 (2019) 976–986. doi: 10.1111/cns.13136.
52. Li Y. Research progress on the role of neutrophils in cerebral ischemia injury. *Chin J Minim Invasive Neurosurg* 22(2017) 571-573.
53. Chen YK, Zhang L, Ni JS, Wang XY, Cheng J, Li YC, Zhen XC, Cao T, Jia J. LLDT-8 protects against cerebral ischemia/reperfusion injury by suppressing post-stroke inflammation. *J Pharmacol Sci* 131(2016) 131-137.
54. Akta HS, Ozel Y, Ahmad S, Halime HP, Betul AA, Ilyas K, Sermin T,Turgut Ş, Ceren CG, Levent K, Hatice KE. Protective effects of resveratrol on hepatic ischemia reperfusion injury in streptozotocin-induced diabetic rats. *Mol Cell Biochem* 460(2019): 217-224.doi: 10.1007/s11010-019-03582-z.
55. Yuan PX, Jiang XJ, Li YY, Wang YX, Zeng M, Xiao X, Lu B. Research progress of microglia mediated inflammatory response in cerebral ischemia-reperfusion injury. *Chin J Geriatr Hert Brain Vessel Di* 21(2019) 1112-1114.

56. Zhang H, Park JH, Maharjan S, Park JA, Choi KS, Park H, Jeong Y, Ahn JH, Kim IH, Lee JC, Cho JH, Lee IK, Lee CH, Hwang IK, Kim YM, Suh YG, Won MH, Kwon YG. Sac-1004, a vascular leakage blocker, reduces cerebral ischemia-reperfusion injury by suppressing blood-brain barrier disruption and inflammation. *J Neuroinflamm* 14(2017) 122-136. doi: 10.1186/s12974-017- 0897-3.
57. Ferrer I, Planas AM. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J Neuropathol Exp Neurol* 62(2003) 329-339. doi: 10.1093/jnen/62.4.329.
58. Zhao YM, Gao WJ. Research Progress on Autophagy-Apoptosis Balance Regulation in Cerebral Ischemia-Reperfusion Injury. *Chinese Archives of Traditional Chinese Medicine* 39(2021) 132-136.
59. Howells CC, Baumann WT, Samuels DC. The Bcl-2-associated death promoter (BAD) lowers the threshold at which the Bcl-2-interacting domain death agonist (BID) triggers mitochondria disintegration. *J Biol Chem* 271 (2011) 114-123. doi: 10.1016/j.jtbi.2010.11.040.
60. Cai L, Zhang BD. Research advances of the PI3K/AKT signaling pathway in cerebral ischemia-reperfusion injury. *Journal of Guangxi Medical University* 38(2021) 2012-2016.

Figures

Figure 1

Zhongfeng capsules(ZFCs) intervention decreased neurologic impairment scores and brain water content in rats model of ischemic/reperfusion injury. (A) Neurological deficit scores measured using Zea–Longa method(n = 10). (B) Brain water content analyzed using drying wet method (n = 10). Mean values±SD represent the value; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs SHAM group; $_{*}p < 0.05$, $^{**}p < 0.01$, vs I/R group.

Figure 2

Zhongfeng capsules(ZFCs) intervention reduced the ischemic areas of brain in rats model ischemic/reperfusion injury. (A) Typical images of cerebral infarct using TTC (n = 5). (B) Cerebral infarct size using a percentage of the infarct hemisphere (n = 5). Mean values±SD represent the value; $^{\#\#}p < 0.01$ vs I/R group; $^{**}p < 0.01$, vs I/R group.

Figure 3

Zhongfeng capsules (ZFCs) intervention restrained the excretion of inflammatory cytokines in rats model ischemic/reperfusion. (A) the IL-1 β concentrations, checked using ELISA (n = 5). (B) the IL-6

concentrations, determined by ELISA (n = 5 in each group). (C) the TNF-α concentrations, checked using ELISA (n = 5). (D) the IL-1 β gene expression, determined using RT-qPCR (n = 3). (E) the IL-6 gene expression, determined using RT-qPCR (n = 3). (F) the TNF-α gene expression, determined using RT-qPCR (n = 3). (G) the IL-1 β protein expression, determined using immune-histochemistry (n = 6). (H) the IL-6 protein expression, determined using immune-histochemistry(n = 6). (I) the TNF-α protein expression, determined using immune- histochemistry (n = 6). (J) the average optic density of IL-1 β protein expression, determined using immune-histochemistry (n = 6). (K) the average optic density of IL-6 protein expression, determined by immune-histochemistry (n = 6 in each group). (L) the average optic density of TNF-α protein expression, determined by immune- histochemistry (n = 6 in each group). Mean values±SD represent the value; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs SHAM group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs I/R group.

Figure 4

Zhongfeng capsules(ZFCs) intervention decreases neural cell apoptosis and in rats model ischemic/reperfusion. (A) Typical images of heamatoxylin and eosin (HE) staining performed in ischemic brains, measured by HE(n = 5). (B) Representative images of TUNEL assay performed in ischemic brains, measured using the TUNEL staining kit(n = 3). (C) The relative neuronal apoptotic rate levels in in ischemic brains in all groups (n = 3). Mean values±SD represent the value; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs SHAM group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs I/R group. on a scale of 50 μm.

Figure 5

Zhongfeng capsules(ZFCs) intervention activated the toll like receptor (TLR)-mediated NF-κB channel in rats model ischemic/reperfusion. (A) Examination of TLR gene level with RT-qPCR method. (B) Examination of NF-κB gene level with RT-qPCR method. . (C) Determination of NF-κB and I-κB α protein bands with western blot method. (D) Determination of NF-κB relative expression levels with Image J software; (E) The relative expression levels Determination of I-κBα relative expression levels with Image J software. Mean values±SD represent the value(n = 3); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus ischemic/reperfusion (I/R) group; $^{*}p < 0.05$, $^{**}p < 0.01$, versus I/R group.

Figure 6

Zhongfeng capsules(ZFCs) intervention upregulated the expression levels of phosphorylated PI3K, and Akt of PI3K/Aktin rats model ischemic/reperfusion. (A) Examination of the Bcl-2 gene level with RT-qPCR method. (B) Examination ofthe Bax gene level with RT-qPCR method. (C) Examination of the Caspase-3 gene level with RT-qPCR method. (D) Determination of PI3K, phospho-PI3K, Akt, phospho-Akt, Bcl-2, Bax, and Casepase-3 protein bands with western blot method. (E)–(I) Determination of pPI3K/PI3K, pAkt/Akt Bcl-2, Bax, and Casepase-3 relative expression levels with Image J software. Mean values±SD represent the value(n = 3); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs SHAM group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs I/R group.

Figure 7

Molecular mechanism of Zhongfeng capsules on cerebral ischemia reperfusion injury. Zhongfeng capsules (ZFC) relieves I/R damage-induced inflammation activation as evidenced by inhibiting the secretion of inflammation cytokines, including IL-1β, IL-6, and TNF-α, which then subsequently downregulated TLR/NF-κB dependent inflammation. ZFC also suppressed PI3K/Akt mediated apoptosis by down-regulating levels of Bax and Caspase-3, while up-regulating expression of PI3K/Akt.
On the other side, activation of PI3K/Akt signaling pathway can inhibit the release of proinflammatory cytokines, like NF-κB, IL-1β, IL-6, TNF-α, which may be another functional mechanism of Zhongfeng capsule against CI/RI.