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Zhongfeng Xingnao prescription alleviates injury of intracerebral hemorrhage via regulating the CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis

Jianyuan Yu [b,1], Huifen Zhou [b,c,1], Jianwen Guo [d,e,***], Tianhang Chen [f], Chongyu Shao [b,c], Zhiyong Pan [a], Jiehong Yang [b,c,**], Haitong Wan [a,b,c,*]

[a] The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China
[b] School of Basic Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, China
[c] Key Laboratory of TCM Encephalopathy of Zhejiang Province (grant no. 2020E10012), Hangzhou, China
[d] Department of Neurology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China
[e] State Key Laboratory of Dampness Syndrome of Chinese Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China
[f] School of Life Sciences, Zhejiang Chinese Medical University, Hangzhou, China

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ABSTRACT

Background and aim: The NLRP3 inflammasome can be activated after intracerebral hemorrhage (ICH), triggering an inflammatory response in the brain. Chinese herbal medicine Zhongfeng Xingnao Prescription (ZFXN) is commonly used in China for intracerebral hemorrhage treatment. However, the underlying treatment mechanism of it is unclear. The purpose of our study is to investigate whether ZFXN alleviates injury after intracerebral hemorrhage by blocking the activation of CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis.

Experimental procedure: We evaluated the protective effect of ZFXN in a rat model of collagenase VII-induced ICH. The neurological deficit score, cerebral hematoma-injury ratio, pathology, and ultrastructure of tissue surrounding the hematoma were evaluated after 5 days of ZFXN treatment, CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis-related protein expression around the hematoma was assessed by Western blot and immunohistochemistry. Meanwhile, ELISA measured the levels of IL-1β, IL-18, IL-6 and TNF-α in serum.

Results and conclusion: After 5 days of ZFXN treatment, the score of neurological deficit and hematoma damage ratio decreased, and the cell destruction such as edema and vacuole conditions around the hematoma improved. The mechanism investigation results showed that ZFXN down-regulated expressions of CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis-related protein around the hematoma area. In addition, ZFXN could attenuate the inflammatory response by regulating the activation of NLRP3 inflammasome after ICH. For the first time, we found that the efficacy of ZFXN on ICH might be related to the regulation of NLRP3 inflammasome.

1. Introduction

Intracerebral hemorrhage (ICH) is a fatal neurological sickness and a subtype of stroke, which is caused by broken cerebral blood vessels and constitutes 10%–15% of all strokes worldwide every year.[1–3] Neuroinflammation is a characteristic of neuroinflammatory diseases and is the result of infection, injury, and autoimmune processes. It can be caused by ICH, if neuroinflammation is not removed in time, it can aggravate the degree of secondary brain injury after ICH.[4,5] After ICH, the leaked blood activates cells involved in immune responses, such as microglia. Activated immune cells release inflammatory factors, which will aggravate the inflammatory response, causing persistent damage to the brain, such as blood-brain barrier (BBB) destruction caused by rupture of vascular endothelial structure, cerebral edema, intracranial

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* Corresponding author. School of Basic Medical Sciences, Zhejiang Chinese Medical University, Binwen Road&548, Hangzhou, 310053, China.
** Corresponding author. School of Basic Medical Sciences, Zhejiang Chinese Medical University, Binwen Road&548, Hangzhou, 310053, China.
*** Corresponding author. The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Da De Road&111, Guangzhou, 510000, China.
E-mail addresses: jianwen_guo@qq.com (J. Guo), yjhong@zcmu.edu.cn (J. Yang), whtong@126.com (H. Wan).
[1] Jianyuan Yu and Huifen Zhou should be considered co-first authors. Contributed equally to this work and share the first authorship.

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hypertension, and neuronal apoptosis.[6–8] Thus, modulating the inflammatory response may serve as a therapeutic after ICH.

The inflammasome, a protein complex, has been discovered to participate in different inflammatory responses, particularly, including the innate immune responses to central nervous system (CNS) injuries such as Alzheimer's disease, ischemic stroke, or ICH. One of the receptor proteins of the inflammasome is the NOD-like receptor family containing pyrin domain-containing protein 3 (NLRP3), NLRP3 inflammasome is the most characteristic and widely studied inflammasome which consists of NLRP3, ASC, and pro-Caspase1. It promotes the pro-inflammatory factors that are secreted immediately and subsequent inflammatory response once the NLRP3 inflammasome is activated.[9,10] The NLRP3 inflammasome can be stimulated by NF-κB.[11] Activated NLRP3 inflammasome will induce caspase-1 be cleaved and induce cell pyroptosis, with the secretion of interleukin-18 (IL-18) and IL-1β. Inhibition of the NLRP3 inflammasome is important for the development of the neuroinflammatory response after intracerebral hemorrhage and is also an important target for the inhibition of pyroptosis.

Except for conventional therapy for ICH, many studies have proved that traditional Chinese medicine (TCM) can alleviate the injury.[12] Zhongfeng Xingnao Prescription (ZFXN) is a well-known and widely used Chinese herb formula for secondary damage of acute stroke treatment in clinics for decades.[13–15] It plays a comprehensive role in stroke. ZFXN can alleviate the injury after stroke by exerting neuroprotective, anti-inflammatory, and antioxidant effects.[16–18] Early studies demonstrated that ZFXN treatment can obviously reduce the disability rate of ICH patients by reducing intracranial pressure, relieving brain edema, and promoting hematoma absorption.[19,20] Besides, ZFXN can distinctly accelerate cerebral hematoma absorption and reduce hematoma volume in experimental animals, increase the number of phagocytes in the hemorrhage focus, and enhance the effect of clearing hematoma and necrotic tissue.[21] Meanwhile, some natural compounds in ZFXN like ginsenoside Rg1, emodin, and ligustilide were approved to have protective effects in the treatment of ICH.[22,23] However, the pharmacological effects of ZFXN in ICH are still unclear.

In this study, we observed the therapeutic efficacy of ZFXN on ICH in rats and explored its potential mechanism in an experimental rat model of ICH. We revealed that ZFXN might reduce inflammation by regulating the CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis after ICH.

2. Materials and methods

2.1. Zhongfeng Xingnao prescription preparation

Zhongfeng Xingnao Prescription (ZFXN) was provided by the Guangdong Provincial Hospital of Chinese Medicine (Guangzhou, China) and it is composed of four dried raw herbs (Table 1), including *Rheum. officinale* Baill. (DH), *Panax ginseng* C.A.Mey. (HS), *Panax notoginseng* (Burk.) F. H. Chen (SQ), and *Ligusticum chuanxiong* Hort. (CX), were mixed at a ratio of 5:30:30:15 (dry weight). We made a UHPLC-MS/MS analysis for ZFXN (Supplemental file).

2.2. Animal model and grouping

We performed the animal experiment according to the Guide for the Care and Use of Animal Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China, Ethics approval reference number was 20220214-25). All healthy male Sprague-Dawley (SD) adult rats, weighing 225–250 g, were provided from the Central Animal Facility of Zhejiang Chinese Medical University and randomly divided into five groups: sham, ICH (model group), ICH + low dose ZFXN (4.5 mL/kg, p.o.) (ZFXN-L group), ICH + medium dose ZFXN (9 mL/kg, p.o.) (ZFXN-M group) and ICH + high dose ZFXN (18 mL/kg, p.o.) (ZFXN-H group). Above the dose of ZFXN was determined based on clinical dosage conversion. We established the rat ICH model based on the literature.[24,25] The rats were fixed on the brain stereo-positioning apparatus after anesthesia with isoflurane. Then, 1 μL of collagenase VII (0.5U/μL, Sigma-Aldrich) was injected at a steady speed of 0.2 μL/min into the rat caudate nucleus (0.5 mm anterior, and 3 mm right side to the bregma, 5.5 mm in depth). When the injection was completed, the needle was placed for up to 8 min which was used to prevent collagenase from overflowing from the skull surface, then microsyringe was removed slowly. Afterward, the scalp was sutured. Put animals back in their cages. Those in the sham-operated group received the same operation but did not inject collagenase VIIs. 24 h after the operation, we tested the Bederson score of each rat. Rats with a score greater than or equal to 1 were regarded as successful models. In treatment groups, ZFXN was given once a day for 5 days. Rats in the sham group and the model group were given physiological saline via gavage. The mortality rate of the animals in this experiment was 0.

Table 1
Herbs in ZFXN.

Chinese name	Latin name	Dosage(g)
Da Huang	*Rheum.officinale* Baill.	5
Hong Shen	*Panax ginseng* C.A.Mey.	30
San Qi	*Panax notoginseng* (Burk.) F. H. Chen	30
Chuan Xiong	*Ligusticum chuanxiong* Hort.	15

2.3. Neurological function evaluation

After 5 days, we assessed the neurological deficit, using the Bederson score. According to the Bederson score, a four-level scale[26,27] is used to assess neurological deficits: 0 = asymptomatic symptoms, 1 = flexion of the forelimb, 2 = decreased resistance to lateral push, and 3 = circling. A higher score indicates the more serious the neurological impairment.

2.4. Measuring hemorrhage volume ratio

Serial sections of the coronal plane were performed at 2-mm intervals to determine the hemorrhage volume ratio. By using Image J, we assessed each section's hematoma and total area. The total hematoma volume (mm^3) and brain volume (mm^3) are calculated by multiplying the hematoma and total brain area by the thickness of each section. The ratio of injury is calculated in accordance with the total hematoma volume/brain volume.

2.5. Pathological examination

After deep anesthesia, the rats were perfused with 0.9% normal saline at 4 °C until the light pink liquid flowed out, then replaced with 4% paraformaldehyde, and the perfusion was stopped when the rat body became stiff. The rats' brains were taken out and immersed in 4% paraformaldehyde for 48 h at 4 °C, which can stabilize the tissue. The brains were then dehydrated and embedded in paraffin wax. Paraffin-embedded tissue specimens were cut into 4 μm sections, stained with hematoxylin-eosin-saffron(H&E), and then observed under an optical microscope for histological study.

2.6. Transmission electron microscopy

Using a transmission electronic microscope (TEM) (H-7650; Hitachi, Tokyo, Japan) to observe the influence of ZFXN on morphological changes around the hematoma area after ICH. A fresh tissue block of 1 mm^3 was obtained from the perihematomal area. Then, the tissues were fixed with 2.5% glutaraldehyde for 24 h and post-fixed in 1% osmic acid subsequently different concentrations of ethyl alcohol were used for dehydration. After the samples had been completely infiltrated and embedded with propylene oxide, they were cut with an ultramicrotome. We stained ultrathin (60 nm) sections with 4% uranyl acetate and lead citrate. Photos were obtained by TEM.

2.7. Measurement of inflammatory cytokines in serums

After day 5 after ICH, blood samples were taken from the hearts, and then whole blood was separated for 10 min at 3000 × rpm at 4 °C to collect serum. We determined serum IL-18, IL-1β, TNF-α and IL-6 levels using Rat Interleukin 18(IL-18) ELISA Kit (MEIBIAO BIOLOGY, MB-1735A, China), Rat Interleukin 1β(IL-1β) ELISA Kit (MEIBIAO BIOLOGY, MB-1588A, China), Rat Interleukin 6(IL-6) ELISA Kit (MEIBIAO BIOLOGY, MB-1731A, China) and Rat Tumor Necrosis Factor-α(TNF-α) ELISA Kit (MEIBIAO BIOLOGY, MB-1721A, China) by the manufacturer's instructions and finally the optical density (OD) was measured at 450 nm.

2.8. Immunohistochemical analysis

In immunohistochemical analysis, to deparaffinization and dehydration, paraffin sections were washed twice with xylene for 10 min each time, followed by two 3-min, two 2-min, 2-min, and 2-min washes with gradient ethanol (100%, 95%, 80%, and 70%) and then washed in distilled water. Antigen repair is achieved by high temperature and high-pressure method, the sections were put into 0.01 M EDTA buffer solution and boiled for 5 min, then they were washed 3 times with distilled water and PBS, and treated with 3% hydrogen peroxide for 10 min. Finally, the sections were incubated with anti-Caspase1(1:50, Abcam, ab74279, UK), anti-NLRP3(1:75, NOVUS, NBP2-12446, USA), anti-GSDMD (1:1000, Abcam, ab219800, UK) primary antibodies at 37 °C for 1 h. The sections were incubated with HRP-linked secondary antibodies for 30 min at 37 °C after washing with PBS for three times. The sections were incubated in diaminobenzidine (DAB) to produce brown products which are positive cells. The integrated optical density (IOD) of Caspase1, NLRP3, and GSDMD immunoreactive cells in the perihematomal area was performed using Image J.

2.9. Western blot analysis

The brains were first chopped and homogenized using RIPA Buffer (Thermo Fisher, 89900, USA). After centrifuging the homogenate, the protein concentration in the supernatant was detected with the BCA Protein Assay Kit (Beyotime, P0010, China) kit. Protein samples (60 μg total proteins) were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gels, then transferred to the polyvinylidenedifluoride (PVDF) (Millipore, IPVH00010, USA) membrane. A 5% BSA/TBST solution was applied for blocking membranes. Subsequently, the protein bands on membranes were separately incubated with the primary antibody overnight at 4 °C: anti-p-NF-κB p65(1:1000, CST, 3033, USA), anti-NF-κB p65 (1:1000, CST, 8242, USA), anti-NLRP3 (1:1000, CST, 15101, USA), anti-Caspase 1 (1:1000, CST, 83383, USA), anti-Cleaved Caspase1 (1:500, Thermo Fisher, PA5-99390, USA), anti-Cleaved IL-1β (1:2000, CST, 63124, USA), anti-GSDMD (1:1000, CST, 39754, USA), anti-Cleaved GSDMD(1:1000, CST, 10137, USA), anti-Pro-Caspase1 (1:1000, CST, 24232, USA), anti-CaMKII(1:1000, Abcam, ab134041, UK) and β-actin(1:10000, Abcam, ab68477, UK). On the next day, membranes were washed for 10 min (3 times) in TBST solution, then incubated them with horseradish peroxidase-conjugated antibodies for 1 h, visualized them using ECL reagent, and analyzed the immunoreactive bands with Image J software. The results of Western blot analysis were normalized to β-actin.

2.10. Statistical analysis

We used GraphPad Prism(8.0.1) software to analyze all the data, and the results were expressed as mean ± standard deviation (S.D.). Firstly, checking the normality of the data, then took one-way analysis of variance (ANOVA), finally, data from more than two groups were compared by Tukey test, or use nonparametric test to determine the results, and $p < 0.05$ was statistically different.

3. Result

3.1. ZFXN relieves neurological impairment and reduces the ratio of hemorrhage volume

To determine the effect of ZFXN on neurological function and hemorrhage volume after intracerebral hemorrhage in rats, we first compared the results of the ZFXN treatment group and sham, model group. As shown in Fig. 1, treatment with the ZFXN alleviated neurological deficits and hemorrhage volume ratio at day 5. ZFXN can reduce the hemorrhage volume ratio at d 5 after ICH. The hemorrhage volume ratio in the model rats was significantly higher than that in the sham-operation group, after 5 days of drug treatment, the final hemorrhage volume of the ZFXN treatment was markedly decreased ($p < 0.05$, $p < 0.01$ or $p < 0.001$ Fig. 1A and B). Additionally, as a comparison between the Model group and Sham group revealed, the rats of ICH showed obvious neurological deficits ($p < 0.001$, Fig. 1C). The Bederson score in the ZFXN-H group was successfully reduced ($p < 0.01$, Fig. 1C) in comparison with the model group, the phenomenon of the forelimb flexing or circling was reduced.

3.2. ZFXN improves histopathology

To determine whether ZFXN could alleviate the histopathology in the perihematomal area, we observed the histopathology in this region by H&E staining. Fig. 2 presents the pathologic changes in all groups. H&E staining of the perihematomal area in the Model showed that the arrangement of brain tissue was disordered and scattered, the cells were swollen with an irregular shape and the vacuoles changed. Meanwhile, the nucleus was pyknotic, and structure unclear. After treatment with varying doses of ZFXN, H&E staining revealed reduced numbers of deformed cells and pyknotic nuclei. ZFXN has a tendency to relieve the lesions in the perihematomal area.

3.3. ZFXN improves brain ultrastructure

To observe the influence of ZFXN on ultrastructure changes around the hematoma area after ICH, tissues around the hematoma were observed by transmission electronic microscope. Fig. 3 shows the ultrastructure in the perihematomal area of the Sham group, Model group, and ZFXN-H group. In the sham group, the cell and tissue ultrastructure were finely packed with visible nuclei. In comparison, on day 5 after ICH, the arrangement of organelle was disordered, swelling cells occurred and the morphology of mitochondrial was altered. Furthermore, the membrane outline was unclear and cells were surrounded by several blebs. In contrast, ZFXN therapy improved the integrity of cells and reduced the degree of brain damage in perihematomal regions.

3.4. ZFXN inhibits pyroptosis and overexpression of inflammatory factors by regulating the CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis in ICH rats

The CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis is closely related to the NLRP3 inflammasome activation and pyroptosis occurrence. We detected the changes in proteins related to the signaling axis by immunohistochemistry and Western blot. After ICH, NLRP3, and Caspase1 were strongly expressed in the cytoplasm, Caspase1 could also be observed in the nucleus. However, a small amount of NLRP3 was observed in the nucleus. Moreover, GSDMD was expressed in the membrane (Fig. 4A). In addition, the integrated optical density (IOD) of Caspase1, NLRP3, and GSDMD immunoreactive cells were performed using Image J. High IOD values indicated that the expression was more positive. The results of immunohistochemistry confirmed the increased expression of NLRP3, GSDMD, and Caspase-1 after ICH (Fig. 4B, $p < 0.05$, $p < 0.01$ or $p < 0.001$). Each ZFXN group expressed less Caspase1, NLRP3, and GSDMD than the model group. 9 ml/kg or 18 ml/kg of ZFXN

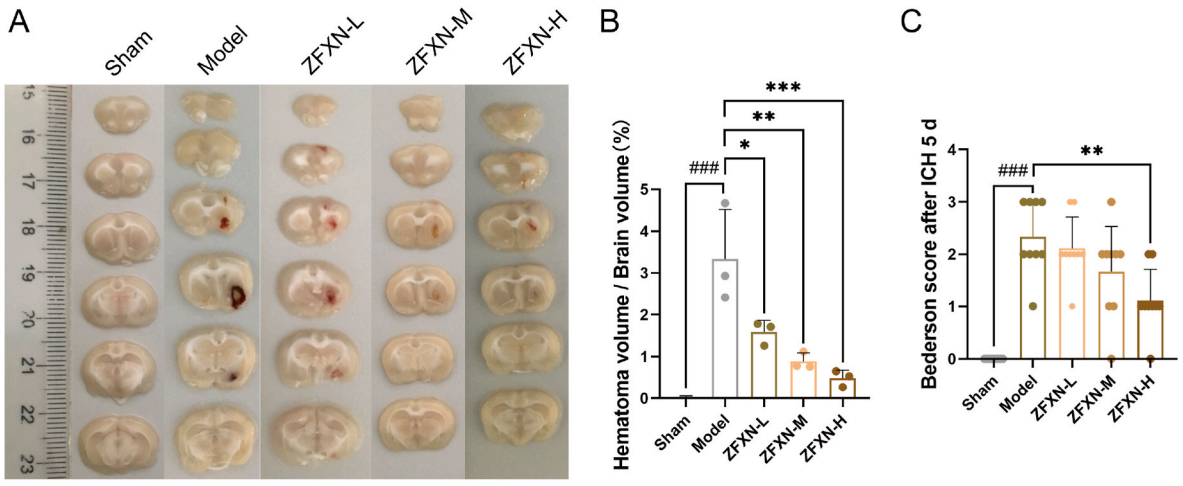

Fig. 1. ZFXN relieves neurological impairment and reduces the ratio of hemorrhage volume. (**A**) Representative photograph of brain slice at 5 days after ICH in rats treated with saline and ZFXN, scale bar 5 mm. (**B**) Statistical graph of hemorrhage volume ratio, Mean ± SD (n = 3). (**C**) The Bederson score for all groups at d 5 after ICH. Mean ± SD (n = 9/group). ###$p < 0.001$: Sham vs. Model group, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$: ZFXN groups vs. model group.

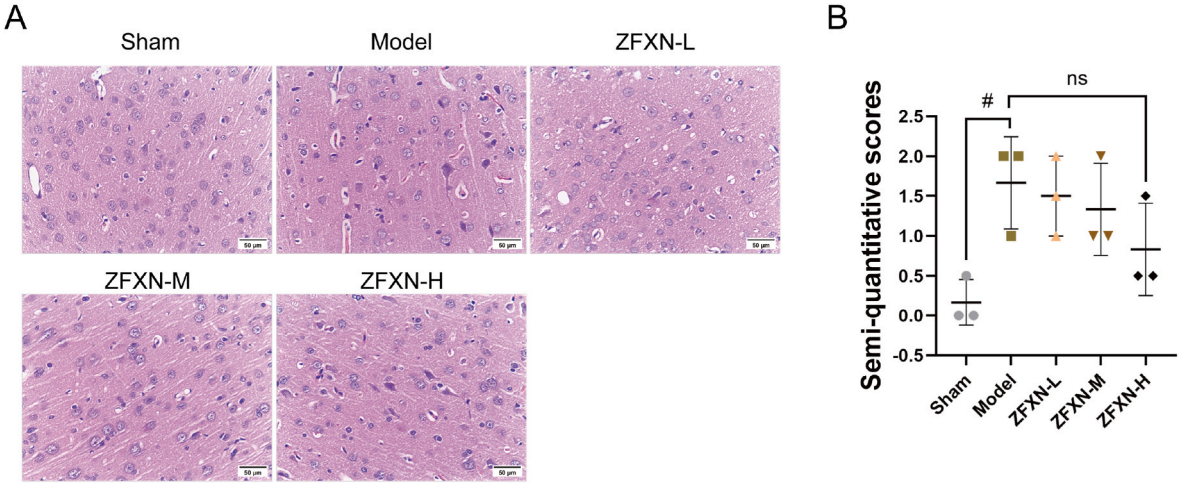

Fig. 2. ZFXN alleviates the histopathology in the perihematomal area at 5 days after ICH. (**A**) H&E staining. scale bar: 50 μm, image at 200 × . (**B**) Semi-quantitative scores of pathological changes in each group, Mean ± SD (n = 3). #$p < 0.05$: Sham vs. Model group.

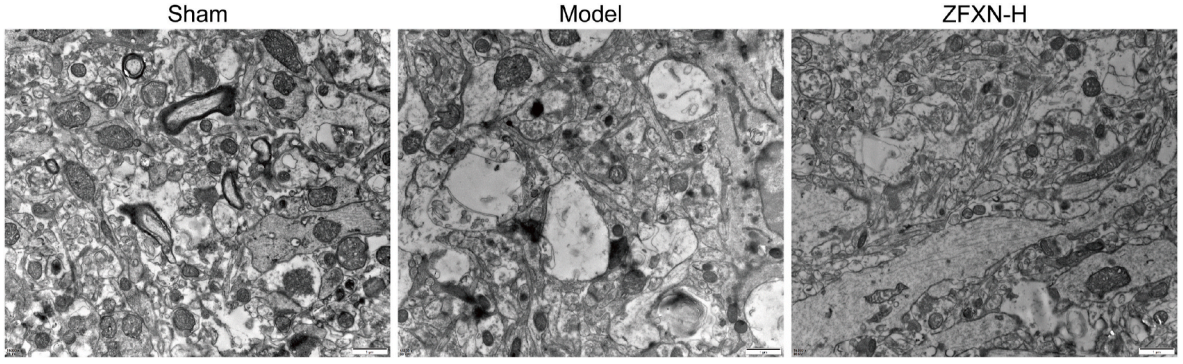

Fig. 3. ZFXN improves brain ultrastructure around the hematoma area after ICH. Representative brain ultrastructure images under TEM in the perihematomal regions. scale bar: 1 μm, image at 15000 × .

significantly down-regulated the expression of Caspase1, NLRP3, and GSDMD (Fig. 4B, $p < 0.05$, $p < 0.01$ or $p < 0.001$).

In the Western blot results (Fig. 5A–D), at d 5 after ICH, the expression of CaMKII, p-NF-κB p65, NLRP3, Pro-Caspase1, Caspase1, Cleaved Caspase1, Cleaved GSDMD, pro IL-1β and Cleaved IL-1β significantly is elevated in rats' brain of model group. Compared to model rats, the levels of p-NF-κB p65, NLRP3, Caspase1 and cleaved IL-1β statistically reduced in ZFXN-M group ($p < 0.05$, $p < 0.01$ or $p < 0.001$), the expression of CaMKII, pro-Caspase1, cleaved Caspase1, pro-IL1β and cleaved GSDMD in ZFXN-L and ZFXN-M groups did not differ

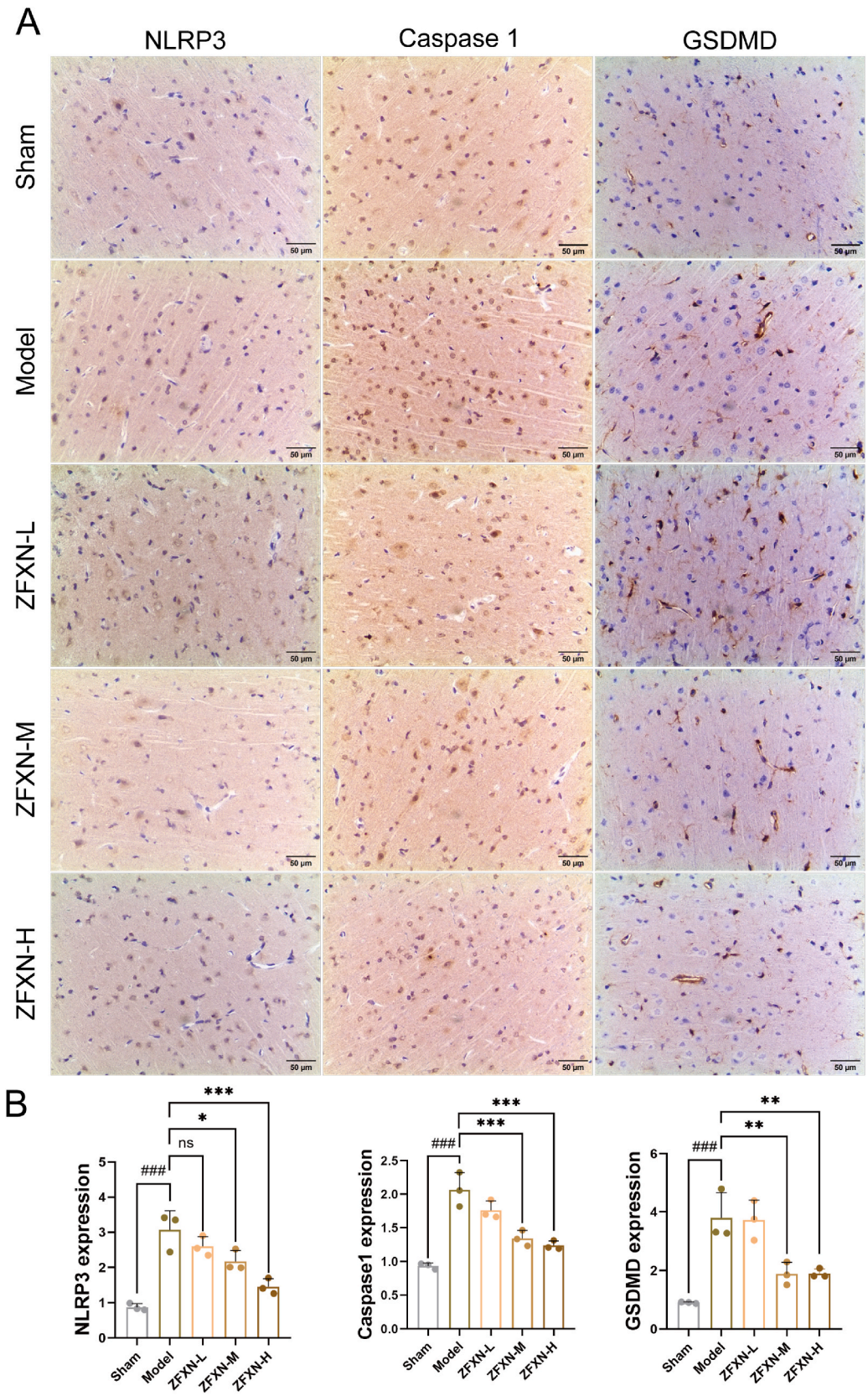

Fig. 4. ZFXN inhibits NLRP3, GSDMD, and Caspase1 activation after ICH. **(A)** Representative immunohistochemical images of NLRP3, GSDMD, and Caspase1 after ICH. scale bar: 50 μm, image at 200 × . (**B**) The quantitative analysis of the expression of NLRP3, GSDMD, Caspase1, Mean ± SD (n = 3). ##$p < 0.01$ and ###$p < 0.001$: Sham vs. Model group, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$: ZFXN groups vs. model group.

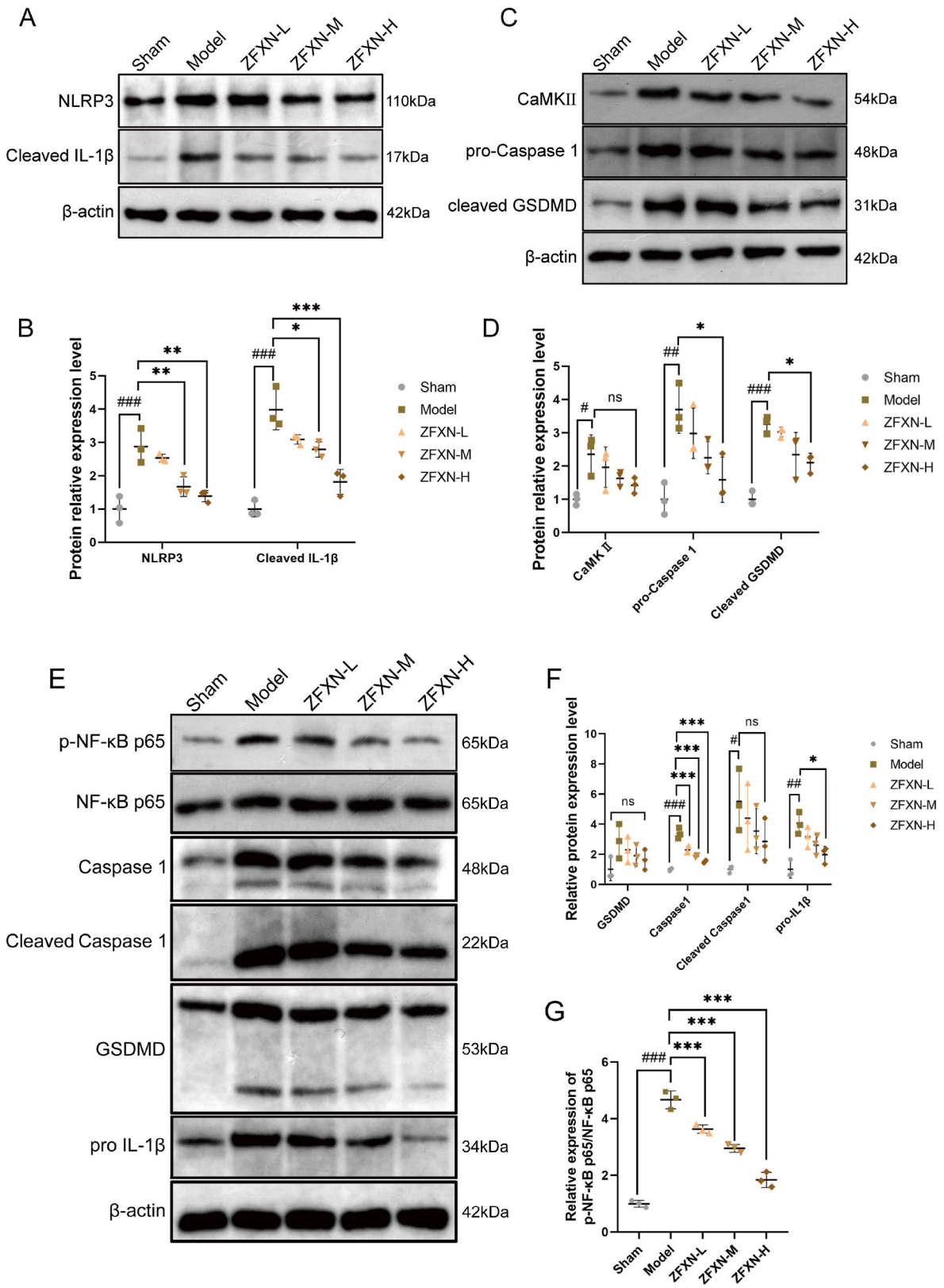

Fig. 5. ZFXN blocks the CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis. **(A, C, E)** The representative Western blot bands of related proteins in the perihematomal regions. (**B, D, F, G**) The quantitative analysis of protein expression of these proteins. Mean ± SD (n = 3). #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$: Sham vs. Model group, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$: ZFXN groups vs. model group.

significantly to model group. A high dosage of ZFXN significantly decreased the expression of the above proteins ($p < 0.05$, $p < 0.01$ or $p < 0.001$) except for CaMKII, cleaved Caspase1 and GSDMD ($p > 0.05$) in comparison to the model group. However, their expression displayed a trend toward a decrease in high-dose ZFXN.

3.5. ZFXN reduces the expression of proinflammatory factors in ICH rats

Pyroptosis is with proinflammatory factors released from ruptured cell membranes and induced inflammation. Here, we examined the secretion of proinflammatory cytokines, IL-1β, IL-18, IL-6, and TNF-α in serum to study the ZFXN effects on the inflammatory response after ICH (Fig. 6). The inflammatory response (IL-18, IL-1β, and IL-6) was improved after ZFXN-H treatment ($p < 0.01$). The level of TNF-α decreased after treatment with ZFXN, but there was no statistical significance ($p > 0.05$). The above results showed that ZFXN can significantly improve the inflammatory response by regulating proinflammatory factors.

4. Discussion

Neuroinflammation mediated by NLRP3 inflammasome exerts a central role in the process of intracerebral hemorrhage.[28,29] In this study, we proved that ZFXN could reduce the NLRP3 inflammasome to alleviate injury in the collagenase VII-induced rat model of ICH. It was first confirmed that ZFXN has the potential to be an anti-inflammation drug might by regulating the NLRP3 inflammasome activation after ICH (Fig. 7).

The activation of NLRP3 inflammasome relies on the NF-κB stimulation. NF-κB is a transcription factor, that can adjust the neurons' important physiological activities, and participate in inflammation, immunity, cell growth and proliferation, and many other physiological processes.[30–32] NF-κB p65 is a member of NF-κB family, a key step in the function of NF-κB p65 is its phosphorylation. Under normal circumstances, NF-κB is located in the cytoplasm, and NF-κB p65 will be phosphorylated after stimulation, such as ICH. In the present study, we examined the expression of p-NF-κB p65 and NF-κB p65 by Western blot to indicate whether ZFXN can block its activation. The result showed that ZFXN can reduce p-NF-κB p65 expression after ICH (Fig. 5A and B). Ca2+/calmodulin–dependent protein kinase II (CaMKII) is a widespread and regulating learning and memory serine/threonine protein kinase in the brain, which is related to synaptic plasticity.[33–35] CaMKII is able to regulate NF-κB p65 activity.[36] In the absence of stimulation, CaMKII remains in an inactive state, it can be activated by an increase in intracellular Ca^{2+} concentration. After intracerebral hemorrhage, the hematoma compresses the brain tissue, causing secondary ischemia and hypoxia. In hypoxia-ischemia conditions, the plasma membrane of neurons depolarizes because oxygen, glucose, and ATP are drastically decreased, K^+ is released into the extracellular space, while Na^+ and Ca^{2+} enter the cell rapidly.[37] Because Ca^{2+} rapidly enters the cell after ICH, CaM can bind to Ca^{2+} and activate CaM-dependent kinase II (CaMKII). Based on the above, we hypothesized that CaMKII activation induces NF-κB p65 activation, which leads to NLRP3 activation. To further test whether ZFXN can block the activation of CaMKII, we tested its expression. We found that the expression of CaMKII increased after ICH, although there was no statistical difference between ZFXN treatment groups and the model group, the expression decreased and there was a dose-dependent relationship.

The inflammatory reaction as part of the innate immune system is believed to be a crucial mechanism in secondary injury following ICH and can be mediated by inflammasomes.[38] Meanwhile, the NLRP3 inflammasome has been extensively and systematically studied in central nervous system immunity, especially its participation in intracerebral hemorrhage-induced inflammatory responses.[39,40] An activator of transcription and an assembly factor are required for activation of the NLRP3 inflammasome, the activated NF-κB p65 specifically binds to NLRP3 to promote NLRP3 transcription and translation, and NLRP3 is then activated and assembled into NLRP3 inflammasome, subsequently activating caspase1. Afterward, pro-IL-1β and pro-IL-18 are cleaved enzymatically, leading to maturation and release.[41] Our results showed that ZFXN can inhibit the activation of NLRP3 inflammasome and induce the reduction of Caspase1 and cleaved Caspase1 level by immunohistochemical staining and Western blot, there is no statistical difference of cleaved Caspase1 level among these groups. Our study also showed that the level of IL-1β, IL-18, IL-6, and TNF-α is lower in ZFXN treatment groups compared to the model group. The phenomenon suggests that ZFXN improves inflammation by inhibiting the NLRP3 inflammasome activation.

There are certain inflammasomes that trigger pyroptosis, which is a form of programmed cell death and can identify the endogenous danger signals and release of inflammatory factors, making the body produce strong inflammatory reaction.[42] Researchers confirmed that GSDM family proteins initiate pyroptosis in response to various stimuli.[43] A buildup of NLRP3 inflammasomes is an important pyroptosis activator, and it is believed that NLRP3 inflammasomes cleave caspase-1 and then trigger the cleavage of GSDM family proteins.[44] NLRP3 inflammasome activation leads to GSDM cleaving into GSDMD N-terminus, then large pores formed in the membrane, releasing mature IL-1β and IL-18 into tissues. According to our in-vivo analysis, the expression of cleaved-GSDMD and GSDMD have increased in model rats because of NLRP3 inflammasomes activation, and the expression of cleaved-GSDMD was reversed after ZFXN treatment. Therefore, ZFXN treatment prevents the process of pyroptosis and inflammation after ICH.

Previous studies have demonstrated that ZFXN has the properties of anti-inflammation, oxidative stress, and anti-excitatory toxicity.[45] In

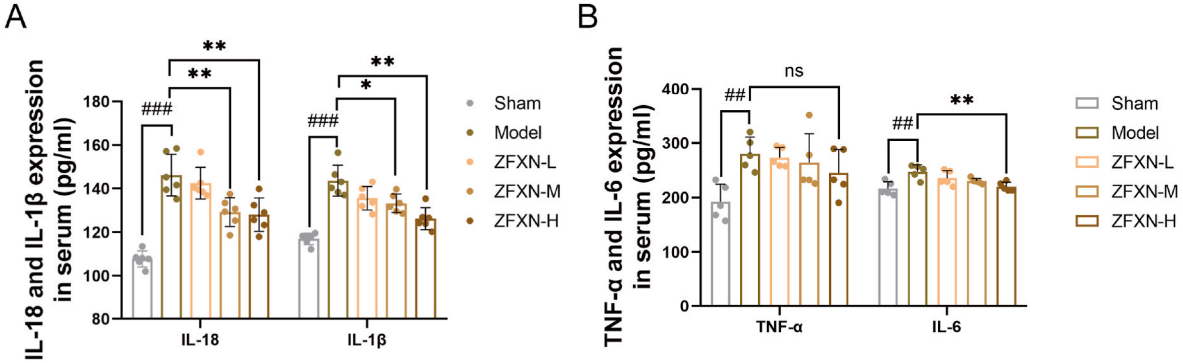

Fig. 6. ZFXN reduces the expression of IL-1β, IL-18, TNF-α, and IL-6. (**A**) The quantitative analysis of the level of IL-1β and IL-18, Mean ± SD (n = 6). (**B**) The quantitative analysis of the level of TNF-α and IL-6, Mean ± SD (n = 5). ##$p < 0.01$, ###$p < 0.001$: Sham vs. Model group, *$p < 0.05$, **$p < 0.01$: ZFXN groups vs. model group.

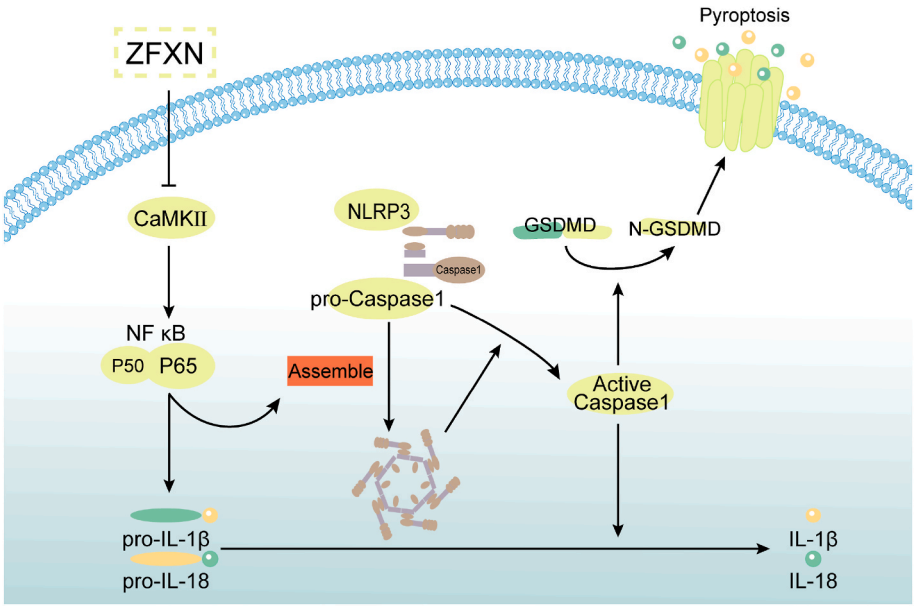

Fig. 7. Schematic summary of the signal pathways involved in this study. The CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis was activated after ICH then leaded to inflammation and pyroptosis in the brain.

this experiment, we used intracranial injection of collagenase VII in rats to construct a model of ICH. The experimental results showed that treating ICH rats with ZFXN for 5 days, it could improve the nerve function and reduce the hematoma volume. Clinical studies had previously found similar results.[16] Zhongfeng Xingnao prescription were developed based on traditional Chinese medicine (TCM) theory and experts' clinical experience. According to the theory of TCM, ZFXN exerts its healing effects on ICH with the effect of tonifying Qi and activating blood. Many active ingredients in ZFXN, especially anthraquinones from rhubarb (Da Huang), have significant anti-inflammatory, antioxidant, and neuroprotective effects.[46–48] Previous studies have suggested that rhubarb anthraquinones by regulating apoptosis in microglial[49] and increasing the expression of zonula occludens-1(ZO-1) expression to protect BBB in ICH.[50] The major components of *red ginseng* (Hong Shen) are ginsenosides. Ginsenosides have been proven to have anti-apoptotic biological functions by down regulate the level of p53 and BAX in subarachnoid hemorrhage.[51] However, no red ginseng study has been performed in the ICH model. *Panax notoginseng* (San Qi) can relieve blood stasis and gather new blood and is used for all kinds of bleeding and bruises in the human body.[52] Saponins extract from *panax notoginseng* has anti-inflammation and anti-apoptosis properties to improve the neurological function of patients with ICH and improve their quality of life.[53] *Ligusticum chuanxiong* Hort (Chuan Xiong) is widely used to treat cerebral vascular diseases in clinic, and its component ligustilide can reduce inflammation by regulating the TLR4/NF-κB signaling pathway in ICH mice. These data suggested that the four herbal drugs contained in the ZFXN have therapeutic effects in ICH. Compatibility of medicines is the soul of traditional Chinese medicine prescriptions, ZFXN treating ICH may be through multicomponent, multiple targets, multiple pathways synergy.

There are a few limitations of this study. This experiment is a preliminary verification that ZFXN can regulate CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis to inhibit the pyroptosis and reduce the inflammatory reaction after ICH. This finding is preliminary and requires further replication and verification. Moreover, ZFXN is composed of four traditional Chinese medicines, and different herbs can also play a synergistic effect to form a multi-target protective effect on the body after ICH. Therefore, further studies are required to elucidate the relationship between the active ingredient contents in ZFXN and their efficacy for ICH. In addition, previous studies showed that autophagy is strongly correlated with NLRP3 inflammasome activation,[54] it can prevent excessive inflammasome activation by clearing the endogenous NLRP3 inflammasome activators,[55] targeting NLRP3 inflammasome components.[56,57] In the next following studies, we will explore the effect of ZFXN on the relationship between autophagy and NLRP3 after ICH.

5. Conclusion

In this study, we found that ZFXN administration is beneficial to collagenase VII induced ICH through protecting neural function, the reduction of the cerebral hematoma-injury ratio, the improvement of the pathological damage of brain tissue, and the regulation of the CaMKII/NF-κB p65/NLRP3/GSDMD signaling axis. Consequently, ZFXN administration might be an effective treatment of intracerebral hemorrhage.

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Availability of data and materials

Data will be made available on request.

Ethics approval and consent to participate

All animal experiments were carried out according to the Guide for the Care and Use of Animal Ethics Committee of Zhejiang Chinese Medical University (Ethics approval reference number was 20220214-25) and international guidelines on the ethical use of animals (Mason and Matthews 2012). Rats were euthanized by isoflurane.

Declaration of competing interest

All authors declare no conflict of interest.

Abbreviation

ZFXN	Zhongfeng Xingnao Prescription
ICH	intracerebral hemorrhage
BBB	blood-brain barrier
CaMKII	Ca^{2+}/calmodulin–dependent protein kinase II
CNS	central nervous system
NLRP3	NOD-like receptor family containing pyrin domain-containing protein 3
GSDMD	Gasdermin D
N-GSDMD	N-terminus of GSDMD
TCM	traditional Chinese medicine
TEM	transmission electronic microscope

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.03.005.

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