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Original article

Zhongfeng Xingnao Liquid ameliorates post-stroke cognitive impairment through sirtuin1 (SIRT1)/nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway

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ABSTRACT

The activation of the sirtuin1 (SIRT1)/nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway has been shown to mitigate oxidative stress-induced apoptosis and mitochondrial damage by reducing reactive oxygen species (ROS) levels. Clinical trials have demonstrated that Zhongfeng Xingnao Liquid (ZFXN) ameliorates post-stroke cognitive impairment (PSCI). However, the underlying mechanism, particularly whether it involves protecting mitochondria and inhibiting apoptosis through the SIRT1/Nrf2/HO-1 pathway, remains unclear. This study employed an oxygen-glucose deprivation (OGD) cell model using SH-SY5Y cells and induced PSCI in rats through modified bilateral carotid artery ligation (2VO). The effects of ZFXN on learning and memory, neuroprotective activity, mitochondrial function, oxidative stress, and the SIRT1/Nrf2/HO-1 pathway were evaluated both *in vivo* and *in vitro*. Results indicated that ZFXN significantly increased the B-cell lymphoma 2 (Bcl2)/Bcl2-associated X (Bax) ratio, reduced terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL)$^+$ cells, and markedly improved cognition, synaptic plasticity, and neuronal function in the hippocampus and cortex. Furthermore, ZFXN exhibited potent antioxidant activity, evidenced by decreased ROS and malondialdehyde (MDA) content and increased superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels. ZFXN also demonstrated considerable enhancement of mitochondrial membrane potential (MMP), Tom20 fluorescence intensity, adenosine triphosphate (ATP) and energy charge (EC) levels, and mitochondrial complex I and III activity, thereby inhibiting mitochondrial damage. Additionally, ZFXN significantly increased SIRT1 activity and elevated SIRT1, nuclear Nrf2, and HO-1 levels. Notably, these effects were substantially counteracted when SIRT1 was suppressed by the inhibitor EX-527 *in vitro*. In conclusion, ZFXN alleviates PSCI by activating the SIRT1/Nrf2/HO-1 pathway and preventing mitochondrial damage.

1. Introduction

Stroke represents a significant global health burden, ranking among the leading causes of death and disability worldwide. It incurs an estimated annual cost of US $721 billion and results in approximately 15 million fatalities [1-3]. In China alone, the number of stroke victims reached 45.9 million in 2019 [4]. A growing body of research indicates that post-stroke cognitive impairment (PSCI) is frequently associated with stroke, particularly ischemic stroke [3, 5, 6]. Notably, varying degrees of PSCI affect approximately two-thirds of ischemic stroke patients [7].

Hypoxia and chronic cerebral hypoperfusion (CCH) are recognized as two primary pathogenic features of ischemic stroke. These characteristics are generally considered hallmarks of ischemic stroke and are likely to cause significant damage to mitochondria [8-10]. Mitochondrial injury leads to excessive reactive oxygen species (ROS) production and oxidative stress. These factors contribute to neuronal death and cognitive impairment, a relationship widely acknowledged as reciprocal causation [8, 11, 12]. Given its critical role in preserving nerve cell survival and mitigating neurological impairments following ischemic stroke, mitochondrial homeostasis is often considered a key therapeutic target [13]. Sirtuin1 (SIRT1), an enzyme dependent on nicotine adenine dinucleotide (NAD^+), has been shown to offer protection against ischemic stroke [14-16]. SIRT1 facilitates the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) from the cytoplasm to the nucleus, activating it and subsequently mediating heme oxygenase 1 (HO-1) transcription [17-21]. Current evidence suggests that activation of the SIRT1/Nrf2/HO-1 pathway en-

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hances the activity of antioxidant enzymes such as superoxide dismutase (SOD), thereby reducing excessive oxidative stress and ROS production [22, 23]. Moreover, this pathway plays a crucial role in maintaining energy metabolism and mitochondrial homeostasis by regulating processes including mitochondrial swelling, bioenergetics, and glucose metabolism [24-27]. One mechanism by which the SIRT1/Nrf2/HO-1 pathway reduces mitochondrial damage is the inhibition of ROS production from the electron transport chain [28]. These findings indicate a strong correlation between the SIRT1/Nrf2/HO-1 pathway and the prevention of oxidative stress, as well as the preservation of mitochondrial function.

CCH is the primary cause of oxidative stress, cognitive impairments, and vascular dementia (VaD) following ischemic stroke [29]. Pharmacological interventions that increase cerebral perfusion, such as nimodipine, aspirin, and Dl-3-*n*-butylphthalide, have been shown to reduce PSCI [30-32]. Recently, traditional treatments and medications, including Dengzhan Shengmai Capsules, Buyang Huanwu Tang, and Dengzhan Xixin Injection, have garnered significant attention [33-35]. Zhongfeng Xingnao Liquid (ZFXN), an in-hospital compound formula of the Hospital of Chengdu University of Traditional Chinese Medicine (CDUTCM), was developed by renowned Chinese traditional medicine expert Professor Shaohong Chen and has been utilized clinically for ischemic stroke treatment for over three decades. ZFXN's formula is derived from Renshen-Dahuang Decoction in *Bian-Zheng-Lu*, a remedy for post-stroke delirium and forgetfulness. It comprises *Panax ginseng* in Araliaceae (Renshen in Chinese), *Ligusticum chuanxiong* in Apiaceae (Chuanxiong in Chinese), *Panax notoginseng* in Araliaceae (Sanqi in Chinese), and *Rheum officinale* in Polygonaceae (Dahuang in Chinese) in a ratio of 6∶3∶2∶1 (Table S1). Clinical trials have demonstrated that ZFXN can significantly ameliorate PSCI, acute cerebral infarction, and ischemic stroke [36-38]. The primary mechanism of ZFXN in preventing ischemic stroke is through inhibition of oxidative stress [39]. However, it remains unclear whether ZFXN can attenuate PSCI by enhancing the SIRT1/Nrf2/HO-1 pathway, which protects mitochondria and synapses. Consequently, this study aims to elucidate the underlying mechanism of ZFXN in treating PSCI, with a particular focus on the SIRT1/Nrf2/HO-1 pathway.

2. Materials and Methods

2.1. Preparation of ZFXN and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis

ZFXN (No. 20210817) was supplied by the Hospital of CDUTCM (Chengdu, China). The clinical medication information is as follows: specification: 100 mL/bottle; concentration: 0.96 g raw medicinal materials/mL; usage and dosage: 50–100 mL·d^{-1}, four times daily, administered orally. The patient dose (50 mL·d^{-1}) was converted to an equivalent rat dose using a conversion ratio of 7 times based on rat/human body weight. Consequently, the dosages for rat treatment were established as 2.8 g·kg^{-1}·d^{-1} (designated ZFXN-L), 5.6 g·kg^{-1}·d^{-1} (ZFXN-M), and 11.2 g·kg^{-1}·d^{-1} (ZFXN-H), respectively. An appropriate amount of ZFXN was filtered using a 0.22 μm microporous membrane, and HPLC-MS/MS (Thermo Fisher, Waltham, MA, USA) was utilized to measure the content of active ingredients in ZFXN as previously described [40].

2.2. Reagents

Nimodipine (#BJ59340) was obtained from Bayer (Leverkusen, Germany), and pentobarbital sodium salt (#69020100) was supplied by Merck (Darmstadt, Germany). Antibodies were purchased from various reagent companies as follows: SIRT1 (#9475T) from Cell Signaling Technology (Beverly, MA, USA), Nrf2 (#ER1706-41) from Huabio (Hangzhou, China), HO-1 (#10701-1-AP), B-cell lymphoma 2 (Bcl2) (#26593-1-AP), and Bcl2-associated X protein (Bax) (#50599-2-IG) from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China), translocase of outer mitochondrial membrane 20 homolog (Tom20) (#L2920) from Santa Cruz Biotechnology (Shanghai, China), neuronal nuclei (NeuN) (#ab177487), postsynaptic density protein 95 (PSD95) (#ab18258), and synaptophysin (SYN) (#ab32127) from Abcam Plc (Shanghai, China), beta-actin (#GB15001), Histone H3 (#GB11102), and secondary antibodies anti-Cy3-rabbit immunoglobulin G (IgG) (#GB21303), anti-488-mouse IgG (#GB25303), anti-rabbit IgG (#GB23303), anti-mouse IgG (#GB23301) from Servicebio Technology Co., Ltd. (Wuhan, China). SIRT1 activity assay kits (#ab156065) were acquired from Abcam Plc (Shanghai, China). Nuclear protein extraction kits (#W038-1-1) and oxidative stress-related biochemical kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), including malondialdehyde (MDA) (#A003-1-2), SOD (#A001-3-2), glutathione (GSH) (#A006-2-1), and catalase (CAT) (#A007-1-1). Mitochondria isolation kits (#C3606), JC-1 kits (#C2006), ROS assay kits (#S0033S), and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) kits (#C1090) were obtained from Beyotime Biotechnology (Shanghai, China). Mitochondrial complex I (#BC0515) and complex III (#BC3245) biochemical assay kits were supplied by Solarbio Science and Technology Co., Ltd. (Beijing, China). EX-527 (#HY-15452) was purchased from MedChemExpress (New Jersey, USA).

2.3. Animals

Sprague-Dawley rats (220 ± 20 g, SPF, Male) were obtained from Byrness Weil Biotech Ltd. (Chongqing, China). The animals were housed in a controlled environment (temperature: 23 ± 1 °C, humidity: 65% ± 5%) at CDUTCM with *ad libitum* access to food and water. The experimental protocols (Fig. 1A) were approved by the Research Ethics Committee of the Institute of Material Medica Integration and Transformation for Brain Disorders, CDUTCM (No. IBD2021008). All animal studies were conducted in strict accordance with the guidelines of the National Institutes of Health (NIH, USA).

A modified bilateral carotid artery ligation (2VO) procedure was employed to generate PSCI rats, as previously described [41]. The forty surviving 2VO rats were randomly allocated into five groups: ZFXN-L group (n = 8, 2.8 g·kg^{-1}, i.g.), ZFXN-M group (n = 8, 5.6 g·kg^{-1}, i.g.), ZFXN-H group (n = 8, 11.2 g·kg^{-1}, i.g.), model group (n = 8, normal saline, i.g.), and nimodipine group (n = 8, NIM, 10 mg·kg^{-1}, i.g.) [42-45]. The sham group comprised eight sham-operated rats (n = 8, normal saline, i.g., carotid artery not occluded). Each rat received the corresponding medication or saline orally at the same time daily for 38 d (Fig. 1A).

2.4. Open field experiment

The open field test (TechMan Soft, China) was conducted to assess the autonomous behaviors of rats in a novel environment over a 5-min period, following previously established methods [46, 47]. The instrument recorded both the movement speed and total distance traveled by the rats.

2.5. Y-maze test

The Y-maze (TechMan Soft, China) was utilized to assess the animals' discriminative learning, working memory, and reference memory, following the method described [48]. The Y-maze consists of three identical dark arms positioned at 120-degree angles from each other. Each rat was placed individually in the

central area of the Y-maze and allowed to explore freely for 5 min. The sequence of arm entries was recorded, and the spontaneous correct alternation rate was calculated using the following formula: spontaneous correct alternation rate = [number of correct alternations/(total number of arm entries − 2)] × 100%.

2.6. Morris water maze (MWM) test

The MWM (Noldus, Netherlands) was utilized to assess the cognitive abilities of rats, following the methodology described in the literature [49]. Briefly, all animals underwent training sessions lasting 1 min·d^{-1} for a period of 5 d. During this training phase, the escape latency of each rat was recorded using a video camera. On the 6th day, the target platform was removed. The rats were then placed into the pool using the same procedure as during training. A video camera integrated into the instrument was used to record two parameters within a 60-sec period: the time spent in the platform/platform area and the number of times the rat crossed the platform/platform area.

2.7. Hematoxylin and eosin (HE) staining and TUNEL analysis

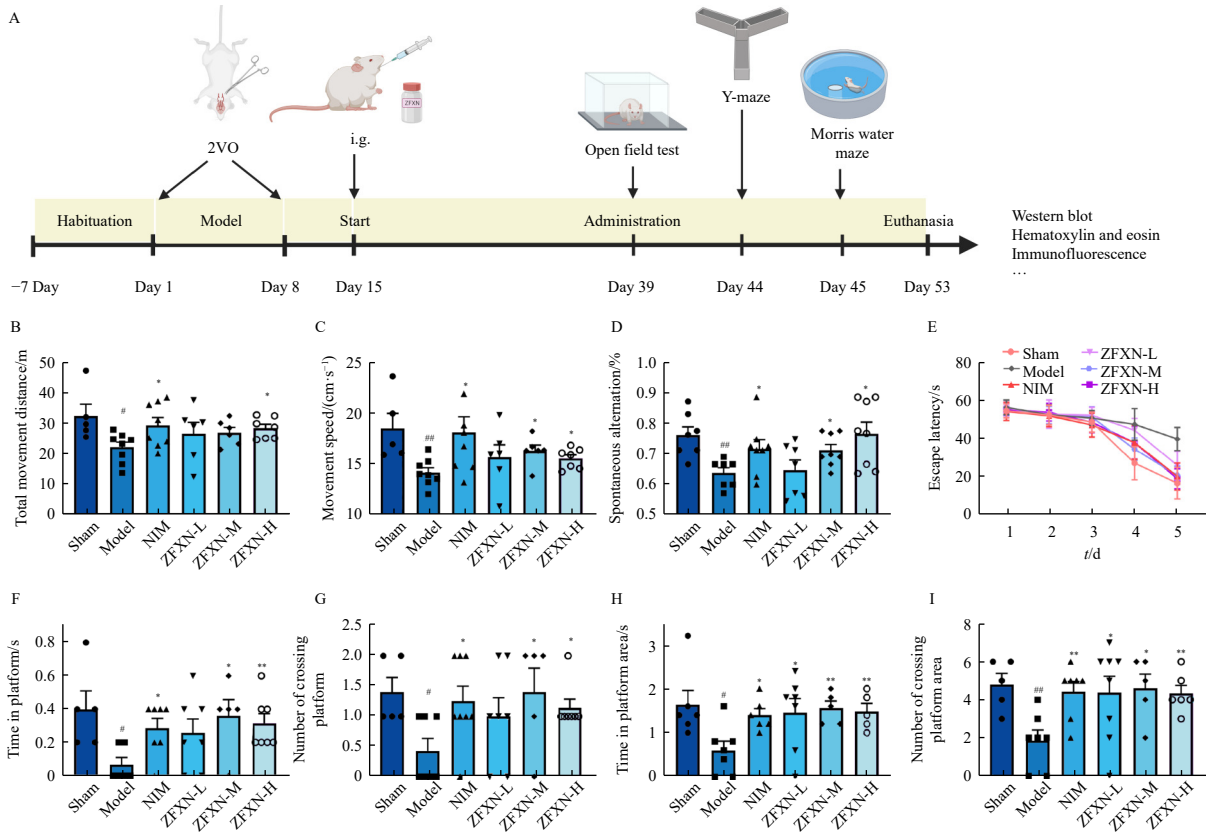
All rats were anesthetized with pentobarbital sodium salt (40 mg·kg^{-1}, i.p.) and euthanized by decapitation. The left hemispheres were rapidly preserved in liquid nitrogen for subsequent analysis. Half of the right hemispheres containing the hippocampus underwent sequential dehydration, paraffin embedding, sectioning (4 μm), and HE staining following standard protocols. The pathomorphological features of the hippocampus and cortex were examined using optical microscopy. Concurrently, after preparing brain tissue slices, neuronal apoptosis was assessed using the TUNEL assay kit as per the manufacturer's instructions. All slices were stained with DAPI (C0065, Solarbio, China) for 10 min and subsequently observed using a fluorescent microscope (SPOTOP, China). The number of TUNEL^{+} cells was statistically analyzed.

2.8. Cell culture

Oxygen-glucose deprivation-induced SH-SY5Y (OGD-SH-SY5Y) cells were established as previously described [45]. SH-SY5Y cells (Shanghai Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in dulbecco's modified eagle medium (DMEM, 8122308, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, SA21101, Cellmax, China) and 1% penicillin/streptomycin (15140-122, Gibco, USA). The cells were incubated under conventional culture conditions to reach 80%−90% confluence. Subsequently, these cells were cultured in an oxygen-glucose deprivation (OGD) medium, which lacks fetal bovine serum (FBS) and glucose, replacing the standard medium. They were then placed in a modular incubator chamber (Billups-Rothenberg, Inc., USA) with 95% nitrogen and 5% CO_2 at 37 °C for 12 h. ZFXN-treated cells were cultured in OGD medium with ZFXN at a concentration of 20 μg·mL^{-1}, which was determined to be a safe and effective dose in our research. To prepare the ZFXN solution, 4 mg (approximately 16 μL of solution) of ZFXN Freeze-dried Powder was precisely weighed and dissolved in 2.5 mL of glucose-free dulbecco's modified eagle medium (DMEM) (1.6 mg·mL^{-1}). ZFXN was filtered *via* a 0.22 μm microporous membrane and diluted to 160 μg·mL^{-1} using glucose-free DMEM, representing the maximum dose. The glucose-free DMEM was then serially diluted to 80, 40, 20, 10, 5, 2.5, 1.25, and 0.625 μg·mL^{-1} to conduct tests on non-toxic concentration and medication efficacy. Ultimately, a dose concentration of 20 μg·mL^{-1} was established. At this concentration, the levels of notoginsenoside R1, ginsenoside Re, ferulic acid, ginsenoside Rg1, ginsenoside Ro and rhein were calculated as 172.8, 213.6, 8.00, 549.6, 78.4 and 0.8 ng·mL^{-1} respectively, based on the results in Table S1. For EX-527-treated cells, intervention with EX-527 at a concentration of 1 μmol·L^{-1} was performed for 60 min prior to OGD.

2.9. Immunofluorescence

Immunofluorescence staining was conducted on brain tissue slices following previously reported methods [19, 50]. Briefly, brain



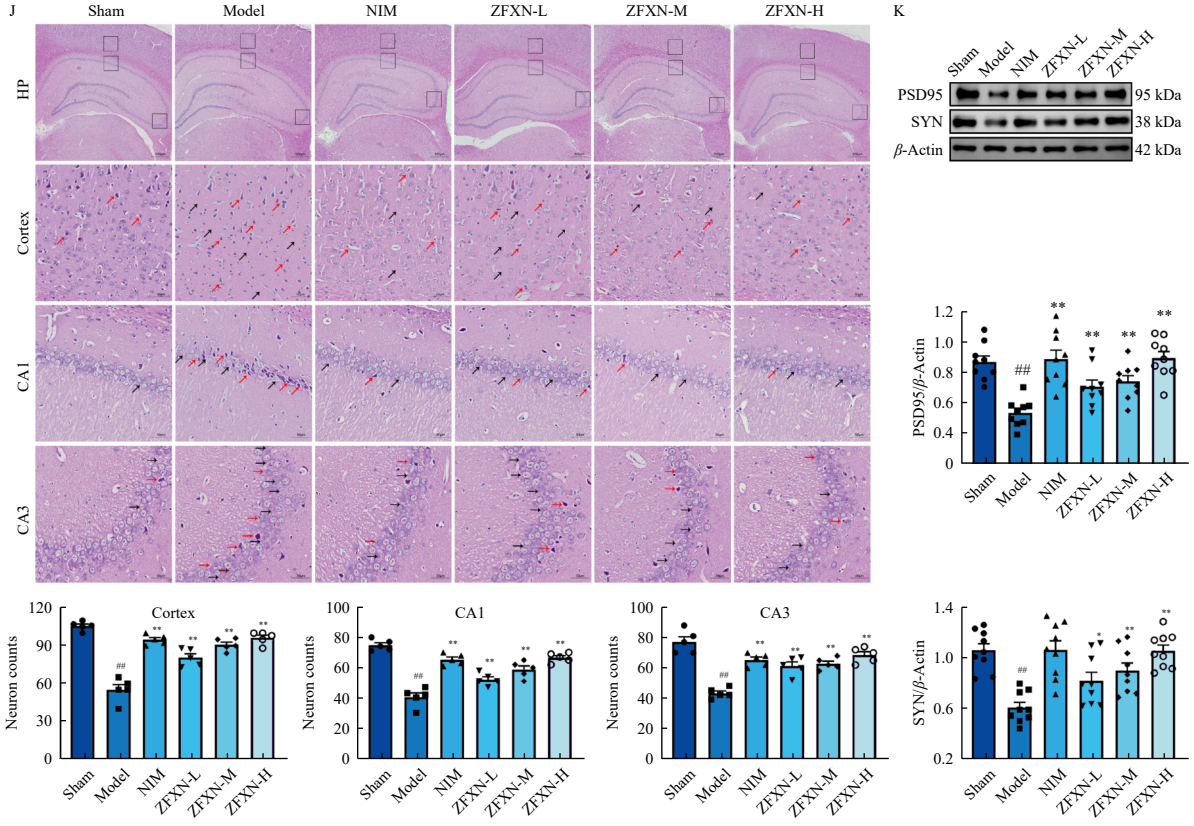


Fig. 1 ZFXN ameliorated cognitive impairment and neuronal damage in 2VO rats. (A) Schematic illustration of the experimental protocols (created in BioRender.com). (B, C) Total movement distance and movement speed of rats in the open field tests (n = 5–8). (D) Spontaneous correct alternation rate of rats in Y-maze tests (%, n = 7–8). (E) Escape latency of rats in the Morris water maze (MWM) tests (n = 5–8). (F, G) Time in platform and number of crossing platform of rats in the MWM tests (n = 5–8). (H, I) Time in platform area and number of platform area crossings by rats in the MWM tests (n = 5–8). (J) Representative image of HE staining and quantitative analysis of neuron counts in the hippocampus (CA1 and CA3) and cortex (n = 5, 20 ×, magnification: 200 ×): enlarged intercellular space (black arrow), atrophy and necrosis of nerve cells (red arrow). (K) Representative western blotting bands and quantitative analysis of PSD95 and SYN (n = 3). Data was presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Model group.

tissue slices were prepared using the same protocol as for HE staining. The slices were incubated with primary antibodies overnight at 4 °C, including Tom 20 (1∶100), NeuN (1∶300), and Nrf2 (1∶100). Subsequently, corresponding secondary antibodies were applied to the slices for 60 min at room temperature. All slices were then stained with DAPI for 10 min. Immunofluorescence images were captured using a fluorescent microscope (SPOTOP, China).

2.10. Oxidative stress analysis

The single-cell suspension of brain tissue or SH-SY5Y cells was suspended in diluted DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate, a cell-permeable probe used to detect intracellular ROS). After the cells were collected, Flow cytometry (BD Biosciences, USA) was employed to measure the level of ROS based on the manufacturer's protocol. Meanwhile, the indicators of oxidative stress (SOD, MDA, GSH, and CAT) in brain tissue and SH-SY5Y cells were detected by commercial kits in strict accordance with the manufacturer's instructions.

2.11. Mitochondrial function analysis

Mitochondrial function was assessed through measurements of mitochondrial membrane potential (MMP), mitochondrial complex activity, and energy generation. Specifically, MMP was evaluated using flow cytometry (BD Biosciences, USA) following the staining of mitochondrial suspensions extracted from fresh brain tissues, in accordance with the JC-1 reagent kit protocol and the method described in document [51]. Additionally, the levels of mitochondrial respiratory chain complexes I and III were quantified using a commercial kit, adhering to the manufacturer's guidelines for both *in vivo* and *in vitro* analyses. Lastly, the concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were determined *in vivo* and *in vitro via* HPLC, as previously detailed [52]. The energy change (EC) was calculated using the formula: EC = ($\text{Content}_{[ATP]}$ + 0.5 × $\text{Content}_{[ADP]}$)/($\text{Content}_{[ATP]}$ + $\text{Content}_{[ADP]}$ + $\text{Content}_{[AMP]}$).

2.12. SIRT1 activity analysis

SIRT1 activity was determined using Abcam's SIRT1 activity assay kit, following the manufacturer's protocols. The relevant reagent solutions were added to the microplate wells as per the described procedure. The reaction was initiated by adding 5 μL of sample to each well, followed by incubation for 30 min at 25 °C. Fluorescence intensity was measured using microplate fluorimeters (SpectraMax iD5, Molecular Devices, USA) with excitation at 350 nm and emission at 450 nm. The SIRT1 activity was calculated as the ratio of fluorescence intensity.

2.13. Western blotting analysis

Western blotting was conducted following a standardized protocol. In brief, after complete lysis of the rat hippocampus and cortex or SH-SY5Y cells, total protein and nuclear protein were extracted separately, and protein concentrations were determined using a commercial bicinchoninic acid (BCA) assay kit (BL521A, Biosharp, China). Target proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride

(PVDF) membranes, and blocked with 5% skimmed milk at room temperature. The primary antibodies used to incubate with the target bands were anti-SIRT1 (1∶1000), anti-Nrf2 (1∶1000), anti-HO-1 (1∶1000), anti-Bcl2 (1∶1000), anti-Bax (1∶1000), anti-Histone H3 (1∶1000), anti-PSD95 (1∶1000), and anti-SYN (1∶100 000). Subsequently, secondary antibodies were used to incubate the target bands for 60 min at 25 °C. After visualizing the target protein bands using a chemiluminescence reagent, imaging software was employed for quantification.

2.14. Data analysis

All data were presented as mean ± standard error of the mean (SEM). The R programming language was utilized to analyze differences between groups. Two-way analysis of variance (ANOVA) was applied to analyze the escape latency of the MWM, while one-way ANOVA was employed for all other data comparisons. Subsequently, Dunnett's post-test was used for multiple comparison analyses. Statistical significance was established at $P < 0.05$.

3. Results

3.1. The representative active ingredients in ZFXN

HPLC-MS/MS analysis was employed to quantify six representative active components in ZFXN (Fig. S1). The analysis revealed the following concentrations: notoginsenoside R1 (2.16 mg·mL^{-1}), ginsenoside Re (2.67 mg·mL^{-1}), ferulic acid (0.10 mg·mL^{-1}), ginsenoside Rg1 (6.87 mg·mL^{-1}), ginsenoside Ro (0.98 mg·mL^{-1}), and rhein (0.01 mg·mL^{-1}) (Table S1).

3.2. ZFXN ameliorated cognitive impairment and neurological damage in 2VO rats

The experimental flow diagram is depicted in Fig. 1A. In open field tests, 2VO rats exhibited significantly reduced total movement distance and speed compared to Sham rats. These effects were notably reversed by NIM and ZFXN-H (Figs. 1B and 1C, $P < 0.05$ or $P < 0.01$). Y-maze tests revealed a marked decrease in the spontaneous correct alternation rate of 2VO rats, which was significantly improved following treatment with NIM, ZFXN-M, and ZFXN-H (Fig. 1D, $P < 0.05$ or $P < 0.01$). In MWM tests, rat escape latency gradually decreased [$F(4, 172) = 28.26$, $P < 0.0001$] as training time increased (Fig. 1E). 2VO rats demonstrated longer escape latencies to reach the target platform compared to Sham rats [$F(4, 34) = 6.038$, $P = 0.0009$]. This increased escape latency was significantly reduced following administration of NIM [$F(4, 34) = 5.168$, $P = 0.0023$], ZFXN-L [$F(4, 33) = 3.775$, $P = 0.0123$], ZFXN-M [$F(4, 33) = 7.008$, $P = 0.0003$], and ZFXN-H [$F(4, 35) = 6.540$, $P = 0.0005$] (Fig. 1E). Furthermore, 2VO rats displayed significantly impaired spatial learning and memory, evidenced by reduced time in the platform/platform area and fewer crossings of the platform/platform area (Figs. 1F–1I, $P < 0.05$ or $P < 0.01$). These reductions were significantly mitigated following NIM and ZFXN treatment (Figs. 1F–1I, $P < 0.05$ or $P < 0.01$). HE staining revealed characteristic pathological changes in the hippocampus and cortex of 2VO rats, including cortical thinning, neuronal reduction and loss, enlarged intercellular spaces (black arrow), and atrophy and necrosis of nerve cells (red arrow). These changes were markedly attenuated following NIM and ZFXN treatment (Fig. 1J, $P < 0.01$). Given the close relationship between synaptic plasticity and learning and memory, PSD95 and SYN expression were assessed to evaluate ZFXN's effect on synaptic plasticity. Results demonstrated significantly down-regulated expression of PSD95 and SYN in 2VO rats, which was significantly reversed by NIM and ZFXN treatment (Fig. 1K, $P < 0.05$ or $P < 0.01$). These findings suggest that ZFXN ameliorates cognitive dysfunction, neuronal damage, and impaired synaptic plasticity in 2VO rats.

3.3. ZFXN suppressed neuronal apoptosis in 2VO rats

Excessive neuronal apoptosis is a critical characteristic of ischemic stroke and PSCI [53]. To assess the level of neuronal apoptosis in 2VO rats, western blotting and TUNEL staining were utilized. The Bcl2/Bax ratio in brain tissue of the Model group significantly decreased compared to the Sham group but markedly increased following NIM and ZFXN treatment (Figs. 2A and 2B, $P < 0.01$). Correspondingly, TUNEL^{+} cells in the hippocampus (CA1, CA3) and cortex regions were notably elevated in the Model group compared to the Sham group. However, this effect was significantly reversed in the NIM and ZFXN treatment groups (Figs. 2C–2F, $P < 0.01$). These results suggest that ZFXN mitigated neuronal apoptosis induced by 2VO in rats.

3.4. ZFXN alleviated oxidative stress injury to brain tissue in 2VO rats

Oxidative stress, characterized by excessive ROS generation, is not only a fundamental cause of neuronal apoptosis but also widely recognized as a crucial factor in the progression and deterioration of ischemic stroke and PSCI [54-56]. Our findings revealed that the ROS level in the brain of Model rats was significantly elevated compared to Sham rats, which was notably reduced following treatment with NIM and ZFXN-H (Figs. 3A and 3B, $P < 0.05$ or $P < 0.01$). Furthermore, the imbalance in the oxidative stress/antioxidant defense system in 2VO rat brains was substantially mitigated by NIM and ZFXN, as evidenced by a decrease in MDA levels in brain tissue, accompanied by an increase in SOD, GSH, and CAT levels compared to Model rats (Figs. 3C–3F, $P < 0.05$ or $P < 0.01$).

3.5. ZFXN ameliorated mitochondrial dysfunction in 2VO rats

MMP and the level of Tom20 in mitochondria are commonly considered markers of mitochondrial damage [57]. Compared to the Sham group, the MMP of brain tissue in the Model group was significantly reduced but markedly increased by NIM and ZFXN treatments (Figs. 4A and 4B, $P < 0.05$ or $P < 0.01$). Additionally, ATP content and EC in the brain tissue of Model rats were substantially lower than those of Sham rats; however, ZFXN-H treatment completely restored these alterations (Figs. 4C and 4D, $P < 0.05$ or $P < 0.01$). Mitochondrial complexes I and III, indicators of mitochondrial function, are known to have neuroprotective effects [58]. Our data revealed that the activity of mitochondrial complex I and III in brain tissue of the Model group was significantly lower than the Sham group; however, their activity increased significantly with NIM and ZFXN treatments (Figs. 4E and 4F, $P < 0.05$ or $P < 0.01$). Likewise, the fluorescence intensity of Tom20 in hippocampal (CA1, CA3) and cortex regions was markedly decreased in Model rats but significantly increased after NIM and ZFXN treatments (Figs. 4G–4L, $P < 0.01$).

3.6. ZFXN enhanced SIRT1/Nrf2/HO-1 pathway in 2VO rats

SIRT1 has been established as a protective factor for ischemic stroke [16]. The SIRT1/Nrf2/HO-1 pathway is strongly associated with anti-oxidation and neuroprotection, as increased SIRT1 expression and activity can decrease ROS formation, while its inhibition can induce oxidative stress by reducing Nrf2 and antioxidant HO-1 levels [25, 26, 59]. In this study, the expressions of proteins associated with the SIRT1/Nrf2/HO-1 pathway, including SIRT1, total Nrf2, nuclear Nrf2, and HO-1, were significantly down-regulated in 2VO rats compared to Sham rats (Figs. 5A–5D,

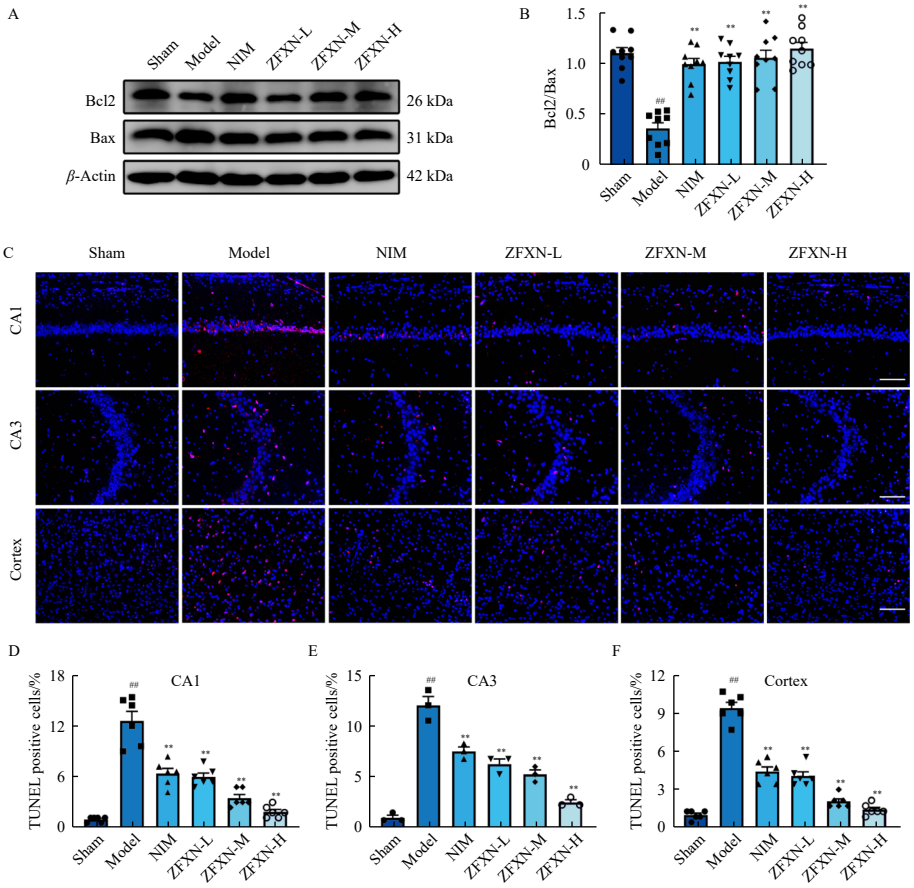


Fig. 2 ZFXN inhibited neuronal apoptosis in 2VO rats. (A) Representative Western blotting images of Bcl2, Bax, and *β*-actin proteins. (B) The ratio of Bcl2/Bax (n = 3). (C) Representative immunofluorescence images of TUNEL staining (magnification: 200 ×, scale bar = 100 μm, TUNEL: red, DAPI: blue). (D–F) Quantification of TUNEL$^+$ cells in the hippocampal CA1, CA3, and cortex regions (n = 3). Data are presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Model group.

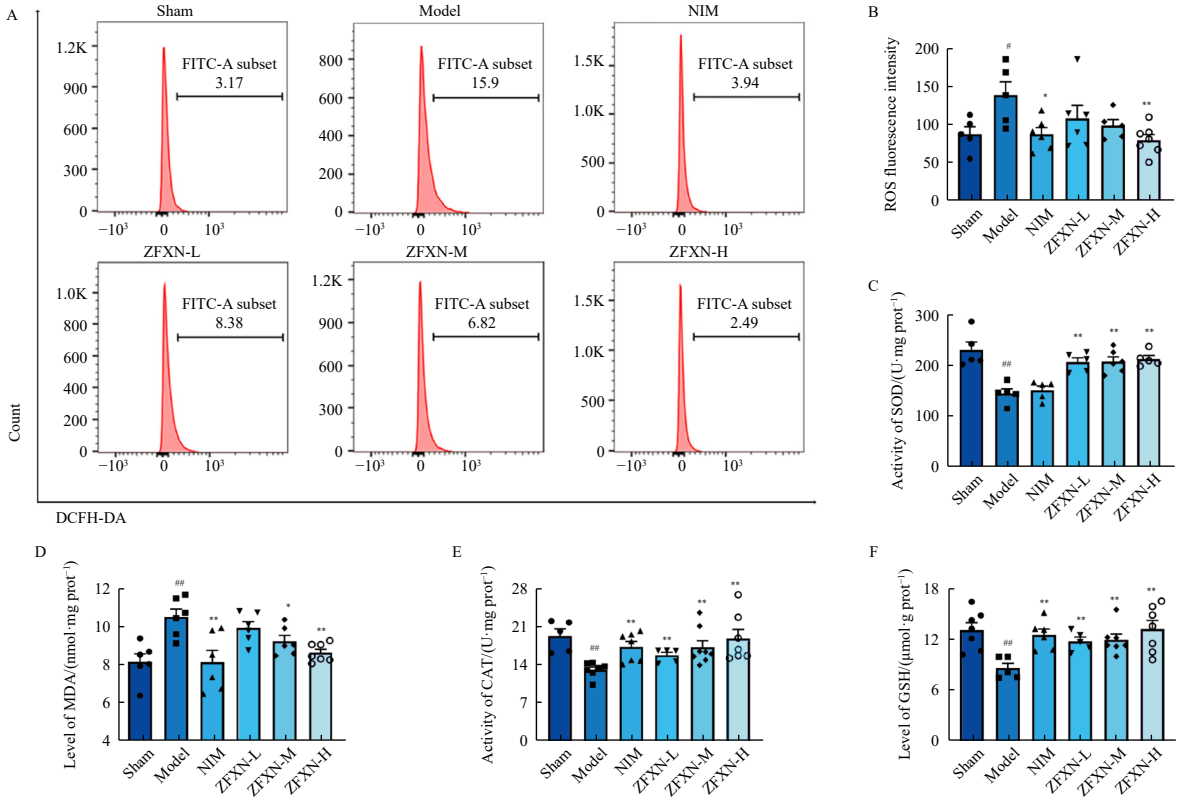


Fig. 3 ZFXN mitigated oxidative stress in brain tissue of 2VO rats. (A, B) Representative fluorescence intensity images and quantification of ROS measured by flow cytometry (n = 5–7). (C) SOD activity (n = 5–7). (D) MDA levels (n = 5–7). (E) CAT activity (n = 5–7). (F) GSH levels (n = 5–7). Data presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Model group.

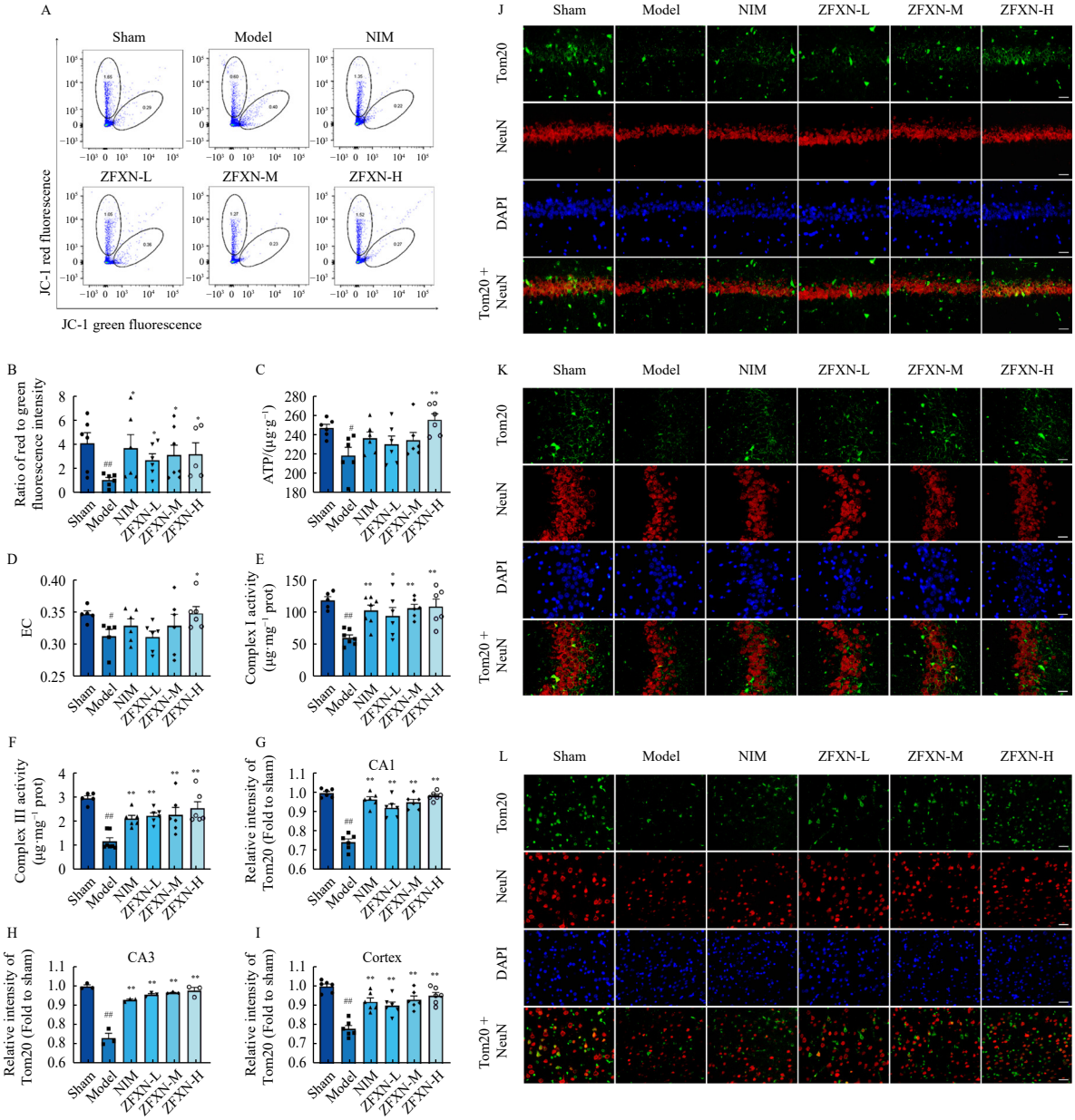


Fig. 4 ZFXN ameliorated mitochondrial dysfunction in 2VO rats. (A) Representative flow cytometry plots of MMP. (B) Quantification of MMP, expressed as the ratio of red/green fluorescence intensity (n = 5–7). (C, D) ATP content and EC of brain tissue (n = 5–6). (E, F) Activity of mitochondrial complex I and III (n = 5–7). Representative immunofluorescence images and quantification of Tom20 in the CA1 regions (G, J), CA3 regions (H, K) and cortex regions (I, L). (magnification: 400 × , scale bar = 50 μm, Tom20: green, NeuN: red, DAPI: blue, n = 5). Data are presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Model group.

$P < 0.01$). However, their protein expressions were significantly up-regulated in the NIM and ZFXN groups (Figs. 5A–5D, $P < 0.01$). As a NAD^{+}-dependent deacetylase, SIRT1's deacetylase activity is closely related to the activation of the downstream transcription factor Nrf2. Our data revealed that SIRT1 activity in the Model group was significantly lower than in the Sham group, while it was markedly reversed by NIM and ZFXN (Fig. 5E, $P < 0.01$). The immunofluorescence co-localization results from the hippocampus (CA1, CA3) and cortex regions corroborated these findings, demonstrating significant enhancement of Nrf2 nuclear translocation in NIM and ZFXN rats compared to Model rats (Figs. 5F–5K, $P < 0.01$). These results suggest that ZFXN activated the SIRT1/Nrf2/HO-1 pathway to prevent mitochondrial dysfunction and neuronal death caused by oxidative stress.

3.7. EX-527 reversed the effects of ZFXN after SIRT1 was blocked in vitro

OGD is an ischemia mimic *in vitro* and has been widely utilized to study the effect and mechanism of ischemia [42, 60, 61]. The non-toxic concentration of ZFXN in SH-SY5Y cells ranged from 0.625 to 160 μg·mL^{-1} (Fig. 6A), with an optimum modeling time of approximately 12 h, yielding a survival rate of about 70% (Fig. 6B). Our data demonstrated that ZFXN significantly increased cell viability in OGD-SH-SY5Y cells at concentrations of 20–80 μg·mL^{-1} (Fig. 6C, $P < 0.01$). Subsequently, 20 μg·mL^{-1} was determined as the optimal ZFXN dosage for cell treatment (Figs. 6A and 6C). In OGD-SH-SY5Y cells, the SIRT1/Nrf2/HO-1 pathway was significantly inhibited, resulting in notable decreases in SIRT1 activity and the expressions of SIRT1, nuclear Nrf2, total Nrf2, and HO-1. However, ZFXN (20 μg·mL^{-1}) significantly counteracted these effects *in vitro* (Figs. 6D and 6E, $P < 0.01$). Similarly, ZFXN significantly reduced ROS and MDA levels while markedly increasing SOD, mitochondrial complex I/III, ATP, and EC levels in OGD-SH-SY5Y cells (Figs. 6F–6L, $P < 0.05$ or $P < 0.01$). Furthermore, the expression of PSD95 and SYN in OGD-SH-SY5Y cells was significantly down-regulated compared to control cells, while ZFXN treatment markedly up-regulated their expression (Fig. 6M,

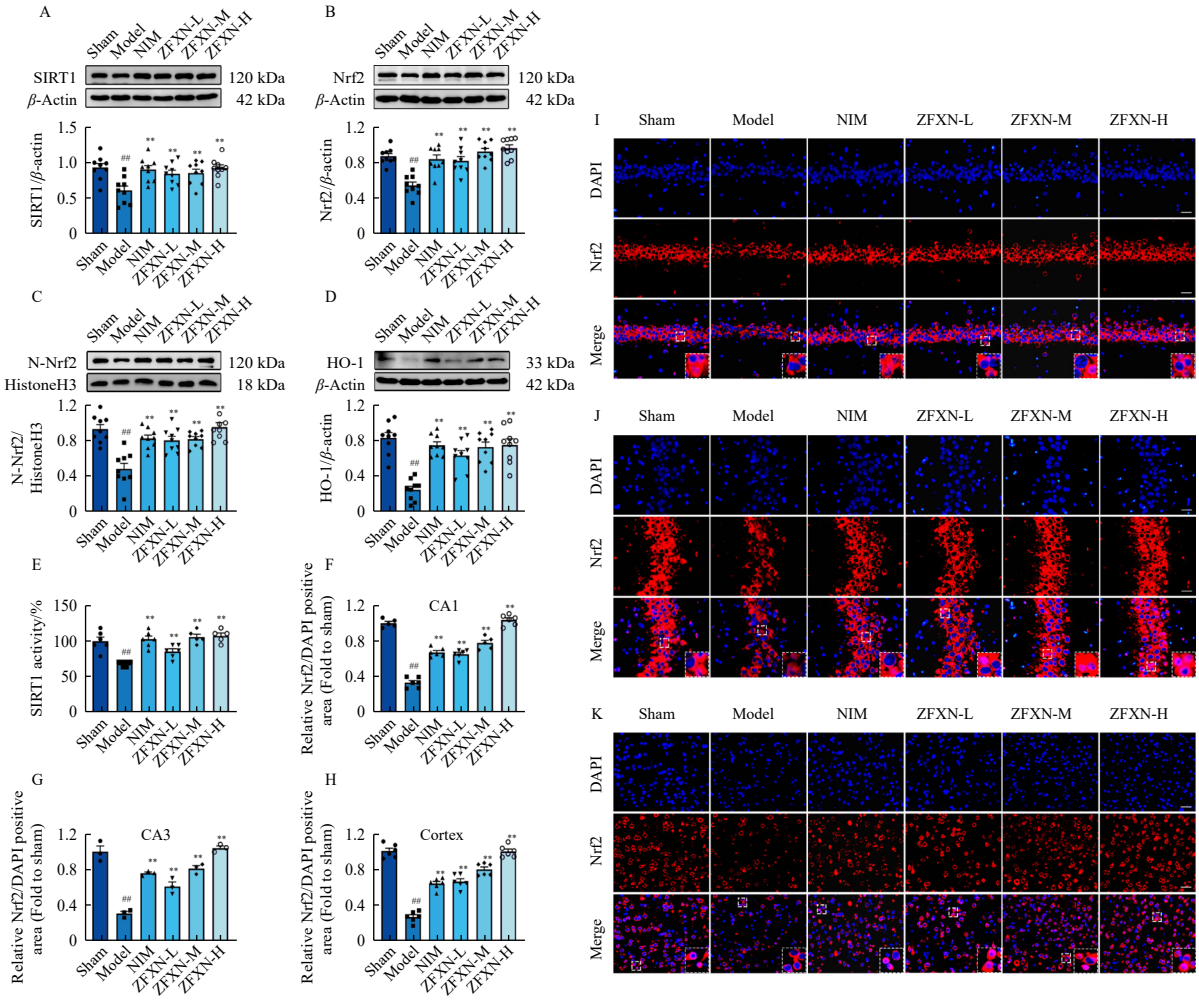


Fig. 5 ZFXN enhanced SIRT1/Nrf2/HO-1 pathway in 2VO rats. (A) Representative western blotting images and quantitative analysis of SIRT1 (n = 3). (B) Representative western blotting images and quantitative analysis of total Nrf2 (n = 3). (C) Representative western blotting images and quantitative analysis of nuclear Nrf2 (n = 3). (D) Representative western blotting images and quantitative analysis of HO-1 (n = 3). (E) The activity of SIRT1 (n = 5–6). (F–H) Relative co-localization positive area of Nrf2 and DAPI in the hippocampal CA1, CA3, and cortex regions, respectively (n = 3). (I–K) Representative immunofluorescence co-localization images of Nrf2 in hippocampal CA1 (I), CA3 (J), and cortex regions (K), respectively. (Magnification: 400 ×. Scale bar = 50 μm. Nrf2: red, DAPI: blue). The data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Model group.

$P < 0.01$). Notably, when SIRT1 was inhibited by EX-527 (a SIRT1 inhibitor) *in vitro*, the effects of ZFXN on the SIRT1/Nrf2/HO-1 pathway, oxidative stress, mitochondrial function, and synaptic plasticity were dramatically diminished (Figs. 6D–6M, $P < 0.05$ or $P < 0.01$). These findings suggest that ZFXN prevents neuronal degeneration by activating the SIRT1/Nrf2/HO-1 pathway, thereby reducing mitochondrial dysfunction and oxidative stress.

4. Discussion

The present study demonstrates that ZFXN mitigates PSCI through the activation of the SIRT1/Nrf2/HO-1 pathway, which serves to preserve mitochondrial function and inhibit neuronal apoptosis. These findings suggest that the upregulation of the SIRT1/Nrf2/HO-1 pathway may constitute a primary mechanism by which ZFXN exerts its therapeutic effects on PSCI.

Alteplase thrombolytic therapy remains the sole approved thrombolytic medication for the initial 4 h following ischemic stroke [62, 63]. While no specific therapeutic treatments exist for PSCI, certain medications are considered beneficial, including acetylcholinesterase inhibitors [64], actovegin [65], and nimodipine [66]. Chinese herbal formulae are gaining increasing attention for their multi-target synergistic effects in alleviating complex disorders such as PSCI [67]. Several Chinese herbs and natural active substances have demonstrated efficacy in ameliorating PSCI in rat models, including icariin [68], alpha-linolenic acid [69], Shuxuening Injection [49], rehmannioside A [70], and okanin [71].

ZFXN is an in-hospital formulation for ischemic stroke and PSCI with demonstrated therapeutic effects, having been clinically applied for over three decades [36-38]. Pharmacological evidence suggests that ZFXN exhibits significant neuroprotective properties, primarily through the regulation of BDNF, microcirculation, and oxidative stress [36, 38, 39]. Research has also indicated that the active ingredients in ZFXN demonstrate synergistic effects across multiple targets [72]. Ginsenoside Rg1 has been shown to alleviate cognitive impairment by maintaining mitochondrial homeostasis [73], reducing oxidative stress [74], enhancing cerebral angiogenesis [75], and suppressing neuroinflammation [76, 77]. Ginsenoside Re exhibits anti-inflammatory, antioxidant, and brain-derived neurotrophic factor regulatory activities, which are strongly associated with its effects on cognitive performance [78-80]. The neuroprotective properties of ginsenoside Ro have been linked to its ability to regulate metabolic disorders [81, 82], modulate oxidative stress [83], and inhibit platelet aggregation [84]. Ferulic acid has demonstrated the capacity to mitigate cognitive impairment and neurological dysfunction following ischemic stroke [85-87]. Notoginsenoside R1 has been reported to enhance learning and memory by increasing blood flow, reducing oxidative stress, promoting neuroprotection, modulating microcirculation, suppressing neuronal excitability, and improving synaptic function [88-92].

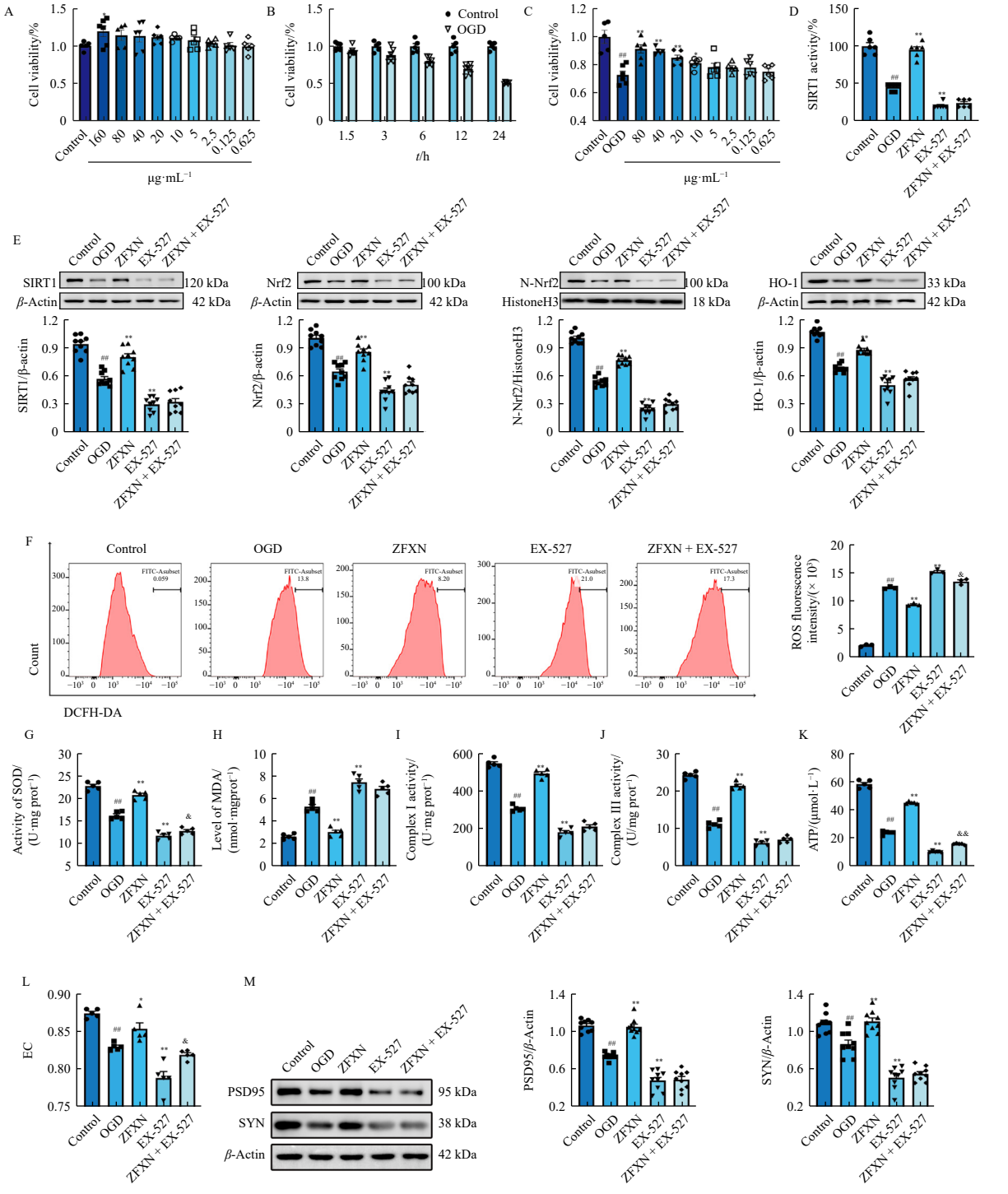


Fig. 6 EX-527 reversed the effects of ZFXN after SIRT1 was blocked *in vitro*. (A) Non-toxic concentration: SH-SY5Y cells were treated with various concentrations of ZFXN, and cell viability was assessed using the CCK-8 assay (n = 5–6). (B) OGD-treated time: SH-SY5Y cells were exposed to OGD conditions for different durations (n = 5–6). (C) Cell viability: SH-SY5Y cells were treated with varying concentrations of ZFXN under OGD conditions (n = 5–6). (D) The activity of SIRT1 (n = 6). (E) Representative western blotting images and quantitative analysis of SIRT1, total Nrf2, nuclear Nrf2, and HO-1 (n = 3). (F) Representative fluorescence intensity images and quantification of ROS (n = 3). (G) The activity of SOD (n = 5). (H) The level of MDA (n = 5). (I, J) The activity of mitochondrial complex I and complex III (n = 5). (K, L) The ATP content and EC in SH-SY5Y cells (n = 5). (M) Representative western blotting images and quantitative analysis of PSD95 and SYN (n = 3). The data was presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* OGD group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ *vs* EX-527 group.

Rhein has been found to improve cognitive function by modulating mitochondrial biogenesis *via* the SIRT1/PGC-1α pathway [93]. Furthermore, numerous ginsenosides, including Rg1 and Re, have been identified as SIRT1 activators [94, 95]. It is hypothesized that the neuroprotective effects of these ginsenosides are associated with the activation of Nrf2 and HO-1 [74, 96]. Multiple studies have also demonstrated that notoginsenoside R1, ferulic acid, and rhein up-regulate the expression of SIRT1 [92, 97, 98]. Their antioxidant activity is closely linked to the promotion of the Nrf2/HO-1 pathway [99-101]. Based on the aforementioned data, it is postulated that ZFXN may mitigate PSCI through the activation of the SIRT1/Nrf2/HO-1 pathway.

CCH, a prominent feature of ischemic stroke, results from brain tissue hypoxia. The 2VO model is widely recognized and accepted for studying CCH and PSCI [102, 103], as it replicates CCH conditions, cognitive deficits, and multiple secondary pathological changes observed in patients with PSCI and VaD. These changes include reduced cerebral blood flow, oxidative stress, and excess-

ive ROS generation [104, 105]. To mitigate the high mortality rate associated with 2VO, a modified bilateral carotid artery ligation approach was employed to establish PSCI in rats [41]. Similarly, the OGD-SH-SY5Y cell model is widely accepted for simulating neuronal damage following ischemic stroke *in vitro*. As anticipated, 2VO rats exhibited cognitive impairment and neuronal injury in the hippocampus and cortex, as demonstrated through open field tests, Y-maze tests, MWM tests, and HE staining. ZFXN significantly ameliorated these characteristics. Synaptic plasticity, crucial for learning and memory, can lead to neurological disorders when compromised [106, 107]. ZFXN notably up-regulated the expression of synaptic markers PSD95 and SYN *in vivo* and *in vitro* [108, 109].

Neuroprotective therapy that inhibits apoptosis is a crucial strategy for addressing cognitive impairment in PSCI, as excessive neuronal apoptosis is a primary mechanism of neuronal loss and cognitive decline [68]. ZFXN significantly increased the Bcl2/Bax ratio in brain tissue and reduced TUNEL$^+$ cells in cortical and hippocampal regions, indicating its neuroprotective effect through the inhibition of neuronal apoptosis. Oxidative stress, characterized by hypoxia-induced overproduction of ROS in brain tissue, is a major contributor to cognitive decline following ischemic stroke [56, 110]. Recent research has demonstrated the efficacy of antioxidants in addressing cognitive impairment, particularly in PSCI [111-113]. Both *in vivo* and *in vitro*, ZFXN substantially reduced ROS generation and MDA levels while elevating SOD, CAT, and GSH levels. These findings suggest that ZFXN prevented neuronal death and rectified the imbalance in the oxidation/antioxidant system in PSCI.

Oxidative stress-induced mitochondrial dysfunction represents a key characteristic of both ischemic stroke and PSCI [13, 114]. Consequently, protecting mitochondria is generally considered the most effective approach for treating these conditions. During CCH, brain tissue experiences mitochondrial destruction and excessive ROS production, leading to oxidative stress. The subsequent reduction in mitochondrial electron transport chain activity results in decreased MMP, ultimately causing the collapse of ATP generation [115, 116]. ZFXN demonstrated efficacy in mitigating mitochondrial dysfunction, as evidenced by improvements in MMP, Tom20 expression, mitochondrial complex I and III activity, and the restoration of EC and ATP synthesis in both *in vivo* and *in vitro* studies.

SIRT1 has been shown to activate and enhance Nrf2 nuclear translocation, which in turn inhibits ROS levels by increasing the expression of its downstream genes HO-1 and quinone oxidoreductase 1 (NQO-1), thereby suppressing oxidative stress-induced tissue injury and apoptosis [25, 27, 117, 118]. In our study of 2VO rats and OGD-SH-SY5Y cells, the data revealed an inhibition of the SIRT1/Nrf2/HO-1 pathway, evidenced by decreased SIRT1 activity and reduced expressions of SIRT1, nuclear Nrf2, total Nrf2, and HO-1. These findings align with previous research [119-122]. Notably, ZFXN significantly mitigated these alterations. However, the beneficial effects of ZFXN on oxidative stress, mitochondrial protection, activation of the SIRT1/Nrf2/HO-1 pathway, and increased expression of PSD95 and SYN were substantially reversed when SIRT1 was blocked by EX-527 (Fig. 7).

The present study demonstrated that ZFXN mitigates PSCI by activating the SIRT1/Nrf2/HO-1 pathway. However, certain limitations necessitate further investigation. While the presence of notoginsenoside R1, ginsenoside Re, ferulic acid, ginsenoside Rg1, ginsenoside Ro, and rhein in ZFXN has been identified, these compounds exhibit poor oral bioavailability and undergo specialized metabolic pathways *in vivo*. For instance, notoginsenoside R1 has a bioavailability of merely 0.25% following oral administration [123], and the body undergoes complex metabolic processes resulting in deglycosylated aglycones and other compounds [89]. The distribution of notoginsenoside R1 or its aglycone in the brain remains unreported. Similar pharmacokinetic characteristics apply to other active compounds. Consequently, while the direct effects of these components were assessed using ZFXN on SH-SY5Y cells, this approach did not account for the intricate processes of ZFXN *in vivo*.

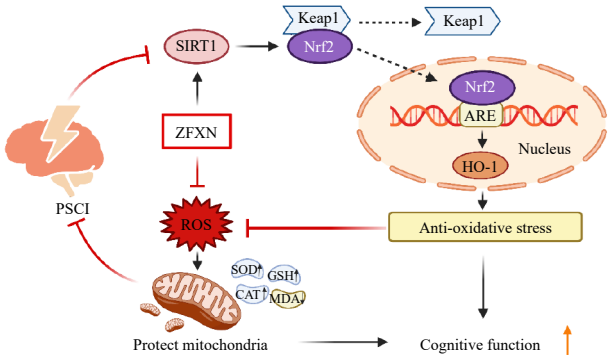


Fig. 7 Schematic illustration of the mechanism underlying ZFXN in alleviating PSCI by enhancing the SIRT1/Nrf2/HO-1 antioxidant pathway to protect mitochondria (Created in BioRender.com).

Moreover, the study demonstrated that inhibiting SIRT1 with EX-527 significantly attenuated the pharmacological effects of ZFXN. Although ZFXN has been shown to modulate SIRT1 function, additional research is required to identify the specific inhibitory molecules in ZFXN that directly interact with SIRT1. This could be achieved through target fishing and other advanced technologies. As previously noted, the metabolites of individual components in ZFXN should also be considered when identifying molecules that act on the SIRT1 protein.

5. Conclusions

In conclusion, ZFXN mitigated PSCI by activating the SIRT1/Nrf2/HO-1 pathway, thereby inhibiting oxidative stress-induced mitochondrial damage and neuronal apoptosis. This finding suggests that ZFXN's capacity to up-regulate the SIRT1/Nrf2/HO-1 pathway represents a promising therapeutic approach for PSCI.

Funding

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Conflict of interest

The authors declare no conflicts of interest.

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