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Original Research

The Role of Formononetin in Cerebral Ischemia-Reperfusion Injury: A New Mediator of c-Fos/IL-10/STAT3 Signaling Pathway

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Abstract

Backgrounds: Inflammation plays a pivotal role in the advancement of ischemic stroke, and Formononetin has been recognized for its potential benefits due to its anti-inflammatory effects. Although Formononetin shows promise for reducing cerebral ischemic injury, its precise effectiveness and the underlying molecular mechanisms still need to be thoroughly explored. The research aimed to investigate Formononetin's impact and mechanisms on ischemic brain damage. **Methods**: In this study, both the ischemia/reperfusion (I/R) mouse model and the oxygen-glucose deprivation/reperfusion (OGD/R) cell model were used. The I/R mouse model was prepared using the middle cerebral artery occlusion (MCAO) method, while the OGD/R SH-SY5Y cell model was established using the oxygen-glucose OGD/R method. Hematoxylin and Eosin (H&E) staining, Tunnel fluorescence staining, and Nissl staining were employed to observe the effects of Formononetin on neuronal damage, apoptosis, and survival in I/R mouse brain tissue. Additionally, the effects of Formononetin on the levels of pro-inflammatory factors in I/R mice and OGD/R cells were detected using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) and Enzyme-Linked Immunosorbent Assay (ELISA) methods. The c-Fos/Interleukin-10 (IL-10)/Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathway in I/R mice and OGD/R cells was examined using RT-qPCR and Western Blot (WB). Furthermore, rescue validation was performed using targeted interventions of IL-10 and c-Fos, confirming that the c-Fos/IL-10/STAT3 signaling pathway is a key target of Formononetin. **Results**: Our findings reveal that Formononetin notably decreased infarct size and neuronal damage *in vivo* ($p < 0.001$). Additionally, Formononetin decreased inflammation and lowered levels of pro-inflammatory cytokines ($p < 0.05$). In cell models, Formononetin effectively suppressed neuronal injury induced by OGD/R and the related inflammatory markers ($p < 0.001$). Mechanistic studies showed that Formononetin enhances IL-10 expression in both models of ischemic brain injury, a process crucial for its protective effects against inflammation ($p < 0.05$). This regulation is facilitated by increased nuclear translocation of c-Fos, highlighting the c-Fos/IL-10/STAT3 pathway as a crucial mechanism of Formononetin's neuroprotective and anti-inflammatory effects in cerebral ischemia ($p < 0.05$). **Conclusion**: We found Formononetin alleviates inflammation associated with I/R injury by activating the c-Fos/IL-10/STAT3 pathway, which highlights the potential of Formononetin as a promising therapeutic approach for I/R injury.

Keywords: ischemic stroke; neuronal injury; Formononetin; molecular mechanisms; protective effects

1. Introduction

Cerebral ischemia poses a significant threat to human health, marked by increased morbidity and mortality rates [1]. Following an ischemic insult, neuronal cell demise ensues, precipitating neurological impairment and secondary tissue damage [2]. The pathogenesis of ischemic stroke is intricate, involving a myriad of interrelated processes such as inflammation, autophagy dysregulation, excitotoxicity, apoptosis, and oxidative stress [3,4]. Despite extensive clinical investigations, therapeutic interventions capable of effectively ameliorating ischemia/reperfusion injury remain notably elusive [5]. Hence, there exists an imperative for further research endeavors aimed at elucidating novel pharmacotherapeutic modalities to mitigate the detrimental effects of ischemic stroke.

Inflammation is a primary characteristic of cerebral ischemia/reperfusion (I/R) injury [6]. The inflammatory response intensifies secondary neuronal damage and substantially impedes the development of tissue injury [6,7]. Following ischemic injury, there is a rise in the expression of various cytokines within neurons, glial cells, and immune system cells [8]. While some of these cytokines exacerbate brain damage, others, including Interleukin-10 (IL-10) which is found in neurons and glia, play a protective role in shielding the brain during stroke by offering neurotrophic support [9]. Studies propose that IL-10 could serve as a protective agent for the brain during episodes of cerebral ischemia/reperfusion [10,11]. Riley and colleagues [12] have shown that phosphorylated IL-10 R1 residues attract Signal Transducer and Activator of Transcription 3 (STAT3) to

the activated receptor. This interaction leads to the phosphorylation and subsequent activation of the dormant transcription factor, phosphorylated-STAT3 (p-STAT3), offering protective benefits to the brain [12].

Isoflavones belong to the flavonoid family. Formononetin, a key isoflavone, constitutes a primary active compound in Tongmai Pills, depicted in Fig. 1 through its chemical structure [13]. Displaying a variety of pharmacological properties, it possesses anti-cancer, anti-apoptotic, anti-inflammatory, and antioxidant capabilities [14]. Research indicates that Formononetin can mitigate H_2O_2-induced cell death by reducing reactive oxygen species (ROS) levels and restraining cellular apoptosis [15]. A recent study indicate that Formononetin alleviates inflammation linked to cerebral I/R injury in rats by regulating the Janus Kinase 2 (JAK2)/STAT3 signaling pathway [11]. According to the study by Hua *et al*. [16], alpha-ketoglutarate (AKG) helps mitigate damage in cerebral I/R by targeting the c-Fos/IL-10/STAT3 signaling pathway. Additionally, there is evidence suggesting Formononetin could exhibit neuroprotective properties in rat models of traumatic brain injury by reducing inflammation in cortical neurons [15,17]. However, the potential of Formononetin to protect against cerebral ischemia via the c-Fos/IL-10/STAT3 signaling pathway has yet to be investigated.

In our research, we examined the anti-inflammatory impact of Formononetin's impact on I/R in mice, elucidating its interaction with the c-Fos/IL-10/STAT3 signaling pathway.

2. Materials and Methods

2.1 Animal Study

Male C57BL/6J mice (6–8 weeks old, weighing 20–25 g, Beijing LiHua Experimental Animal Technology Co., Ltd., Beijing, China) were randomly divided into four groups (n = 6 per group): (1) Control group (n = 6): Mice underwent sham surgery without middle cerebral artery occlusion (MCAO) and received no treatment; (2) MCAO group (n = 6): Mice underwent MCAO surgery without any additional treatment; (3) MCAO+DMSO group (n = 6): Mice underwent MCAO surgery and received vehicle solution (0.5% CMC-Na containing 1% DMSO, 10 mL/kg) by oral gavage once daily; (4) MCAO+Formononetin group (n = 6): Mice underwent MCAO surgery and received Formononetin (30 mg/kg, dissolved in 0.5% CMC-Na containing 1% DMSO, 10 mL/kg, Cat. No. F805983, Macklin, Suzhou, China) by oral gavage once daily. All mice were housed under specific pathogen-free (SPF) conditions with controlled temperature (22 ± 2 °C), humidity (55 ± 5%), and a 12-hour light/dark cycle, with free access to standard laboratory chow and water. Drug administration began immediately after MCAO reperfusion and continued for the duration of the experiment. The MCAO technique was executed following established protocols cited in the literature [18]. Mice were anesthetized with sodium pentobarbital (1.5%, 40 mg/kg, intraperitoneal injection). After reperfusion, Formononetin (dissolved in 0.5% CMC-Na containing 1% DMSO) was administered by oral gavage at a dose of 30 mg/kg body weight once daily, with the dosing volume maintained at 10 mL/kg. The control group received an equal volume of vehicle solution (0.5% CMC-Na containing 1% DMSO) by the same route. Subsequent neurological deficit evaluations were carried out to assess the degree of neurological impairment, using the previously outlined methods [18]. At the end of the experiment, mice were deeply anesthetized with sodium pentobarbital (3.0%, 150 mg/kg, intraperitoneal injection) and euthanized by cervical dislocation. Brain tissues were immediately collected for subsequent analyses. All experimental procedures were approved by the Animal Ethics Committee of Jiangsu Kanion Pharmaceutical Co., Ltd. (Experimental Animal Ethics Number: 20240312) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Histopathological Analysis, Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay, and Nissl Staining

Three mice were randomly selected from each group to measure the infarct volume. The remaining mice had their brain tissues divided into two parts: one part was used for histopathological analysis, and the other part was used for molecular biology analysis.

The infarct volume in 1 mm sections of brain was assessed by 2,3,5-tribenzotetrazole staining. All infarcts extended into both cortical and subcortical areas. Hematoxylin and Eosin (H&E) staining was used to assess brain tissue pathology. Tissue sections, embedded in paraffin and sliced to 5 micrometers, were stained and examined histologically. TUNEL assay was performed with an *In Situ* Cell Death Detection Kit (Cat. No. 11684817910, Roche, Germany) to identify apoptotic neurons at injury sites. Fluorescence microscopy (BX53, Olympus, Tokyo, Japan) was utilized to visualize and capture images of TUNEL-positive cells. Paraffin-embedded sections were subjected to a xylene dewaxing process for five minutes and subsequently stained with Nissl stain. The sections were then immersed in 95% ethanol until the Nissl substance appeared dark blue against a colorless or pale blue background. Nissl bodies within the cortical neurons were visualized under a microscope, providing insights into cellular morphology and structure.

2.3 Cell Culture and Oxygen-Glucose

The SH-SY5Y cell line was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China) and confirmed by Short Tandem Repeat (STR) for purity. Mycoplasma, bacteria, and fungi were all negative. The cells were cultured in DMEM/F12 medium with 10% fetal bovine serum and antibiotics at 37 °C with 5% CO_2. Before the oxygen-glucose deprivation (OGD) proce-

dure [19,20], cells were pretreated with Formononetin (10 or 50 mmol/L) or stattic (10 µmol/L) for one hour. OGD involved transferring cells to glucose-free DMEM and exposing them to 95% N_2 and 5% CO_2 at 37 °C for four hours. After OGD, cells were returned to standard culture conditions for a twelve-hour reoxygenation period.

2.4 siRNA Transfection

Cells were transfected with *IL-10* siRNA or scramble control siRNA (GenePharma, Shanghai, China). siRNA sequences were: *IL-10* siRNA sense: 5′-UAAGCUCCAAGAGAAAGGCdTdT-3′, anti-sense: 5′-GCCUUUCUCUUGGAGCUUAdTdT-3′; scramble siRNA sense: 5′-AACAGUCGCGUUUGCGACUGGdTdT-3′, anti-sense: 5′-CCAGUCGCAAACGCGACUGUUAdTdT-3′. *c-Fos* siRNA: sense: 5′-GCAAGGUGGAACAGUUAUC dTdT-3′ and anti-sense: 3′-dTdT CGUUCCACCUUGUCAAUAG-5′. Cells were seeded in 6-well plates (2×10^5 cells/well) and grown to 60–70% confluence. For transfection, 50 nM siRNA and 5 µL Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) were separately diluted in 125 µL Opti-MEM (Gibco, Carlsbad, CA, USA), combined, and incubated for 15 minutes at room temperature. The mixture was added to cells in antibiotic-free medium, and replaced with complete medium after 6 hours. After 48 hours of incubation (37 °C, 5% CO_2), knockdown efficiency was verified by Western blot. Transfected cells then underwent OGD treatment as described previously.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA) Assay

SH-SY5Y cells and mice brain tissues were homogenized in cold PBS and centrifuged at 14,000 rpm. IL-10 (Cat. No. ab255729), IL-1β (Cat. No. ab197742), IL-6 (Cat. No. ab222503), and tumor necrosis factor-α (TNF-α) (Cat. No. ab208348) levels were quantified using specific ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.6 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from SH-SY5Y cells and peripheral mouse brain tissue near the infarct area using TRIzol reagent (Cat. No. 15596026, Invitrogen, USA). SYBR Green (Cat. No. RR420A, Takara, Ostu, Japan) was used for real-time PCR, with GAPDH serving as the normalization reference. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate, and the results are presented as mean $\pm$ standard deviation (SD). The primer sequence list is shown in **Supplementary Tables 1**,**2**.

2.7 Western Blot (WB) Assay

Cell samples and brain tissues were lysed in Radioimmunoprecipitation Assay buffer (RIPA) buffer supplemented with phosphatase and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined using the BCA Protein Assay Kit (Cat. No. 23225, Thermo Fisher Scientific, USA). Equal amounts of protein (30 µg) were separated by 10% Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against: IL-1β (1:1000, Cell Signaling Technology, Danvers, MA, USA, #12242); TNF-α (1:1000, Cell Signaling Technology, #3707); IL-6 (1:1000, Abcam, ab229381); IL-10 (1:1000, Abcam, ab133575); p-STAT3 (1:1000, Abcam, ab267373); STAT3 (1:1000, Abcam, ab68153); c-Fos (1:1000, Abcam, ab222699); GAPDH (1:5000, Cell Signaling Technology, #5174); Histone H3 (1:1000, Cell Signaling Technology, #4499). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (goat anti-rabbit IgG, 1:5000, #7074; goat anti-mouse IgG, 1:5000, #7076; Cell Signaling Technology) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA) and captured using the ChemiDoc XRS+imaging system (Bio-Rad). Band intensities were quantified using Image Lab software (Version 6.0, Bio-Rad, Hercules, CA, USA). GAPDH was used as the loading control for cytoplasmic proteins, while Histone H3 served as the loading control for nuclear proteins. The relative protein expression was calculated as the gray value ratio of target protein to their respective loading controls, and results were expressed as fold change relative to control. Each experiment was performed in triplicate.

2.8 Immunofluorescence Staining

For the immunofluorescence study, procedures from prior reports were adapted [19,20]. Initially, the brain samples were promptly rinsed with cold PBS and then fixed using a 4% paraformaldehyde solution for 15 minutes at room temperature. After this stabilization step, the samples underwent a 3–5-minutes rinse in PBS. Subsequently, the brain sections were treated for 30 minutes at room temperature with a blocking solution made up of 0.2% powdered milk, 2% normal goat serum (Merck Millipore, NS20L), 0.1 M glycine, 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA, A7906), and 0.01% Triton X-100. For brain tissue immunofluorescence: primary antibody IL-10 (1:200, Abcam, ab133575) for 90 minutes at room temperature in a humid chamber. After that, the brain sections were washed three times in PBS and then incubated with fluorescent secondary antibodies (Alexa Fluor 647-conjugated goat anti-rabbit IgG (1:500, Invitrogen, A21245)) for 45 minutes at room temperature. The nuclei were highlighted by staining the sections with DAPI

(4,6-diamidino-2-phenylindole). A laser scanning confocal microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany) was utilized to capture and document the resulting fluorescence.

2.9 Measurement of Intracellular ROS Levels

The intracellular ROS levels were measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe (Sigma-Aldrich, D6883). SH-SY5Y cells were seeded in 24-well plates at a density of 1×10^5 cells/well and divided into four groups: Control, OGD/R, OGD/R+DMSO, and OGD/R+Formononetin (20 µM). After respective treatments, cells were washed twice with PBS and incubated with DAPI for 12 h, following 10 µM DCFH-DA in serum-free medium for 30 minutes at 37 °C in the dark. The cells were then washed three times with PBS to remove excess probe. Fluorescence images were captured using a fluorescence microscope (Olympus IX73, Japan) with excitation at 488 nm and emission at 525 nm. The fluorescence intensity was quantified using ImageJ software (1.52U, National Institutes of Health, Bethesda, MD, USA) and normalized to the control group.

2.10 Statistical Analysis

The data involved in this study are all continuous variables. A normality test was performed first with Shapiro-Wilk methods and data that followed a normal distribution were presented as mean ± SD, then, differences between two groups were assessed using the Student's *t*-test, while comparisons among more than two groups were made using analysis of variance (ANOVA) following post Bonferroni test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Formononetin Reduced Brain Infarct Volumes in Mice that Underwent Middle Cerebral Artery Occlusion (MCAO)

The structural depiction of Formononetin is illustrated in Fig. 1A. To determine the therapeutic potential of Formononetin, the study implemented the MCAO model in mice, administering Formononetin at a concentration of 30 mg/kg. 2,3,5-tribenzotetrazole staining assay conducted 24 hours after MCAO induction revealed that Formononetin markedly decreased the brain infarct volumes in mice (Fig. 1B,C, $p < 0.001$).

3.2 Formononetin Reduced Neuronal Damage and Cell Death in the Brains of Mice Subjected to MCAO

The effect of Formononetin on neuronal damage in mouse brain tissues after MCAO was assessed. Post-I/R, extensive neuronal damage was observed in the ischemic penumbra of the cerebral cortex, as demonstrated by H&E staining (Fig. 2A). In the sham operation group, the neuronal cells were arranged regularly, the structure was clear, and the nuclei were round and regular. In contrast, most of the neurons in MACO are disorganized and severely atrophy. Conversely, Formononetin notably ameliorated these detrimental effects. Nissl staining showed that compared with the sham operation group, the number of Nissl bodies decreased significantly in the drug-loading group and increased significantly after Formononetin treatment (Fig. 2B,C, $p < 0.001$). Apoptosis within the brain tissues was quantified using the TUNEL assay, which suggests that neuronal apoptosis was markedly elevated in the MCAO group compared to the control group, whereas Formononetin application reduced apoptosis levels, depicted in Fig. 2D,E ($p < 0.001$). Furthermore, Formononetin reduced the MCAO-induced increase in the pro-apoptotic gene Bcl-2-associated X protein (*BAX*) and the anti-apoptotic protein B-cell lymphoma 2 (*BCL-2*), as demonstrated through RT-qPCR analyses (Fig. 2F,G, $p < 0.001$). Collectively, these results demonstrate that Formononetin significantly attenuated neuronal damage and brain cell apoptosis in mice subjected to MCAO.

3.3 Formononetin Successfully Attenuated Inflammation in MCAO Mice and in SH-SY5Y Cells Treated With Oxygen-Glucose Deprivation/Reperfusion (OGD/R)

In the present study, mRNA expression conducted 24 hours post-reperfusion revealed a notable elevation in pro-inflammatory cytokine levels due to MCAO, which Formononetin treatment significantly attenuated (Fig. 3A–C, $p < 0.05$, or $p < 0.01$). ELISA results confirmed a significant elevation in the expressions of IL-1β, TNF-α, and IL-6 in MCAO mice (Fig. 3D–F, $p < 0.001$), and in OGD/R-treated SH-SY5Y cells (Fig. 3G–I, $p < 0.001$). Formononetin effectively mitigated these changes, restoring cytokine levels to their baseline. To investigate the effect of Formononetin on ROS generation during OGD/R injury, we performed DCFH-DA fluorescence staining (Fig. 3J). The results showed that OGD/R treatment significantly increased intracellular ROS levels compared to the control group ($p < 0.001$). The OGD/R+DMSO group showed similar elevated ROS levels to the OGD/R group, indicating that the vehicle had no significant effect. Notably, treatment with Formononetin (20 µM) markedly attenuated the OGD/R-induced ROS accumulation ($p < 0.001$ vs. OGD/R+DMSO group). The fluorescence intensity in the Formononetin-treated group was reduced by approximately 60% compared to the OGD/R+DMSO group, suggesting that Formononetin effectively suppressed oxidative stress in SH-SY5Y cells during OGD/R injury.

3.4 Formononetin Targeted IL-10 to Alleviate Cerebral I/R Injury in Mice

Formononetin administration was shown to increase IL-10 production, enhancing survival in mice compared to non-treated controls. This underscores IL-10's vital role in modulating immune responses, suggesting that Formononetin's neuroprotective effects against cerebral I/R injury may involve increased IL-10 levels. Fig. 4A–C shows

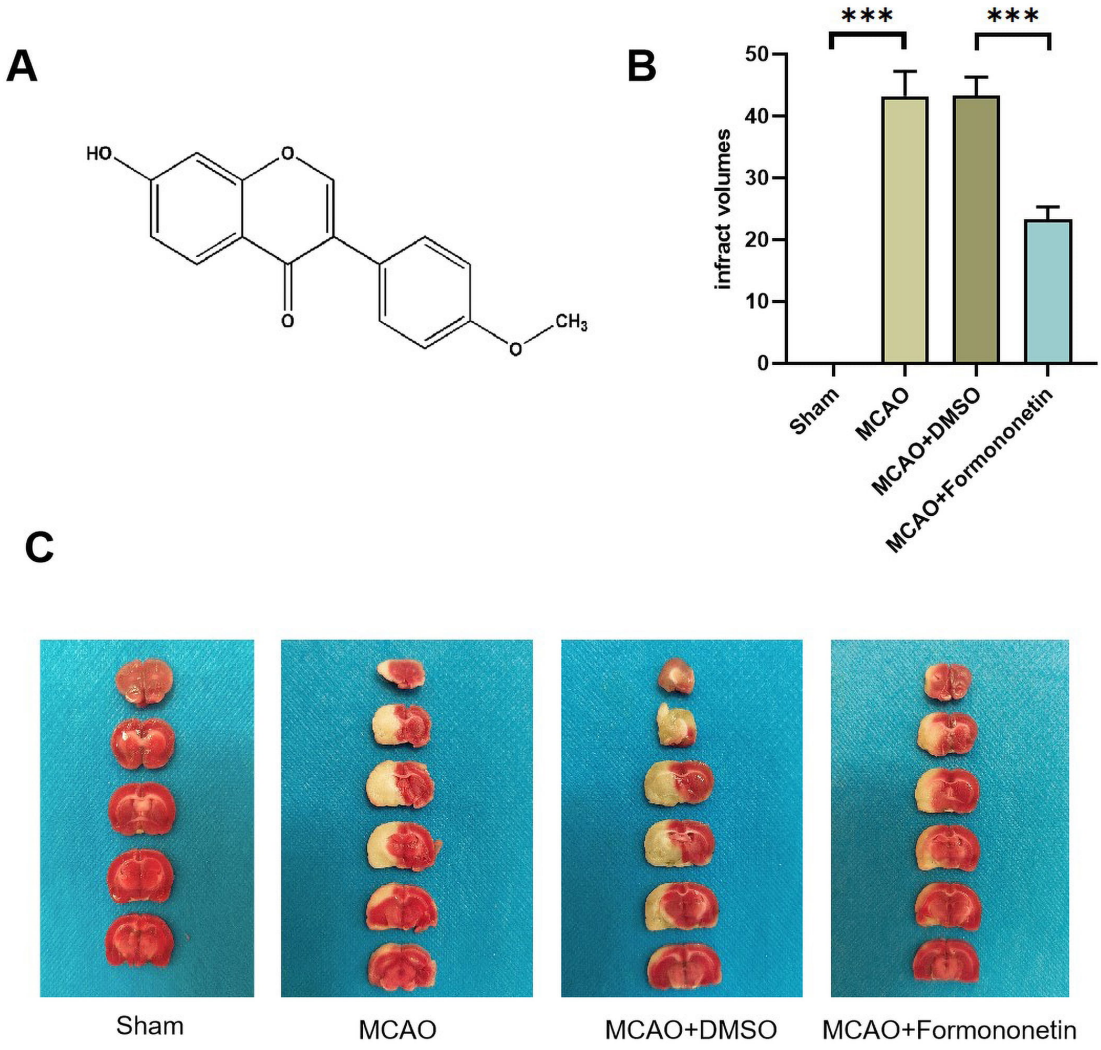

Fig. 1. Formononetin therapy reduced infarct volumes in middle cerebral artery occlusion (MCAO) mice. (A) Formononetin's molecular structure. (B,C) Effects of Formononetin on brain infarct volumes in mice following cerebral ischemia/reperfusion (I/R). ***$p < 0.001$. n = 3, data are represented as mean ± standard deviation (SD).

that after Formononetin treatment, IL-10 levels and STAT3 phosphorylation levels were significantly increased ($p < 0.001$). Fig. 4D–F also showed that IL-10 increased in the MCAO+Formononetin group ($p < 0.001$). The effect of Formononetin was further validated in SH-SY5Y cells *in vitro* (Fig. 4G–K, $p < 0.05$, or $p < 0.01$, or $p < 0.001$). However, using stattic, a STAT3 inhibitor, negated Formononetin's protective effects, and potentially reverses the suppression of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, as well as apoptosis (Fig. 5A–H, $p < 0.05$, or $p < 0.01$, or $p < 0.001$). Collectively, these results indicate Formononetin's protective effects against OGD/R-induced inflammation are mediated via the IL-10/STAT3 pathway.

To elucidate IL-10's contribution to Formononetin's modulation of SH-SY5Y cellular responses, IL-10-targeted siRNAs were utilized to suppress IL-10 expression prior to subjecting cells to 24 hours of OGD/R treatment. WB verified the successful reduction of IL-10 (Fig. 6A–C, $p < 0.01$). Inhibition of IL-10 expression notably compromised Formononetin's ability to mitigate expression of IL-10, STAT3, and the diminution of inflammatory markers (Fig. 6D–I, $p < 0.01$, or $p < 0.001$). These outcomes highlight IL-10's pivotal function in facilitating Formononetin's neuroprotective effects in SH-SY5Y cells following OGD/R insult.

3.5 Inhibited c-Fos Greatly Reduced Expression of IL10/STAT3 Pathway and the Protective Effect of Formononetin

The Activator Protein-1 (AP-1) transcription factor complex, consisting of Fos and Jun family members, boosts IL-10 transcription. Given this, it's suggested that Formononetin's neuroprotective effects against cerebral I/R injury may involve the modulation of c-Fos [21,22]. In our study, we observed a significant decrease in c-Fos expression in the nucleus of the OGD/R group. However, Formononetin reversed this effect, promoting the nuclear translocation of c-Fos (Fig. 7A–C, $p < 0.001$). To further

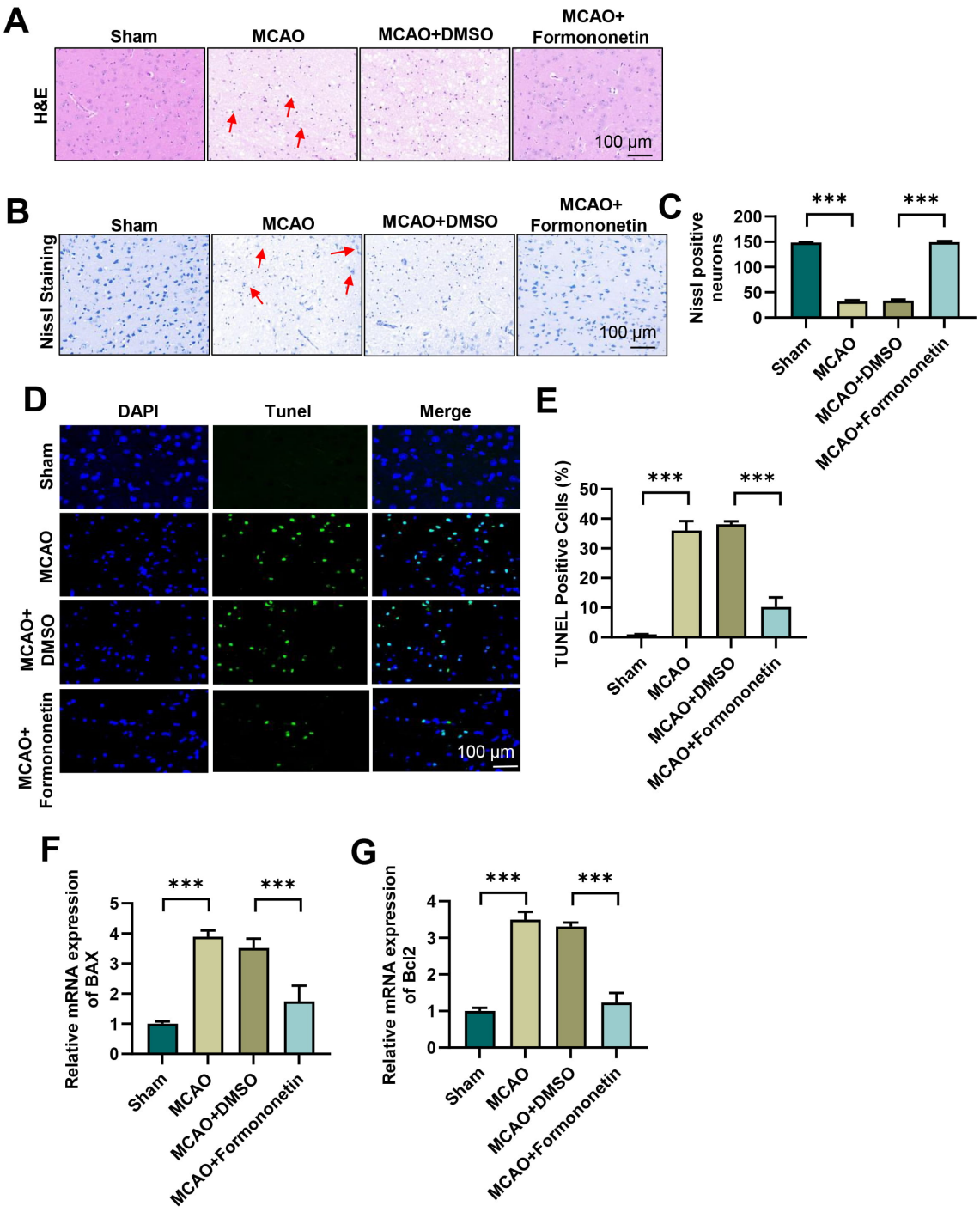

Fig. 2. Formononetin therapy reduced nerve injury and brain cell death in MCAO mice. (A) Hematoxylin and eosin staining experiment. Scale bar, 100 μm. Red arrows: indicate the lesion area or structural changes. (B,C) Nissl staining assay. Scale bar, 100 μm. Red arrows: indicate the neuronal damage and structural destruction. (D,E) Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) assay, Scale bar, 100 μm. (F,G) Bcl-2-associated X protein (*BAX*) and B-cell lymphoma 2 (*BCL-2*) mRNA levels were determined by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). ***$p < 0.001$. n = 3, data are represented as mean ± SD. H&E, Hematoxylin and Eosin.

explore the function and role of c-Fos, we suppressed its expression through siRNA interference experiments, with the efficiency of interference evaluated by mRNA and protein results (Fig. 7D–F, $p < 0.001$). The research results demonstrated that inhibiting c-Fos markedly suppressed the activation of IL-10 and STAT3 expression induced by Formononetin (Fig. 7G–J, $p < 0.05$, or $p < 0.01$, or $p < 0.001$). Further investigation revealed that knockdown of c-Fos sig-

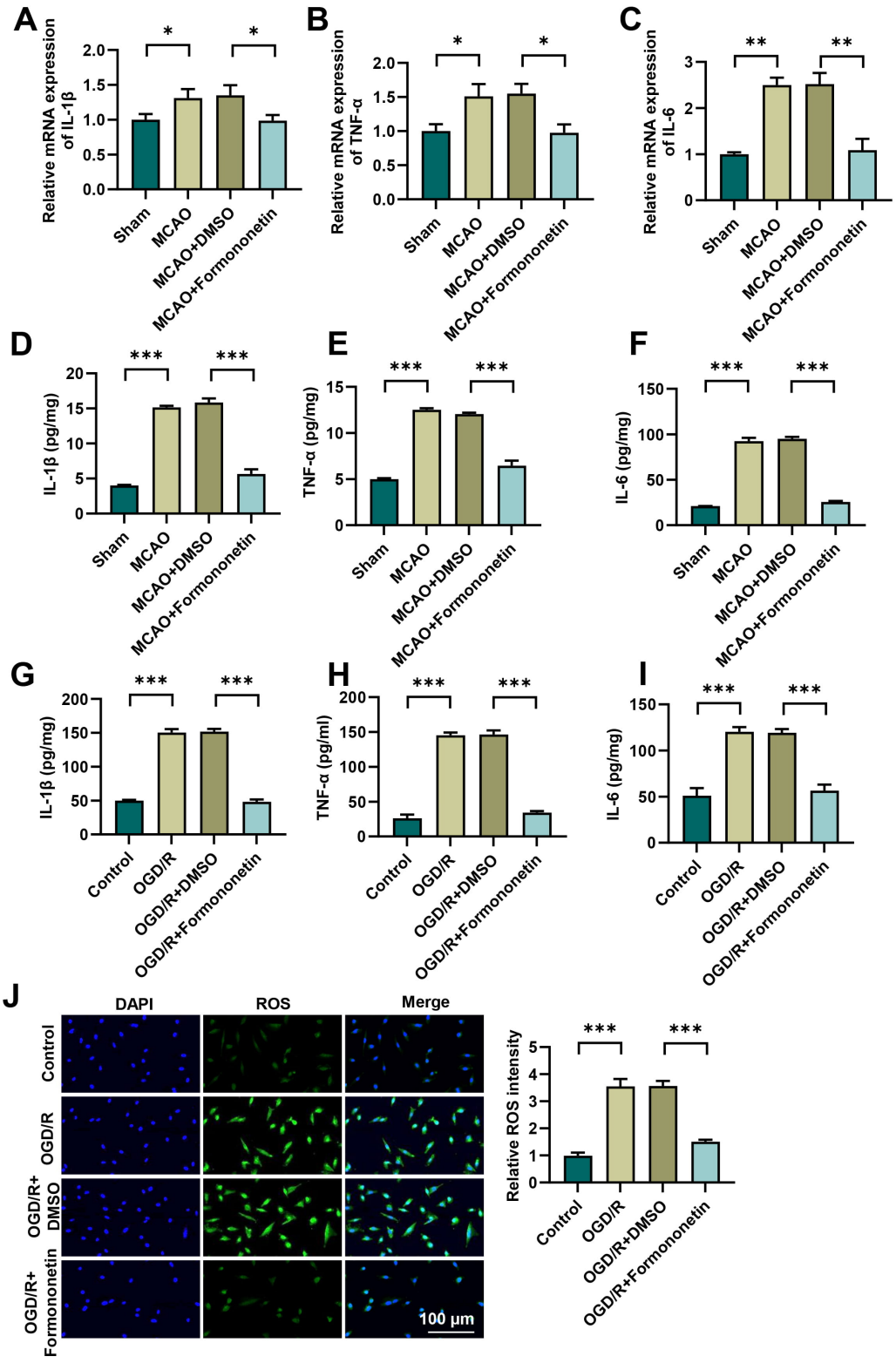

Fig. 3. Formononetin therapy decreased inflammatory response. (A–C) The mRNA expression of pro-inflammatory cytokines was determined by RT-qPCR assay in MCAO mice. (D–F) The levels of pro-inflammatory cytokines were measured using Enzyme-Linked Immunosorbent Assay (ELISA) in MCAO mice. (G–I) The levels of pro-inflammatory cytokines were measured using ELISA in oxygen-glucose deprivation/reperfusion (OGD/R)-treated SH-SY5Y cells. (J) Representative fluorescence images showing intracellular reactive oxygen species (ROS) levels detected by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) staining in SH-SY5Y cells under different treatments, Scale bar, 100 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 3, data are represented as mean ± SD.

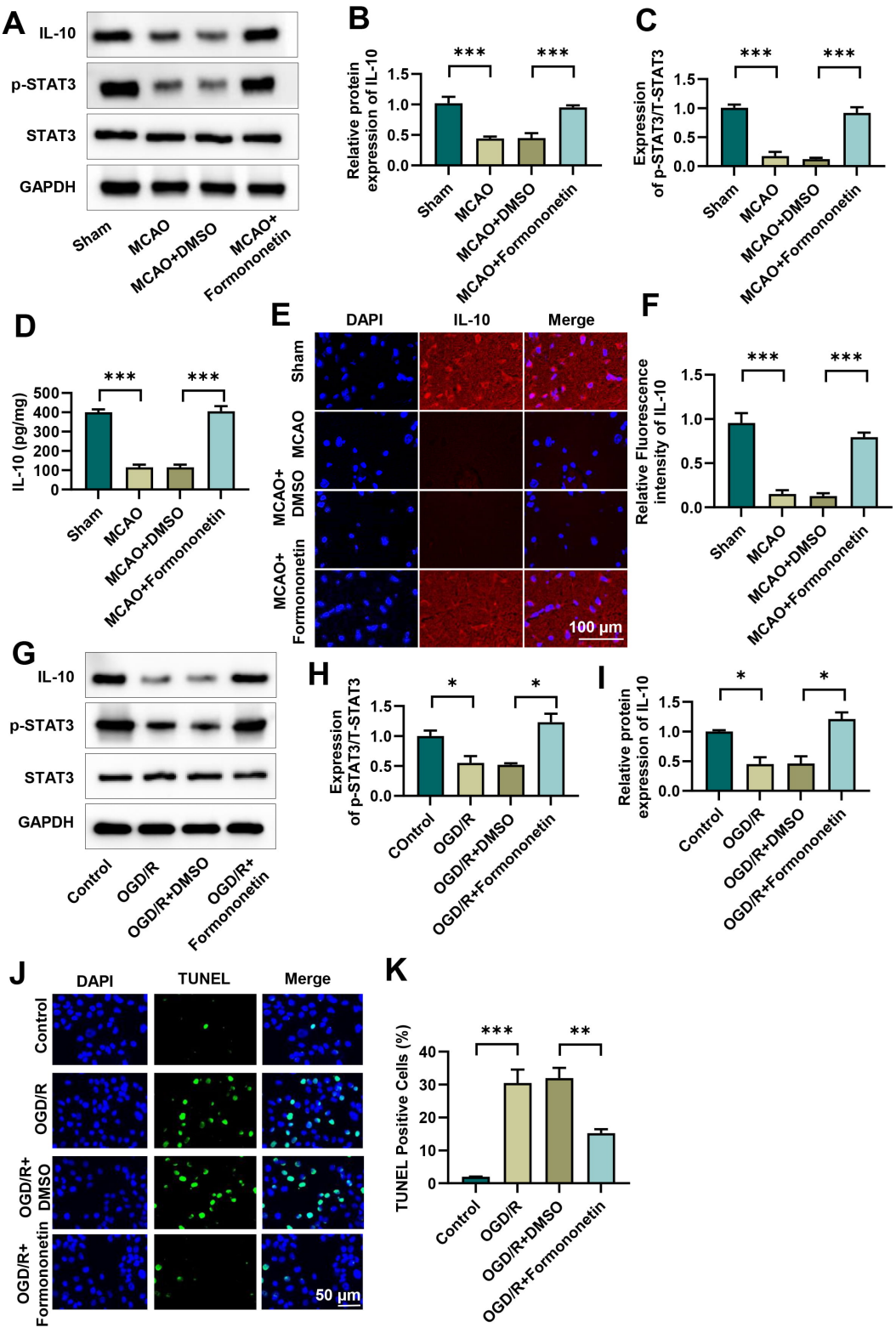

Fig. 4. Formononetin's protective effect against I/R damage was associated with the activation of Interleukin-10 (IL-10)/Signal Transducer and Activator of Transcription 3 (STAT3) signaling. (A–C) protein expression of IL-10 and phosphorylated-STAT3 (p-STAT3)/STAT3 in the indicated groups in the MCAO model. (D) IL-10 levels were measured using an ELISA kit in the *in vivo*. (E,F) Immunofluorescence staining of IL-10 pro-inflammatory cytokines levels was measured *in vivo*. Scale bar, 100 μm. (G–I) protein expression of IL-10 and p-STAT3/STAT3 in SH-SY5Y cells in the indicated groups. (J,K) Quantification of TUNEL-positive cells in SH-SY5Y cells in the indicated groups (TUNEL, green; DAPI, blue). Scale bar, 50 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n = 3, data are represented as mean ± SD.

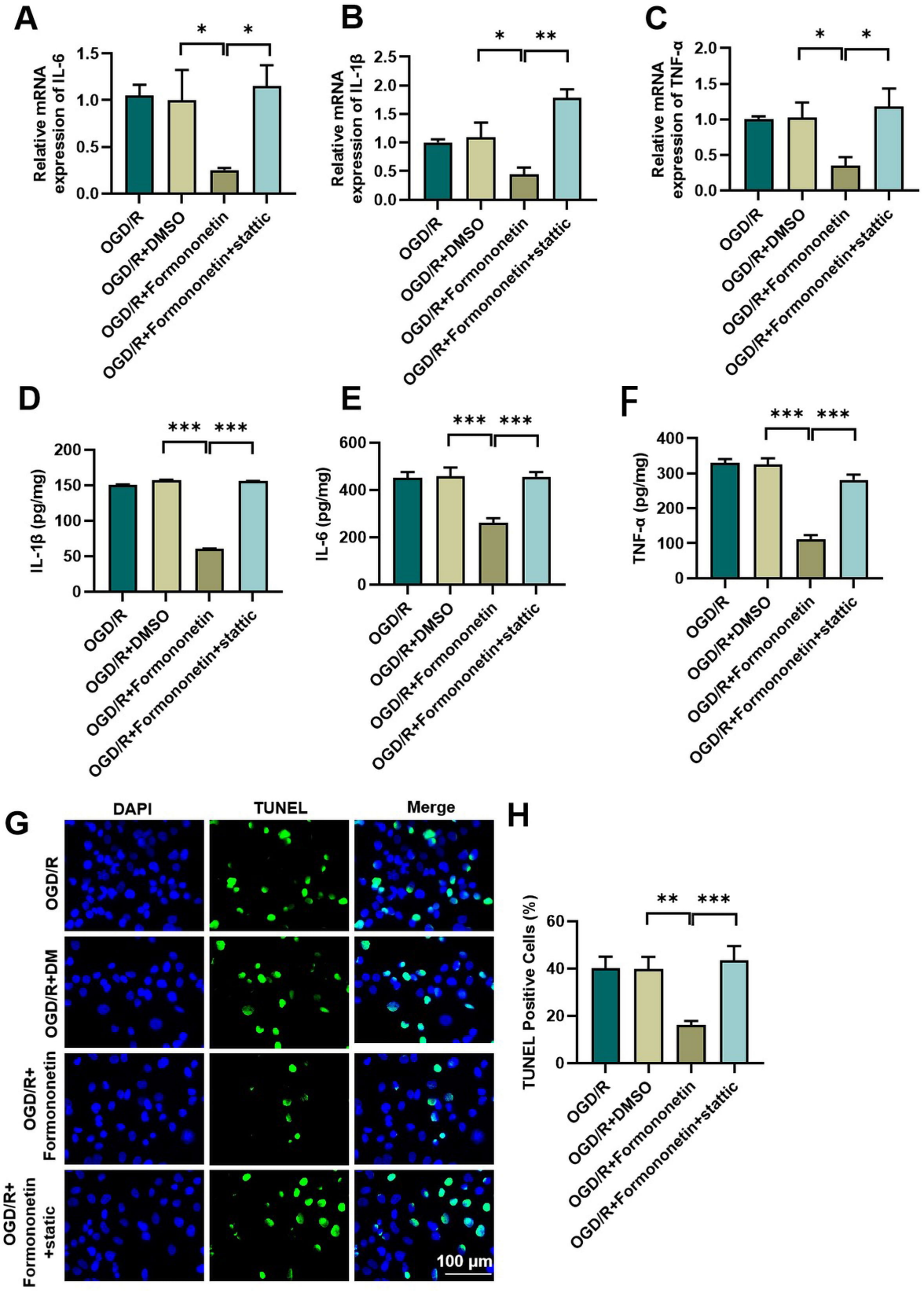

Fig. 5. Reducing STAT3 diminished Formononetin's protective effect. (A–C) mRNA expression of pro-inflammatory cytokines measured by RT-qPCR assay. (D–F) Pro-inflammatory cytokines levels were measured using an ELISA kit. (G,H) Quantification of TUNEL-positive cells in SH-SY5Y cells in the indicated groups (TUNEL, green; DAPI, blue). Scale bar, 100 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 3, data are represented as mean ± SD.

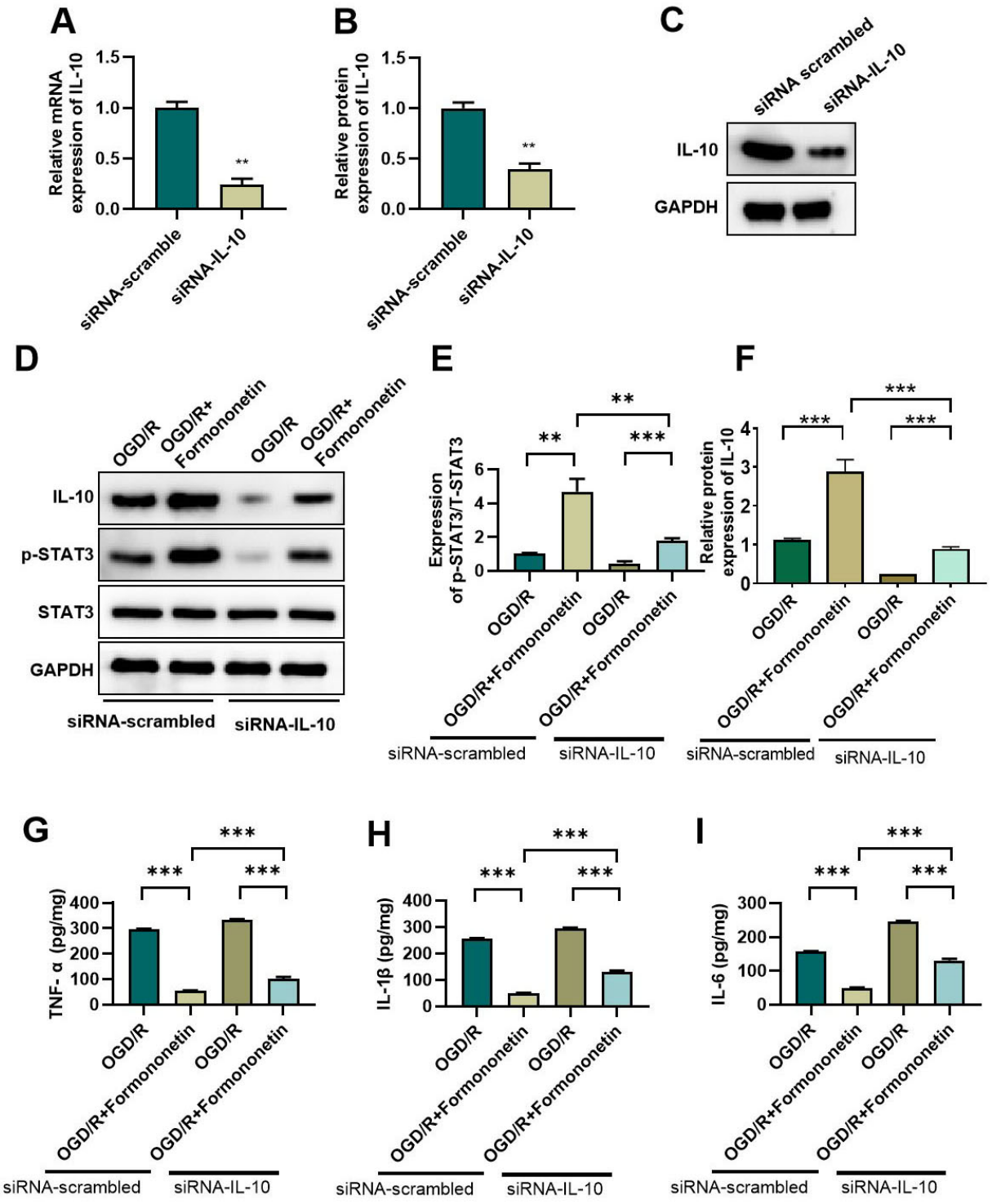

Fig. 6. Inhibiting IL-10 greatly reduced the protective effect of Formononetin. (A–C) RT-qPCR and Western Blot (WB) experiments were conducted to verify the efficiency of gene interference. (D–F) Protein expression of IL-10 and p-STAT3/STAT3 were detected by WB experiments. (G–I) The pro-inflammatory cytokines were measured using ELISA. $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3, data are represented as mean ± SD.

nificantly reversed the activation of inflammatory factor expression induced by Formononetin (Fig. 7K–M, $p < 0.001$). Collectively, these results suggested that Inhibited c-Fos greatly reduced the expression of the IL-10 /STAT3 pathway and the protective effect of Formononetin.

4. Discussion

Our study delineated the neuroprotective mechanism of Formononetin in mitigating cerebral ischemia, highlighting its role in upregulating IL-10 expression. *In vivo* experiments demonstrated that Formononetin supplementa-

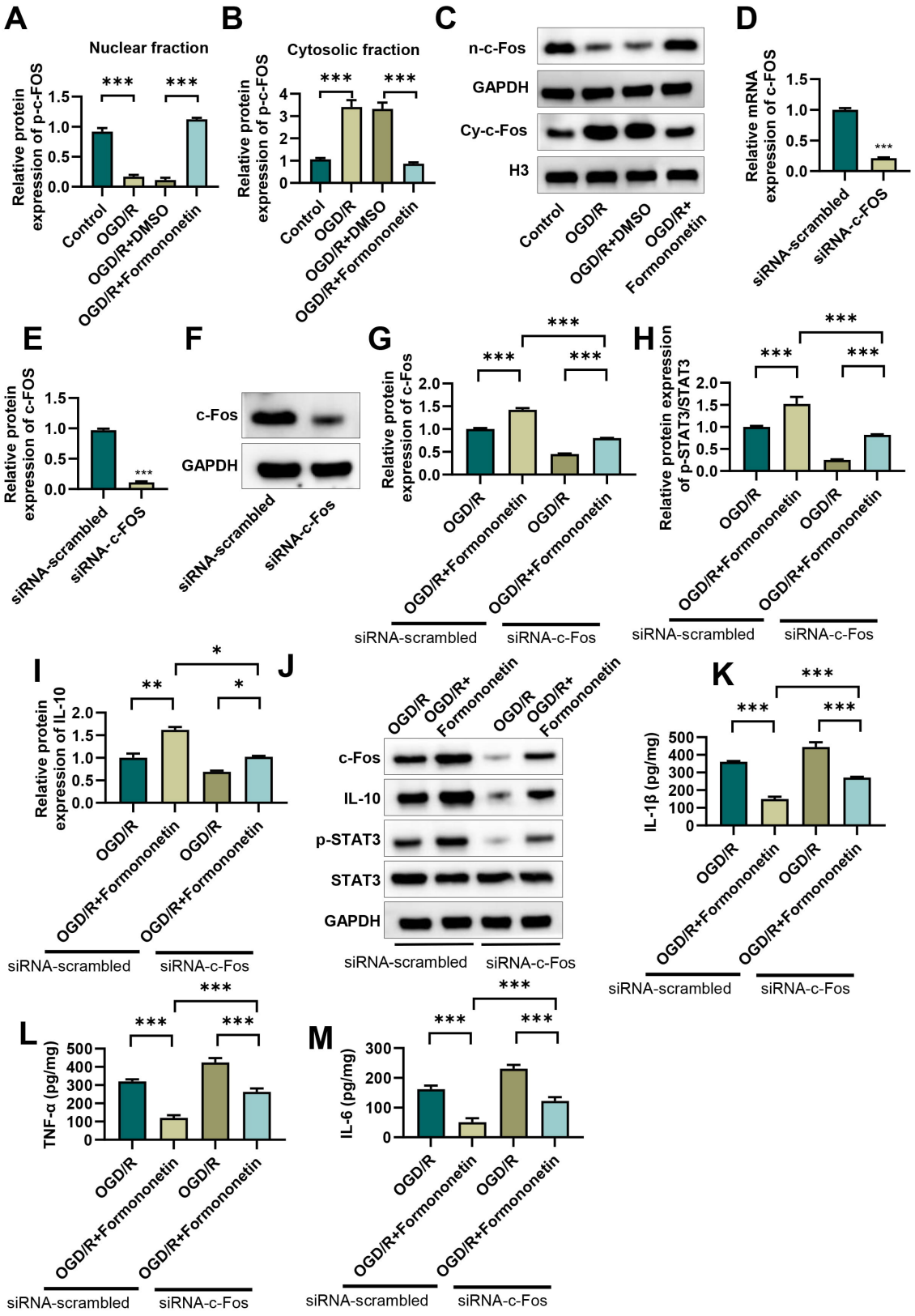

Fig. 7. Inhibited c-Fos greatly reduced expression of IL-10/STAT3 pathway and the protective effect of Formononetin. (A–C) Protein and mRNA expression of cy-c-Fos and n-c-Fos expression. (D–F) Knockout efficiency of c-Fos in sic-Fos knockdown experiment. (G–J) Protein expression of c-Fos/IL10/STAT3 in sic-Fos knockdown experiment. (K–M) The pro-inflammatory cytokines were measured using ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 3, data are represented as mean ± SD.

tion mitigated neurological impairments, decreased infarct sizes, and reduced neuronal damage and apoptosis in mice subjected to MCAO. *In vitro* analyses further revealed that Formononetin treatment effectively attenuated neuronal injury induced by OGD/R in SH-SY5Y cells. Formononetin's neuroprotective mechanism against cerebral I/R injury was attributed to its modulation of the IL-10/STAT3 signaling pathway. *In vitro* silencing of IL-10 significantly compromised the protective efficacy of Formononetin. The study underscored the significance of the c-Fos/IL-10/STAT3 signaling axis in Formononetin's mechanism of action, providing a molecular basis for its protective effects against I/R injury.

Cerebral I/R injury is a complex condition that leads to significant death and disability rates [23], often accompanied by impairments in cognitive function [22]. In our study, we found that Formononetin was effective in reducing neurological deficits and the volume of infarcts resulting from I/R injury in the MCAO model. These findings are consistent with other studies [12,15]. Our results also showed that Formononetin significantly attenuated brain cell apoptosis in mice subjected to MCAO. Studies suggested that apoptosis is involved in the progression of I/R injury [3,7,23]. Zhao *et al*. [24] indicated that inhibiting apoptosis can mitigate the occurrence of I/R injury. These results indicated Formononetin may alleviate the occurrence of I/R injury by inhibiting cell apoptosis.

Inflammation plays a crucial role in the development of cerebral I/R injury [6,7], TNF-α, a principal initial inflammatory cytokine, stimulates neutrophil and lymphocyte activation, enhances endothelial cell permeability, modulates extracellular metabolic processes, and promotes cytokine synthesis and secretion [25]. IL-6 is pivotal in B cell differentiation, antibody production, and T cell growth, contributing to defense mechanisms and initiating inflammation [26]. IL-18 and IL-1β, members of the IL-1 family, regulate immune responses, cellular growth, specialization, and signaling processes [27]. In the current study, Formononetin notably decreased the elevated levels of IL-1β, TNF-α, and IL-6 in SH-SY5Y cells subjected to OGD/R. Additionally, Formononetin promoted the expression of IL-10. IL-10 serves as a potent anti-inflammatory cytokine, playing a key role in regulating the immune system's response. Similarly, Yu *et al*. [15] suggested that Formononetin reduced IL-1β and TNF-α expression while increasing IL-10 levels, thus suppressing inflammation to safeguard against I/R injury. IL-10 serves as a potent inhibitor of inflammatory responses, crucial for immune system homeostasis [28]. Studies suggested that administering recombinant IL-10 intracerebrally was significantly effective in mitigating damage associated with cerebral I/R injuries, whereas the absence of IL-10 modestly elevated infarct volumes and exacerbated neurological deficits [29,30]. Aligned with these results, our research demonstrated that Formononetin mitigated the effects of I/R injury by increasing IL-10 expression. Blocking IL-10 effectively reversed Formononetin's suppressive effect on the inflammatory response in SH-SY5Y cells subjected to oxygen-glucose deprivation/reperfusion. Therefore, our study indicates that the elevation of IL-10 levels induced by Formononetin plays a crucial role in providing protection by regulating inflammatory responses [31]. Besides, other research proposes that activating the c-Fos/IL-10/STAT3 signaling pathway is crucial in mitigating the effects of I/R injury [16]. The AP-1 transcription factor complex is known for its ability to activate the IL-10 gene and consists of components from the Fos and Jun families. In our study, we found that inhibiting c-Fos significantly reduced the anti-inflammatory effects of Formononetin. Similarly, Hua *et al*. [16] indicated that AKG treatment markedly upregulated the translocation and expression of p-c-Fos to facilitate IL-10 transcription. Thus, our study suggested that Formononetin's protective effect against cerebral ischemia via the c-Fos/IL-10/STAT3 signaling.

In conclusion, the innovative aspects of this study lie in several key areas. First, we demonstrated for the first time that Formononetin exhibits neuroprotective effects through modulating the IL-10 signaling pathway in cerebral I/R injury. This novel mechanism extends our understanding of both Formononetin's therapeutic potential and the regulation of inflammatory responses in ischemic stroke. Additionally, our systematic approach combining *in vivo* and *in vitro* experiments provides comprehensive evidence for the molecular mechanisms underlying Formononetin's protective effects.

However, we acknowledge several limitations in our study. While our research demonstrated the effectiveness of Formononetin in an animal model, the translation of these findings to clinical applications requires further investigation. The complexity of stroke pathophysiology and individual patient variations may influence treatment outcomes. Moreover, our study focused primarily on the AP-1/IL-10 pathway, while other potential molecular mechanisms and signaling cascades might also contribute to Formononetin's neuroprotective effects. Several aspects warrant further investigation. Future studies should explore the optimal therapeutic window and dosing regimen for Formononetin administration in different stroke scenarios. Additional research is needed to evaluate potential drug interactions and long-term safety profiles. Furthermore, clinical trials will be essential to validate these findings in human patients and establish Formononetin as a viable therapeutic option for ischemic stroke treatment.

5. Conclusion

To conclude, our study demonstrated that Formononetin alleviates inflammation associated with I/R injury. Importantly, we observed that Formononetin mitigates I/R injury by activating the c-Fos/IL-10/STAT3 pathway. These findings highlight the potential of Formononetin as a promising therapeutic approach for I/R injury.

Availability of Data and Materials

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

Author Contributions

MY and JZ designed the research study. MY, FN, XX, and CZ performed the experiments and analyzed the data, JZ provided technical guidance and assistance with data collection. MY drafted the initial manuscript, FN, XX, CZ, and JZ did important revisions. All authors read and approved the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Animal Ethics Committee of Jiangsu Kanion Pharmaceutical Co., Ltd. (Experimental Animal Ethics Number: 20240312) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Conflict of Interest

All authors declare no conflict of interest. Despite several authors (Ming Yan, Fuyong Ni, Xue Xie, Chenfeng Zhang) are from Jiangsu Kanion Pharmaceutical Co., Ltd., this research does not involve any products of the company, and the judgments in data interpretation and writing were not influenced by this relationship.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL26274.

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