Inhibition of the JAK2/STAT3 Signaling Pathway by Piperine Confers Neuroprotection in Rats with Ischemic Stroke

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Research Article

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Abstract

Pepper is a traditional Chinese herbal medicine used for different inflammatory, fever, and bacterial infections. Our previous research shown that the principal pepper active component, piperine, reduces cerebral ischemia-reperfusion (I/R) damage, but the underlying mechanism was not completely understood. In this study, SH-SY5Y cells and Sprague–Dawley rats were pretreated with piperine, AG490, or vehicle. Oxygen–glucose deprivation of SH-SY5Y cells and a rat middle cerebral artery occlusion model were used to mimic cerebral I/R injury. In vitro results from CCK-8 and LDH assays showed that piperine and AG490 mitigated I/R injury induced by OGD and WB showed that piperine lowered the levels of p-JAK2 and p-STAT3. The infarction volume and neuronal apoptosis were reduced by piperine and AG490 in vivo, as demonstrated by TTC, HE, and TUNEL staining. The mNSS, beam-walking test, and MST test indicated that piperine and AG490 promoted recovery of neurological function after I/R injury in rats. These in vivo and in vitro findings demonstrate that the neuroprotective action of piperine on brain I/R injury is mediated by the JAK2/STAT3 signal pathway. It offers a viable therapeutic target for cerebral I/R injury and piperine could be turned into a potential JAK2-specific inhibitor.

1 Introduction

Acute ischemic stroke accounts for 6.5 million deaths and greater amount of disability per year globally [1]. Recanalization of the occluded artery through thrombolysis and/or endovascular thrombectomy is effective, but has many restrictions [2]. Thus, research of neuroprotective agents is important. Inflammation plays a significant part in cerebral ischemia/reperfusion (I/R) injury. After cerebral ischemia and reperfusion, levels of inflammatory factors such as Janus tyrosine kinase (JAK) and signal transducer and activator of transcription (STAT) [3], nuclear factor-κB (NF-κB) [4], tumor necrosis factor [5], matrix metalloproteinase-9 (MMP9) [6], interleukin-1 beta (IL-1β) [7], which intensify inflammation, increase. Accumulated evidence has confirmed that the JAK2/STAT3 pathway is activated by cerebral ischemia and is a key player in the pathophysiology of stroke [8]. Furthermore, mice treated with the JAK2/STAT3 inhibitor AG490 show significantly less neurological deficit, a smaller infarct volume, and decreased astrocytic differentiation in the ipsilateral brain following middle cerebral artery occlusion (MCAO) [9].

Piperine is the key alkaloid components of black pepper, a traditional Chinese herbal medicine owing to its antioxidant, anti-microbial, and gastro-protective properties [10]. Piperine contains a wide range of biological properties, including antioxidant [11], anti-microbial [12], anti-inflammatory [13, 14], anti-cancer [15], and anti-depressant [16], activities. Piperine has been found to have anti-inflammatory properties in numerous in vitro and in vivo experiments. In gastric cancer cells, piperine has been shown to reduce p38 MAPK and STAT3 activation, hence inhibiting IL-1-induced IL-6 production [6].

Piperine is a potential therapeutic agent in ischemic disease. Piperine salvages ischemic penumbral zone neurons by reducing the levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α [17]. Additionally, piperine pretreatment reduces neuronal apoptosis by inhibiting mitochondrial dysfunction [18]. We had
previously demonstrated that piperine has a neuroprotective effect in MCAO models that may be mediated via modulating complement and coagulation cascades [19]. Additionally, to evaluate whether piperine reduced cerebral I/R injury by blocking the JAK2/STAT3 signaling pathway and to assess the potential of piperine as a treatment for ischemia, we conducted in vitro and in vivo investigations in the present study.

2 Materials And Methods

2.1 Cell culture and oxygen–glucose deprivation (OGD) insult

The SH-SY5Y cells were a kind gift from Dr. Qi.Wan (Qingdao University, Qingdao, Shandong). For cell cultures, the SH-SY5Y cells were cultured as previously described [20]. Briefly, SH-SY5Y cells were seeded in 6 cm culture dish at 37°C in 5% CO2/95% air. When the Cells reached approximately 80% confluence, they were treatment with PIP or DMSO for 2h before OGD, and passaged at a 1:4 ratio.

OGD cell model was used to simulate ischemia in vitro, replaced the cell culture medium with glucose-free medium (11966025, Gibco, NY, USA) and place it in the humidified chamber (Lansing Plas Labs, USA), and incubate at 37°C, 5% CO2 and 95% N2 for 4 hours, followed by the reintroduction of oxygen and glucose by replaced the medium with fresh maintenance medium (8119174, Gibco, NY, USA) for the whole period at 37°C in a 21% O2/5% CO2 incubator for 24 h. The control cells were cultured for 4 hours in a glucose-contain medium (8119174, Gibco, NY, USA), and placed in normoxic incubator at 37°C in 5% CO2 and 21% O2.

2.2 Cell counting kit (CCK-8)

SH-SY5Y cells were seeded at an appropriate density of cells/well in 96-well plates (8 × 10^3 cells/well) to determine cell viability after treatment with piperine, AG490, or DMSO using the CCK-8 assay. According to the instructions, the Cell Counting Kit-8 (CCK-8) (Dojindo, CK04-3000, Japan) was used to measure the vitality of the cells. Data on the absorbance were collected using a 96-well plate reader from Molecular Devices in the United States at 450 nm. Additionally, the survival ratio is [(As − Ac)/(Ac − Ab)] * 100%. A blank group is represented by Ab, a control group by Ac, and an experimental group by As.

2.3 Lactate dehydrogenase (LDH) Assay

According to the manufacturer's instructions, the CytoTox 96 Cytotoxicity kit (Promega, USA) was used to quantify the LDH release. After the cell is completely lysed by lysis, the amounts of maximum LDH release were determined. A 96-well plate reader (made by Molecular Devices, USA) was used to measure the absorbance at 490 nm. Division of the experimental LDH release by the maximum LDH release was used to get the LDH release (%).

2.4 Animal and durgs
Male Sprague-Dawley (SD) rats (250–280 g, 2–3 per cage) purchased from the Wuhan University Center for Animal Experiments were maintained at a temperature of 23 to 25°C with a 12-hour light/dark cycle and provided enough food and water. The Animal Care and Ethics Committee of Wuhan University School of Medicine (Ethical batch number: AF268) and the IACUC rules were both followed in the approval and execution of all animal usage and experimental protocols. In order to distribute samples among the experimental groups and to gather and process data, randomization was used. Researchers conducting the tests were unaware of the groups to which each animal had been assigned. The experimental rats were randomly assigned and ARRIVE guidelines are followed for reporting on animal research. [21, 22].

The rats were randomly divided into the following groups: sham group (killed 24 h after surgery), MCAO + vehicle (Ctr) group, MCAO + PIP group, MCAO + AG490 group, and MCAO + AG490 + PIP group. The animals of Ctr group received 0.1% dimethyl sulfoxide (DMSO) once daily p.o. for 14 days before operation. Animals of MCAO + PIP group were administered with PIP (Sigma, P49007, St. Louis, MO, USA) (20 mg/kg, dissolved in 0.1% DMSO) once daily p.o. for 14 days. While animals in MCAO + AG490 group were received 0.1% DMSO once daily p.o. for 14 days and administered with AG490 (Tyrphostin, B42) (14 mg/kg, dissolved in 0.1% DMSO) one time before operation. For MCAO + AG490 + PIP group, animals were administered with PIP once daily p.o. for 14 days and received AG490 one time before operation.

2.5 Transient focal cerebral ischaemia and tissue extraction of penumbra

Male SD rats (250–280 g) were put to anaesthetize using a mask and 4% isoflurane in a mixture of 70% N₂O and 30% O₂. Throughout the experiment, a rectal probe was used to measure core body temperatures, which were kept constant at 37°C using a heating pad and heating lamp. After carefully exposing the right common carotid artery and its branches, a 3–0 monofilament was inserted from the external carotid artery into the internal carotid artery (about 22mm) to block the blood supply of the right middle cerebral artery. The monofilament was retracted after 2 hours of blockage to allow reperfusion before the rats were put under anesthesia once again. Local cerebral blood flow (CBF) was monitored with a Doppler flowmeter (PeriFlux System 5010; Perimed), A CBF loss of more than 70% during the ischemic phase, followed by a recovery to at least 60% of the baseline CBF, was defined to be a successful MCAO. Unsuccessful MCAO models were excluded in this study. Animals in the sham-operated group received similar surgical and anesthetic procedures without MCAO. Rats were anesthetized before being killed after 24 hours of reperfusion, brain was removed and stored in -20°C for sever hours, the method of penumbra tissue extraction refers to our published articles [19].

2.6 Western blotting

The equal amount of total proteins was separated on 10% SDS-PAGE gel and transferred to the polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) by electrophoresis. The membranes were promptly blocked for one hour in 5% non-fat milk/1x TBST, then incubated overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-pJAK2 antibody (1:1000, Abcam, ab219728); mouse monoclonal anti-pSTAT3 antibody (1:1000, Cell Signal Technology, 4113); rabbit polyclonal anti-caspase-
3 antibody (1:1000, Cell Signal Technology, 9662); rabbit monoclonal anti-GAPDH antibody (1:5000, Cell Signal Technology, 2118); mouse monoclonal anti-STAT3 (1:1000; Santa Cruz sc-8019); rabbit monoclonal anti-JAK2 (1:1000, Cell Signal Technology, 3230). After being washed with 1x TBST, the membranes were incubated with corresponding secondary antibody for 1 hour at room temperature. The Tanon 5200 chemiluminescent imaging system (Shanghai) was used to see protein bands, and Image J was used to evaluate the results.

2.7 Triphenyltetrazolium chloride (TTC) staining and infarct measurement

To gauge the infarct size, TTC (Sigma, V900570, St. Louis MO, USA) staining was done. After a 24-hour period of reperfusion, rats were given 1% pentobarbital sodium (50 mg/kg, I.P.) to induce a deep anesthesia. Following this, 150 ml of 0.9% saline was quickly injected into the heart, the brains were removed, and each brain was divided into 8 cerebral coronary slices. These slices were then immediately incubated in 2% TTC at 37°C for 30 min, and finally fixed in 4% paraformaldehyde. The next day, the brain slices were scanned used a scanner (Microtek Phantom V700 Plus) and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). The ipsilateral hemisphere's infarct area divided by the whole ipsilateral hemisphere area was multiplied by 100% to determine the infarct volume percentage.

2.8 Hematoxylin-eosin (HE) and Nissl staining

To create paraffin-embedded slices (4 mm), the rat's brain was extracted and treated in 4% paraformaldehyde. To investigate PIP's neuroprotective effects, HE staining was performed. The sections were dewaxed, stained for 3 minutes with hematoxylin, differentiated for 1 second in 1% hydrochloric alcohol, then stained for 3 seconds with eosin. After neutral resin was used to dry the dyed pieces, Aperio VERSA 8 was used to scan them (Leica, Germany). Analyzing the neuronal loss was done using Image J.

Nissl staining was performed to examine neuronal damage in brain sections as described previously [23], paraffin sections were were deparaffinized and then stained with 0.1% cresyl violet for 2 minutes. Following dehydration, xylene clearing, and mounting media coverslipping, brain slices were prepared. By using Aperio VERSA 8, the stained slices were scanned (Leica, Germany). Analyzing the neuronal loss was done using Image J.

2.9 Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL)/ Neuronal Nuclei (NeuN) double staining

Deparaffinized sections were incubated with 3% Bovine Serum Albumin (BSA, Sigma, B2064, St. Louis, MO, USA) for 30 minutes after endogenous peroxidase was suppressed with 0.3% H2O2 in PBS-3% methanol for 10 minutes. The sections were incubated with the anti-NeuN primary antibody (1:200, ab177487, Abcam, Cambridge, MA, USA) and secondary antibody (1:400, B100802, Wuhan Baiqiandu Technology, Wuhan, China). We used TUNEL staining in accordance with the manufacturer's
recommendations. The TdT incubation buffer (ddH2O 34 mL, 5 Equilibration Buffer 10 mL, Alexa Fluor 647-12-dUTP Labeling Mix 5 mL, Recombinant TdT Enzyme 1 mL) was applied to hydrated and permeable cross cryosections for 60 min at 37°C. Following incubation, slices were washed 3 times in PBS, after which DAPI was used to stain the nuclei.

2.10 Immunofluorescence

For Immunofluorescence, deparaffinized brain sections were incubated with 3% BSA for 30 min after blocked with 0.3% H2O2-PBS methanol for 10 min. Sections were incubated with anti-pSTAT3 antibody (1:100, Cell Signal Technology, 4113) overnight at 4°C, then 2 hours of room temperature incubation with the subsequent second antibody: anti-pJAK2 antibody (1:100, Cell Signal Technology, 3776), GFAP antibody (1:100, Cell Signal Technology, 80788), Iba1 antibody (1:100, Cell Signal Technology, 17198), MAP2 antibody (1:50, Cell Signal Technology, 4542), NeuN antibody (1:200, ab177487, Abcam). Additionally, the staining was visualized using 3,3'-diaminobenzidine (DAB, D8001, St. Louis, MO, USA). The inverted fluorescent microscope (Olympus, IX73, Japan) was used to capture the staining pictures.

2.11 Neurological Severity Scores

The modified neurological severity score (mNSS) test, which has been previously described, was performed on the rat [24]. These tests, which mimic human contralateral neglect tests, are a mix of motor, sensory, reflex, and balance assessments. On a scale of 0 to 18, neurological function was rated; the greater the score, the more serious the injury.

2.12 Beam Walk Test

To assess fine motor coordination, the beam walk test was used. As the animal traversed a (10 × 2 cm) beam, it was timed. At one end of the beam, an enclosure was set up so the animal might feel secure. The animal was encouraged to approach and enter the box by making a loud noise. The amount of time it took the rat to enter the box determined the score. The degree of the neurological deficiency is inversely correlated with the score.

2.13 Adhesive-Removal Test

The modified sticky-tape (MST) test was used to assess forelimb function. Briefly, created a sleeve with a 3.0 x 1.0 cm piece of yellow paper tape, wrap it around the front paw, secure it with tape, and stick the finger just barely out of the sleeve. Rats often react by making a determined effort to remove the sleeve with their mouth or the opposite front paw. 30 seconds were spent observing the rats after they were placed in the cage. The first timer was set up and operated continuously. The second timer was only activated when the animal tried to remove the tape sleeve. The performance of the right (unaffected) and left (affected) forelimbs was compared. Separate tests were conducted on the ipsilateral and contralateral limbs. Every test day, the test was administered three times, and the top two results from each attempt were averaged. The severity of the neurological deficiency correlated negatively with the score. [25].

2.14 Data analysis
The SPSS for Windows 21.0 software suite was used to conduct the statistical analysis. Image J software was used to analyze the pictures (National Institutes of Health, Bethesda, MD, USA). Student’s t-tests were used for comparisons between two groups, while ANOVA tests were used for comparisons of three or more groups. Differences with P values < 0.05 or lower were considered significant.

3 Results

3.1 Effect of piperine on apoptosis after I/R injury in vitro

To verify the effect of piperine on cerebral ischemia-reperfusion injury, SH-SY5Y cells pretreated with various concentrations of piperine for 2 hours underwent OGD for 4 hours and then reperfusion for 24 hours. CCK-8 (Fig. 1A) and LDH (Fig. 1B) assays showed that 25 µM piperine mitigated the injury induced by OGD. We extracted proteins from cells pretreated with 25 µM piperine for WB analysis and found that piperine reduced the expression of p-JAK2 after I/R injury (Fig. 1C&D). Furthermore, single or combined use of piperine and JAK2 inhibitor AG490 reduced nerve cell death after OGD (Fig. 1E&F).

3.2 Piperine reduces apoptosis after I/R injury by regulating the JAK2/STAT3 pathway in vitro

Western blotting was used to assess changes in caspase 3, p-JAK2, and p-STAT3 protein levels after pretreatment with piperine and AG490 (Fig. 2). After OGD, expression of caspase-3, p-JAK2, and p-STAT3 were increased, and piperine, AG490, or both significantly reduced the expression of caspase-3, p-JAK2, and p-STAT3 (p < 0.05).

3.3 Piperine reduces the infarction volume and neuronal loss after I/R injury

To examine the effect of piperine on cerebral I/R injury in vivo, TTC staining was conducted to analyze the cerebral infarction volume (Fig. 3A-B). Compared with the control group (28.35%), the infarct volume was 10.17% in the piperine group (p < 0.05), 12.73% in the AG490 group (p < 0.05), and 9.74% in the piperine + AG490 group (p < 0.01).

Histopathological changes of all groups were revealed by HE and Nissl staining (Fig. 3C). In the sham group, there were no histopathological changes and neurons were round, well structured with clear cytoplasm, a visible nuclear structure, and even distribution of chromatin and the Nissl structure was regular. In the control group, the structure of the Nissl body was irregular, nucleus had pyknosis, the nucleolus was deeply stained, chromatin was condensed, and necrosis was observed. After treatment with piperine and/or AG490, necrotic cells were decreased, the Nissl body had a complete outline, and chromatin was relatively distributed evenly.

3.4 Piperine reduces neuronal apoptosis after I/R injury in vivo
Assessing neuronal apoptosis was done using TUNEL staining. No obvious apoptosis was seen in the sham group, whereas neuronal apoptosis was increased significantly after cerebral I/R injury. After administration of piperine and/or AG490, the proportion of apoptotic cells was decreased, and the combination of piperine and AG490 had a better effect (Fig. 4).

### 3.5 Piperine promotes recovery of nerve functions in rats after I/R injury

Next, we analyzed the effect of piperine on long-term recovery of brain nerve function at 1 day, 3 days, 1 week and 2 weeks after I/R injury. We selected a set of commonly used scoring criteria to measure motor, sensory, reflex, balance, forelimb condition and complex neurological function in rats. The mNSS (Fig. 5A), balance beam-walking test (Fig. 5B) and adhesive strip removal test (Fig. 5C) were included. Piperine and AG490 treatment groups showed significantly lower mNSS at days 7 and 14 after MCAO compared to the control group (Fig. 5A), as well as lower beam-walking test scores at days 3, 7, and 14 after MCAO and a higher ratio on the MST test at days 7 and 14 after MCAO (Fig. 5C). Additionally, the piperine + AG490 treatment group had higher ratios of the MST test at days 7 and 14 after MCAO and significantly lower mNSS and scores in the beam-walking test at days 3, 7, and 14 after MCAO (Fig. 5A-C).

### 3.6 Piperine exerts a neuroprotective effect after I/R injury by regulating the JAK2/STAT3 pathway in vivo

Western blotting was then used to analyze the levels of caspase 3, p-JAK2, and p-STAT3 in protein isolates from the ischemic penumbra. The protein expression of p-JAK2, p-STAT3, and caspase 3 was higher in the control group as compared to the sham group. Compared with the control group, protein expression of p-JAK2, p-STAT3, and caspase-3 was significantly decreased in piperine, AG490, and piperine + AG490 groups (Fig. 6).

Double immunofluorescence labeling of p-JAK2 and p-STAT3 also showed that their expression was increased after cerebral ischemia, whereas piperine and AG490 reduced the expression of p-JAK2 and p-STAT3 (Fig. 7), which was consistent with WB results. Additionally, p-JAK2 and p-STAT3 positive cells are mostly located in the nucleus.

### 3.7 Piperine mainly plays a regulatory role in neuronal cells

Next, we double labeled p-STAT3 with astrocyte marker glial fibrillary acidic protein, microglial marker ionized calcium binding adaptor molecule-1 (Iba-1), neuron dendritic marker microtubule-associated protein-2, and neuron marker NeuN (Fig. 8), and found that p-STAT3 essentially overlapped with NeuN.

### 4 Discussion

In adults, stroke is the most common cause of mortality and long-term disability, and over 80% of strokes are ischemic [26]. The major goals of modern ischemic stroke therapies are to restore blood flow through
mechanical thrombectomy or TPA thrombolysis. However, because of the small time window, only a low percentage of patients can receive these treatments, which associated with an increased risk of symptomatic intracerebral hemorrhage [27]. Therefore, finding a viable treatment for people who have had an ischemic stroke is urgent. Our previous research established that piperine has neuroprotective effects by reducing cerebral I/R damage and the cerebral infarction volume after MCAO. Additionally, isobaric tags for relative and absolute quantitation-based proteomics identified numerous significantly differentially expressed proteins [19], but no underlying mechanism was explored. In this work, we found that cerebral ischemia activated the JAK2/STAT3 signaling pathway, whereas piperine therapy blocked the JAK2/STAT3 signaling pathway, decreased the volume of cerebral infarction and neuronal death, and helped MCAO rats recover their long-term functional abilities (Fig. 9).

Pepper is a traditional Chinese herbal medicine used for arthritis, bronchitis, gastritis, diarrhea, snake bite, menstrual pain, fever, and bacterial infections [13], these are also recorded in the traditional Chinese medicine classic Tang Ben Cao (pinyin in China) and Ben Cao Gang Mu (pinyin in China). Its functions may be related to the anti-inflammatory and antioxidant effects of its main component, piperine (1-peperoylpiperidine). As a natural aromatic hydrocarbon, piperine has good safety and bioavailability. Using the modern drug safety evaluation system, Bastaki et al. performed a 90-day GLP compliance toxicity study in SD rats to evaluate the safety of daily intake of piperine in food using multiple doses of piperine (5–50 mg/kg body weight /day). It should be noted that no adverse reactions were observed even after administration of piperine at the highest dose (50 mg/kg body weight /day), indicating that piperine has good biosafety [28]. Furthermore, the blood–brain barrier is the main barrier for drug delivery to the central system. Piperine is quickly absorbed into plasma and through the brain whether orally or intraperitoneally administered and has a very high absorption rate [18]. Piperine has important medicinal properties including anti-tumor, anti-hepatotoxic, anti-diarrheal, anti-depressant, analgesic, and immunomodulatory effects [29, 30]. Another important clinical application is that piperine enhances the solubility and bioavailability of many drugs [31, 32]. The pharmacological properties of piperine have garnered interest for the treatment of metabolic and gastrointestinal disorders.

In a study of cerebral ischemic diseases, piperine inhibited the expression of COX-2, NOS-2, and nuclear factor-kappa B in a MCAO rat model [17] and exerted beneficial neuroprotective effects by inhibiting protein expression of caspase 3 and 9 [33]. Kaushik et al. found that piperine reduces mitochondrial dysfunction and neurological impairment, and enhances neuronal survival during ischemic stroke [18]. In a prior investigation, we found that piperine controls the complement and coagulation pathways to prevent cerebral ischemia injury [19]. The neuroprotective properties of piperine were validated in the current investigation, and it was discovered that these properties may be correlated with suppression of the JAK2/STAT3 signaling pathway.

Excitotoxicity, oxidative stress, inflammation, and apoptosis-like cell death are all components of the complicated mechanism underlying cerebral I/R damage [34]. The occurrence and progression of cerebral ischemia are significantly influenced by the JAK2/STAT3 signaling pathway. Numerous studies have demonstrated that the JAK2/STAT3 signaling pathway participates in the development of the central
nervous system, including nerve cell proliferation, survival, and differentiation. This pathway is largely inactive in the central nervous system under physiological settings. Additionally, the JAK2/STAT3 signaling pathway is activated under ischemic stimulation and participates in pathological processes such as apoptosis, inflammatory responses, vascular remodeling, oxidative stress, and autophagy [35]. After cerebral ischemia, high levels of proinflammatory substances including IL-1, IL-6, and TNF-JAK2 stimulate JAK2 and its downstream STAT3 signaling. Binding of proinflammatory factors to their receptors induces receptor-related JAK2 phosphorylation and leads to the phosphorylation of downstream Stat3. Subsequently, two phosphorylated STAT3 dimers are transferred to the nucleus to regulate the expression of corresponding inflammatory target genes and aggravate cerebral ischemia-reperfusion injury [36, 37]. Previous studies including our own have shown that inhibition of the JAK2/STAT3 signaling pathway by JAK2 inhibitor AG490 or other drugs inhibits the inflammatory response and reduces cerebral ischemic injury [38, 39]. Activation of the JAK2/STAT3 pathway in MCAO rat. However, some studies have reached the opposite conclusion. Dong et al. discovered that IL-22 therapy raises JAK2 and STAT3 phosphorylation levels, lowering oxidative stress and neuronal death in mice following brain I/R injury. IL-22 also has a protective effect on the brain [40], and studies have also shown that large doses of diosmin protect against brain I/R injury by activating the JAK2/STAT3 signaling pathway [41]. This difference in experimental results may be related to differences in the inhibitor, dosage, and time of administration.

The main focus of the current investigation was on determining how neuronal death in the ischemic penumbra affected the prognosis of cerebral ischemia. Following cerebral ischemia, neurons in the ischemic core area die within minutes, while this process takes several hours in the penumbra area [42]. Although neurons in the penumbra function abnormally, neuronal cells remain alive. Improvement in the neuronal status and function in the ischemic penumbra directly affects stroke prognosis [43]. Our findings suggested that piperine improved the prognosis of cerebral ischemia by lowering neuron death and inflammatory factor expression in the penumbra.

AG490 is a type of tyrosine kinase receptor inhibitor that specifically inhibits the JAK2/STAT3 signaling pathway [44]. In neural cells, AG490 provides robust protection against cytokine and peroxide-induced cell death by targeting the JAK2/STAT3 signaling pathway [45]. In a transient MCAO rat model, AG490 induces significant neuroprotection and improves neurological recovery [36]. Our previous study also showed that AG490 reduces brain water content, decreases BBB permeability, and improves neurological functions [38]. Our current data showed that piperine had a similar effect to AG490. After piperine pretreatment, p-JAK2 and p-STAT3 were decreased, which reduced the cerebral infarction volume and promoted recovery of neurological functions. These data indicate that piperine is a potential inhibitor of the JAK2/STAT3 signaling pathway.

In conclusion, our study demonstrates that piperine pretreatment inhibits neuronal apoptosis, reduces the cerebral infarct volume, and contributes to amelioration of neurological deficits, which may be related to the inhibitory effect of piperine on the JAK2/STAT3 signaling pathway. We will investigate the protective mechanism of piperine against cerebral I/R injury in more detail in a forthcoming investigation. Taken
together, our current research suggests that piperine may represent a potential therapeutic alternative for the management of cerebral I/R injury.

Declarations

Consent for Publication

All the authors read the manuscript carefully and gave their consent for publication.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Ethics approval: Not applicable

Consent to participate Not applicable

Consent for Publication Not applicable

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Competing interests The authors declare no competing interests.

References


Figures
Figure 1

Piperine increased cell activity and decreased p-JAK2 expression. a) CCK-8 showed that piperine (10-100 μM) increased neuronal survival rate in OGD-induced neuronal injury. b) Piperine (10-100 μM) could reduce OGD-induced increase of LDH release (n = 5, *P < 0.05 vs. 0 μM, **P < 0.01 vs. 0 μM). c+d) Western blots showed that piperine (25 μM) could inhibit JAK2 phosphorylation (n = 5, *P < 0.05 vs. Ctr). e) CCK-8 showed that piperine (25 μM) and AG490 increased survival rate in OGD-induced neuronal injury.
injury. f) Piperine (25 μM) could reduce OGD-induced increase of LDH release. (n = 5, *P < 0.05 vs. Ctr, **P < 0.01 vs. Ctr)

**Figure 2**

Administration of piperine (25 μM) reduced the expression of p-JAK2 and p-STAT3 in SH-SY5Y cells after OGD/R. a) Western blots showed that piperine and AG490 decrease the expressions of P-JAK2, P-STAT3 and Caspase3 in SH-SY5Y cells after OGD. b-d) Quantification results of P-JAK2, P-STAT3 and Caspase3 protein levels. (n = 5, *P < 0.05, vs. Ctr, **P < 0.01, vs. Ctr)
Figure 3

Piperine reduced the volume of cerebral infarction, alleviated the neurologic deficits and protected the neurons against I/R injury. a) Representative rat brain slices stained with TTC, unstained areas indicate injured tissue. b) Quantification of the infarct volumes in rat with 2-h tMCAO and 24-h reperfusion (n = 5 mice per experimental group, *P < 0.05, vs. Ctr, **P < 0.01, vs. Ctr). c) Nissl and HE staining showed apparent brain edema, abnormal neurons, and irregularly arranged Nissl substance after cerebral infarction, the use of piperine and/or AG490 can reduce brain edema and increase the survival of neurons.
and the number of Nissl substance (40x magnification, scal bar = 100 μm, Arrowheads indicate necrotic nucleis).

Figure 4

Piperine repressed apoptosis in the brain after I/R injury. TUNEL and NeuN immunofluorescence double staining suggested that piperine and / or AG490 could reduce neuronal apoptosis after cerebral ischemia-reperfusion injury (40x magnification, scal bar = 100 μm).
Figure 5

**Piperine improved neurological function in ischaemia–reperfusion injury.** a) Compared with the control group, the modified neurological severity score (mNSS) of PIP+AG490 group decreased significantly at days 3, 7 and 14 after MCAO, and the scores of PIP group and AG490 group decreased significantly at days 7 and 14 after MCAO; b) Compared with the control group, the piperine and / or AG490 pretreatment group had lower scores in the beam-walking test at days 3, 7 and 14 after MCAO. c) Compared with the
control group, animals pre-injected with piperine and/or AG490 had a higher ratio in the modified sticky-tape (MST) test at days 7 and 14 after MCAO. (n = 7, *P < 0.05, PIP vs. Ctr, #P < 0.05, AG490 vs. Ctr, &P < 0.05, PIP+AG490 vs. Ctr, &&P < 0.01, PIP+AG490 vs. Ctr)

Figure 6

Piperine increased the expression of Caspase3 and inhibited the JAK2/STAT3 signal pathway in vivo. a) Western blots showed the expressions of t-JAK2, p-JAK2, t-STAT3, p-STAT3, Caspase3 in different groups. The levels of p-JAK2, p-STAT3 and Caspase3 increased after I/R injury, which were reduced by piperine and/or AG490. b-d) Quantified levels of p-JAK2, p-STAT3 and Caspase3 in different groups, t-JAK2, t-STAT3 and GADPH were used as loading controls respectively (n = 5, *P < 0.05, vs. Ctr, **P < 0.01, vs. Ctr).
Figure 7

Immunofluorescence double labeling showed that piperine could inhibit the JAK2/STAT3 signal pathway.

a) Immunofluorescence double staining showed that p-JAK2 and p-STAT3 positive cells increased after cerebral I/R injury, which were reduced by PIP and/or AG490. b, c) Quantified levels of p-JAK2 and p-STAT3 positive cells in different groups. (n = 5, *P < 0.05, vs. Ctr, 40x magnification, scal bar = 100 μm)
**Figure 8**

**P-STAT3 is mainly expressed in NeuN.** a-d) Immunofluorescence double staining of P-STAT3 with astrocyte activation marker GFAP, microglia marker Iba-1, neuron cytoskeleton marker MAP2 and neuron marker NeuN, which found that p-STAT3 mainly existed in neurons (40x magnification, scal bar = 100 μm).
Figure 9

**Schematic summary.** After cerebral ischemia-reperfusion injury, the JAK2/STAT3 signaling pathway is activated to promote the release of inflammatory cytokines and apoptotic factors, resulting in neuronal cell death. Piperine can inhibit the JAK2/STAT3 signaling pathway by inhibiting the phosphorylation of JAK2, thereby inhibiting neuronal apoptosis and reducing reperfusion injury.

**Supplementary Files**

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