Neurochemistry International 124 (2019) 51–61

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Quantitative iTRAQ-based proteomic analysis of piperine protected cerebral ischemia/reperfusion injury in rat brain

Yichun Zou[a], Pian Gong[b], Wenyuan Zhao[a], Jianjian Zhang[a], Xiaolin Wu[a], Can Xin[a], Zhongwei Xiong[a], Zhengwei Li[a], Xiaohui Wu[a], Qi Wan[c], Xiang Li[d], Jincao Chen[a,e,*]

[a] Department of Neurosurgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, 430071, China
[b] Department of Neurosurgery, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, China
[c] Institute of Neuroregeneration and Neurorehabilitation of Qingdao University, Qingdao, Shandong, 266071, China
[d] Queensland Brain Institute of the University of Queensland, St Lucia, Queensland, Australia
[e] Department of Neurosurgery, Tongji Hospital of Huazhong University of Science and Technology, Wuhan, Hubei, 430030, China

ARTICLE INFO

Keywords:
Complement and coagulation cascade
iTRAQ
Proteome
Piperine
Ischemia penumbra

ABSTRACT

Piperine is the key bioactive factor in black pepper, and has been reported to alleviate cerebral ischemic injury. However, the mechanisms underlying its neuroprotective effects following cerebral ischemia remain unclear. In this study, rats were administered vehicle (dimethyl sulfoxide) or piperine, 20 mg/kg, daily for 14 days before focal cerebral artery occlusion. After occlusion for 2 h followed by reperfusion for 24 h. Histological examinations were used to assess whether piperine has a neuroprotective effect in the rat model of cerebral ischemia/reperfusion injury. The levels of proteins in the ischemic penumbra were evaluated by isobaric tags for relative and absolute quantitation-based proteomics. A total of 3687 proteins were identified, including 23 proteins that were highly significantly differentially expressed between the control and piperine groups. The proteomic findings were verified by immunofluorescence and western blot analysis. Interestingly, piperine administration downregulated a number of critical factors in the complement and coagulation cascades, including complement component 3, fibrinogen gamma chain, alpha-2-macroglobulin, and serpin family A member 1. Collectively, our findings suggest that the neuroprotective effects of piperine following cerebral ischemia/reperfusion injury are related to the regulation of the complement and coagulation cascades.

1. Introduction

Stroke is the second leading cause of death and most common cause of disability worldwide (Bronner et al., 1995). In 2010, 16.9 million people suffered their first stroke, and there were 5.9 million stroke-related deaths (Feigin et al., 2014). These numbers represented significant increases from 1990, by 68% and 26%, respectively. Currently, rapid restoration of vascular flow is the only effective therapy for acute stroke treatment (Meairs et al., 2012). However, this treatment is limited by a narrow therapeutic time window (Donnan et al., 2008). Penumbra is a research hotspot in cerebral ischemia and this region represents a much larger volume of brain tissue than the ischemic core, and may be rescued with timely treatment, resulting in a reduction in post-stroke disability. To date, the majority of stroke studies have focused on the inflammatory and oxidative stress mechanisms related to cerebral ischemia/reperfusion (I/R) injury (Lakhan et al., 2009; Margaill et al., 2005). However, there is evidence for important roles of the complement and blood coagulation systems in cerebral I/R injury (Oikonomopoulou et al., 2012; Wang et al., 2018). Indeed, hypercoagulability is an important prognostic factor in stroke (Cote et al., 2000).

Black pepper is widely used in food spices and traditional herbal medicine, as an antipyretic and anti-inflammatory agent, and in treatment for epilepsy and snake venom poisoning (Szallasi, 2005). Piperine (1-peperoylpiperidine) is the key active factor in black pepper, and a number of recent *in vitro* and *in vivo* studies have reported that it can prevent the growth of breast cancer cells by inhibiting cell cycle

Abbreviations: A2M, alpha-2-macroglobulin; Apoa1, apolipoprotein A1; C3, complement component 3; DEPs, differentially expressed proteins; FGG, fibrinogen gamma chain; GO, gene ontology; HE, Hematoxylin-eosin; I/R, ischemic/reperfusion; iTRAQ, isobaric tags for relative and absolute quantification; KEGG, kyoto encyclopedia of genes and genomes; MAP2, microtubule-associated protein 2; PPI, protein–protein interaction; TF, transferring; TTC, triphenyltetrazolium chloride; TUNEL, transferase Mediated dUTP Nick End Labeling

* Corresponding author. Department of Neurosurgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, 430071, China.
E-mail address: chenjincao2012@hotmail.com (J. Chen).

https://doi.org/10.1016/j.neuint.2018.12.010
Received 8 October 2018; Received in revised form 26 November 2018; Accepted 20 December 2018
Available online 21 December 2018
0197-0186/

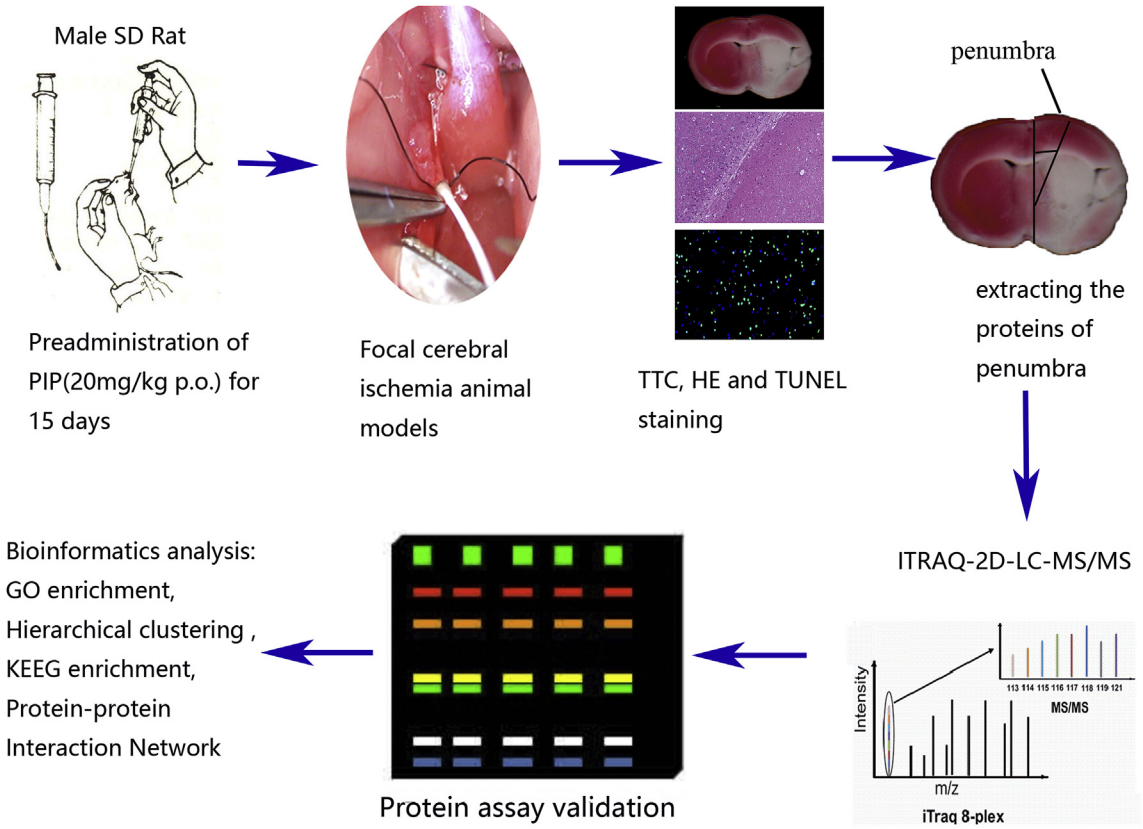

Fig. 1. Overall workflow of the histological analysis, protemics and verification research of the effects of piperine (PIP) on cerebral ischemia/reperfusion (I/R) injury.

progression, inducing activation of mitochondrial caspase-dependent apoptosis, and decreasing cell migration by reducing matrix metalloproteinase 2 and matrix metalloproteinase 9 expression (Greenshields et al., 2015). Piperine was also reported to significantly inhibit lipopolysaccharide-induced nuclear factor kappa B activation in acute lung injury (Lu et al., 2016), and to decrease cellular oxidative stress induced by a high-fat diet (Vijayakumar et al., 2004). Furthermore, piperine exerts anticonvulsant effects by increasing striatal gamma aminobutyric acid levels (Da et al., 2013), and reduces astrocyte activation in a chemical kindling model of epilepsy (Anissian et al., 2018).

In cerebral ischemic stroke, piperine was reported to significantly improve neurobehavioral deficits and reduce proinflammatory cytokine expression (Vaibhav et al., 2012). In other ischemic diseases, pretreatment with piperine protects against myocardial ischemia via its antioxidant activity (Dhivya et al., 2017), while intraperitoneal piperine administration just prior to reperfusion can reduce I/R injury to the small intestine (Akyildiz et al., 2013).

Despite these studies supporting a neuroprotective effect of piperine, there are no comprehensive proteomic analyses of the mechanisms of action of the drug. Thus, in the present study, we performed isobaric tags for relative and absolute quantification (iTRAQ)-based proteomics to examine the proteome-wide changes in the cerebral ischemic penumbra after treatment with piperine following cerebral I/R injury.

2. Methods and materials

2.1. Animals

Male Sprague-Dawley rats (250–280 g) were group housed with 2–3 rats per cage on a 12 h light/dark cycle in a temperature-controlled room (23–25 °C) with free access to food and water. All animal use and experimental protocols were approved and carried out in compliance with the IACUC guidelines and the Animal Care and Ethics Committee of Wuhan University School of Medicine. Randomization was used to assign samples to the experimental groups and to collect and process data. The experiments were performed by investigators blinded to the groups for which each animal was assigned. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2011; McGrath and Lilley, 2015). For our study, we designed three groups: sham group, control group and piperine group. Piperine for 20 mg/kg (Sigma, P49007, St. Louis, MO, USA) was given to the rats of piperine group once daily for 15 days, and the concentration of piperine is according to Vaibhav's study (Vaibhav et al., 2012). Vehicle (dimethyl sulfoxide) was given to the sham and control group.

2.2. Transient focal cerebral ischemia and tissue extraction of penumbra

Male SD rats weighing 250–280 g, were anesthetized with 4% isoflurane in 70% N_2O and 30%O_2 by using a mask. A midline incision was made in the neck, the right external carotid artery (ECA) was carefully exposed and dissected and a 3–0 monofilament nylon suture was inserted from the ECA into the right internal carotid artery to occlude the origin of the right middle cerebral artery (approximately 22 mm). After 120 min of occlusion, the suture was removed to allow reperfusion, the ECA was ligated and the wound was closed (Zhang et al., 2017). Sham-operated rats underwent identical surgery and isoflurane anesthesia except that the suture was inserted and withdrawn immediately. Rectal temperature was maintained at 37.0 ± 0.5 °C using a heating pad and heating lamp. After 24 h of reperfusion, rats were killed after being anesthetized. Brains were removed and stored in −20 °C for serveral hours. Then the penumbra tissue were defined and separated according to the method of Ashwal (Ashwal et al., 1998).

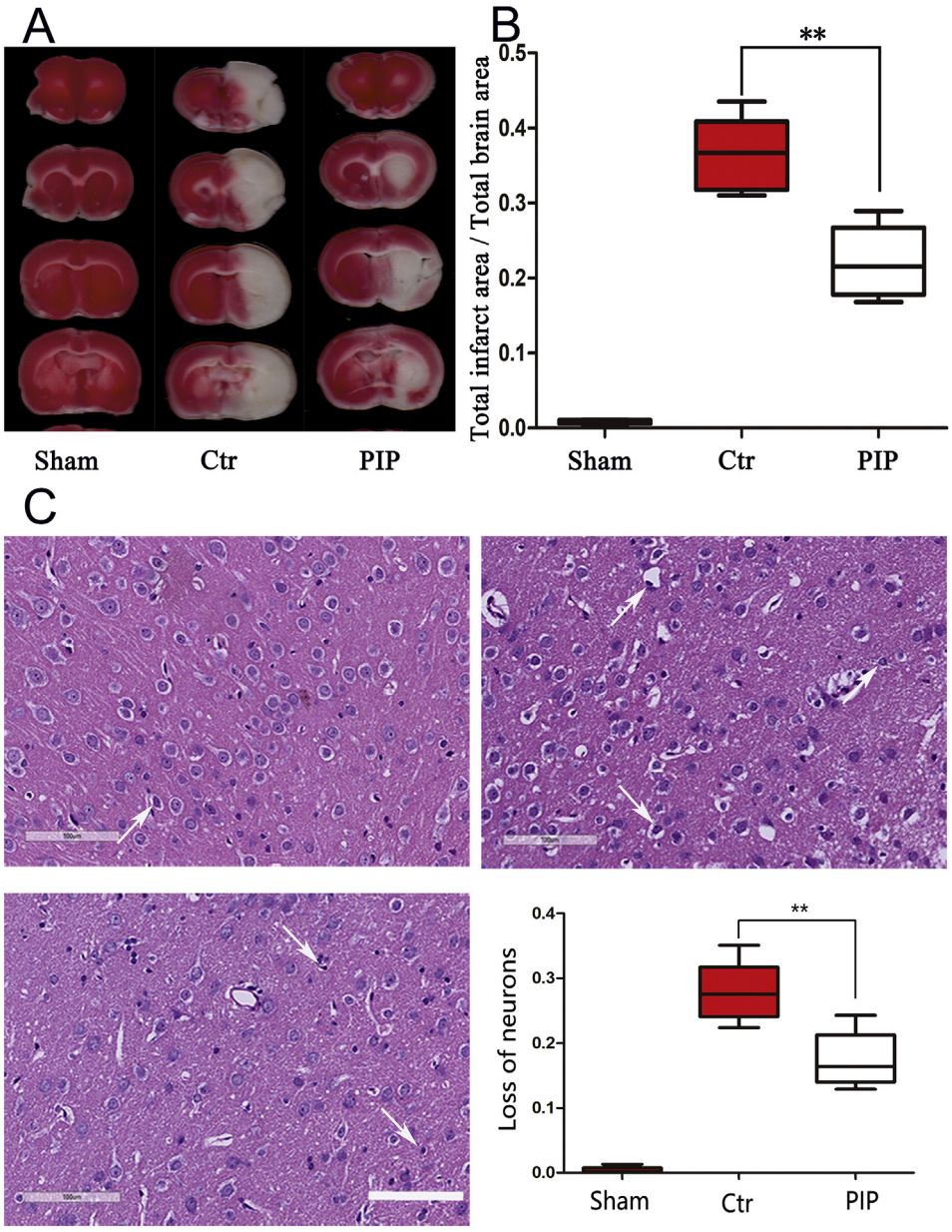

Fig. 2. Histological study of the neuroprotective effect of PIP on cerebral I/R injury. (**A, B**) TTC staining showing that PIP treatment significantly decreased the infarct volume (36.38%) 24 h after ischemia, compared with the control group (22.1%; $^{**}p < 0.01$, $n = 5$ per group). (**C**) HE staining showing that PIP treatment significantly reduced neuronal loss (17.38%) compared with the control group (27.84%) ($^{**}p < 0.01$, $n = 5$ per group). Arrowheads indicate necrotic nuclei. Scale bar, 100 μm. PIP, piperine group; Ctr, control group.

2.3. Measurement of infarct volume (TTC)

2,3,5,6-triphenyltetrazolium chloride (TTC, Sigma, V900570, St. Louis, MO, USA) staining was performed to measure the infarct size. After 24 h of reperfusion, rats were deeply anesthetized with 1% pentobarbital sodium (50 mg/kg, I.P.), then, they were rapidly injected with 150 ml 0.9% saline form heart. Their brains were removed and cut into 8 cerebral coronary slices by BRAIN SLICERS (2 mm). The slices were immediately incubated in 2% TTC at 37 °C for 30 min, and then fixed in 4% paraformaldehyde overnight at 4 °C. Next day the brain slices were scanned by scanner (Microtek Phantom V700 Plus). The infarct volume percentage was calculated as the infarct area of the ipsilateral hemisphere/total area of ipsilateral hemisphere. Student's t-tests were used for comparisons between control and piperine groups. Difference with P values < 0.05 were considered significant.

2.4. Hematoxylin-eosin staining

Hematoxylin-eosin (HE) staining was used to evaluate the neuroprotective effect of piperine against I/R injury. The brains were removed and fixed in 4% paraformaldehyde to do paraffin-embedded sections (4 μm). For HE staining, the sections were deparaffinized, hydrated, stained with hematoxylin for 3 min, 1% hydrochloric acid alcohol differentiated for 1s, and stained with eosin for 3 s. The stained sections were dehydrated with neutral resin and scanned by Aperio VERSA 8 (Leica, Germany). Image J was used to analysis the neuronal loss. Student's t-tests were used for comparisons between control and piperine groups. Difference with P values < 0.05 were considered significant.

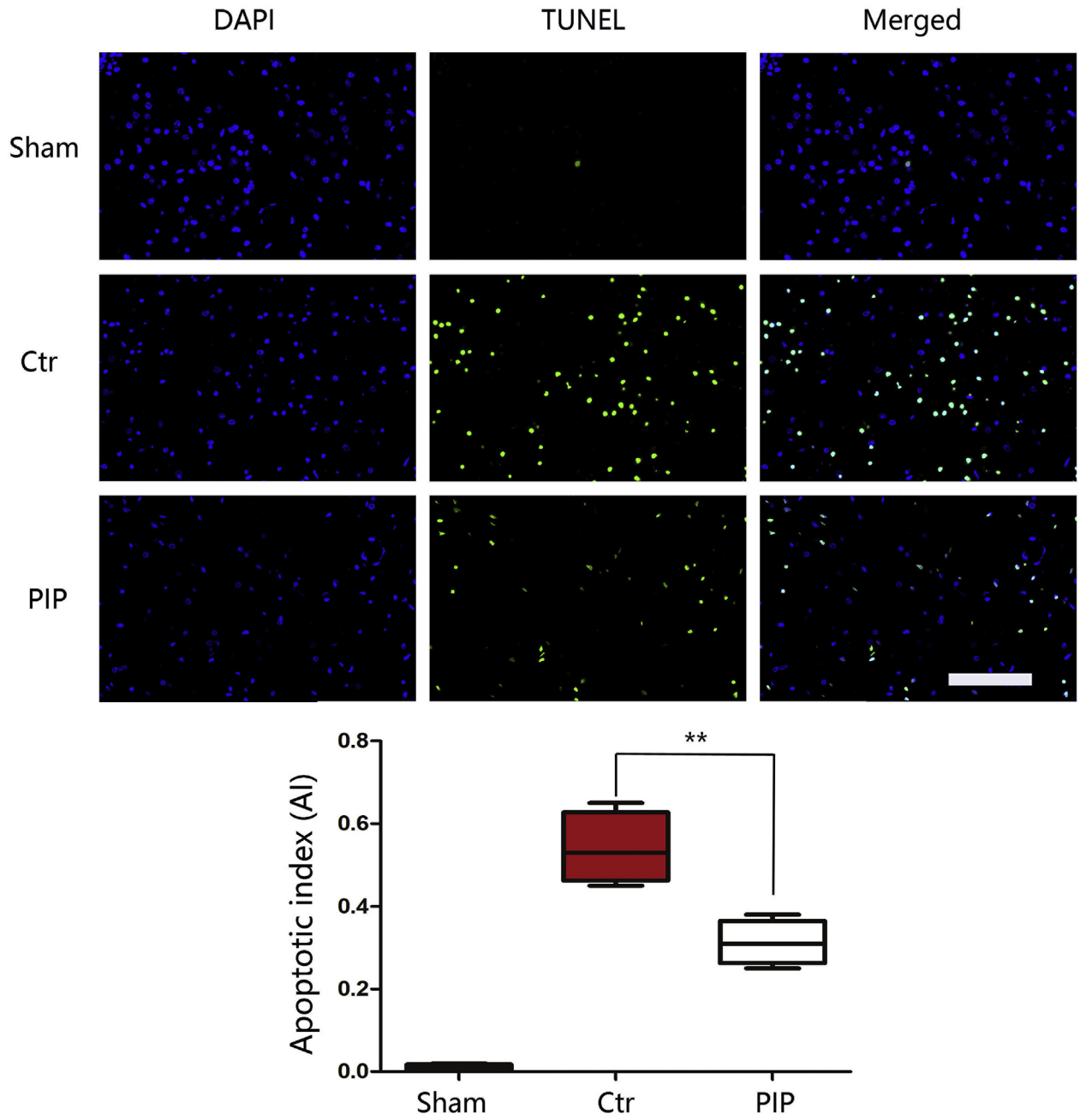

Fig. 3. TUNEL staining for cell death in the rat brain. The PIP group showed a lower apoptotic index (31.25%) than the control group (54%). (**$p < 0.01$, n = 4 per group). Scale bar, 100 μm.

2.5. TUNEL and immunofluorescence

For Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) staining, conversion reagent was added according to the manufacturer's instructions. Hydrated and permeabilized cross cryosections were treated for 1 h at 37 °C in TdT incubation buffer (ddH2O 34 μL, 5 × Equilibration Buffer 10 μL, Alexa Fluor 647-12-dUTP Labeling Mix 5 μL, Recombinant TdT Enzyme 1 μL). After incubation, sections were washed with PBS for 3 times, the nuclei were labeled with DAPI. The TUNEL-positive cells were evaluated using a microscope and quantified under high-power magnification (× 400), and the apoptotic index (AI) of each sample was equal to the mean value of each visual field marker index.

For Immunofluorescence, deparaffinized sections were endogenous peroxidase inhibited with 0.3% H_2O_2 in PBS-3% methanol for 10 min, incubated with 3% Bovine Serum Albumin (BSA, sigma, B2064, St, Louis, MO, USA) for 30min. Sections were incubated with the primary antibody MAP2 (Servicebio, GB11128-2, CHN) overnight at 4 °C, then sequentially incubated with the second antibody at room temperature for 30 min. 3,3'-diaminobenzidine(DAB,D8001, St. Louis, MO, USA) was used to visualize the staining. The staining images were taken by inverted fluorescence microscope(Olympus,IX73,Japan).

2.6. Sample preparation

Lysis buffer (2% SDS, 7 M urea, 1x Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland)) was added into the ischemic penumbra samples. The lysis was performed by sonication on ice for 3 min and kept on ice for 30 min. After centrifugation at 15000 rpm for 15 min at 4 °C, the supernatant was collected and transferred to a new Eppendorf

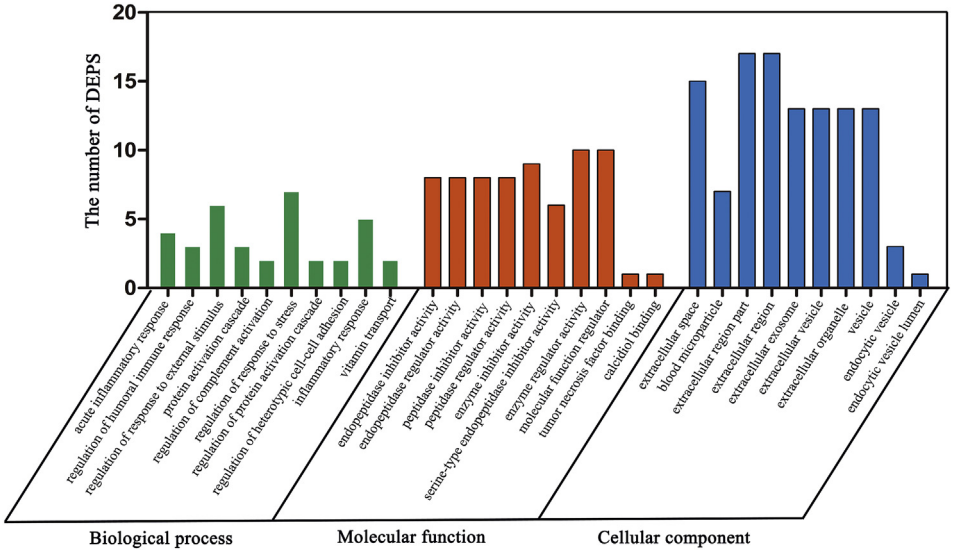

Fig. 4. Gene ontology enrichment analysis of differentially expressed proteins (DEPs) between the piperine group and control group (n = 3 per group).

tube.

2.7. Proteindigestion and iTRAQ labeing

Proteins were redissolved in 500 mM triethylammonium bicarbonate (TEAB). Determine the protein concentration of the supernatant using the BCA protein assay, and then transfer 100 μg protein per condition into a new tube and adjust to a final volume of 100 μL with 8M ureas. Add 11 μL of 1M DTT and incubate sample at 37 °C for 1 h, then transferred into 10K ultrafiltration tube (Millipore). To remove urea, samples were centrifuged by adding 100 Mm TEAB for three times. 120 μL of 55 mM iodoacetamide was added to the sample and incubate for 20 min protected from light at room temperature. Then proteins were tryptic digested with sequence-grade modified trypsin (Promega, Madison, WI). The resultant peptide mixture was labeled with iTRAQ 8-plex reagent (AB Sciex) following the manufacture's instruction. The labeled peptide samples were then pooled and lyophilized in a vacuum concentrator.

2.8. High PH reverse phaseseparation

The peptide mixture was redissovled in the buffer A (buffer A: 20 mM ammonium formate in water, pH10.0, adjusted with ammonium hydroxide), and then fractionated by high pH separation using Ultimate 3000 system (Thermo Fisher scientific, MA, USA) connected to a reverse phase column (XBridge C18 column, 4.6 mm x 250 mm, 5 μm, (Waters Corporation, MA, USA). High pH separation was performed using a linear gradient. Starting from 5% B to 45% B in 40 min (B: 20 mM ammonium formate in 80% ACN, pH 10.0, adjusted with ammonium hydroxide). The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 1 mL/min and column temperature was maintained at 30 °C. Twelve fractions were collected; each fraction was dried in a vacuum concentrator for the next step.

2.9. Low PH nano-HPLC-MS/MS analysis

The fractions were resuspended with 30 μl solvent C respectively (C: water with 0.1% formic acid; D: ACN with 0.1% formic acid), separated by nanoLC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on an Easy-nLC 1000 system (Thermo Fisher Scientific, MA, USA) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) equipped with an online nano-electrospray ion source. 10 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm x 2 cm), with a flow of 10 μl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm x 15 cm) with a linear gradient, from 3% D to 32% D in 120 min. The column was re-equilibrated at initial conditions for 10 min. The column flow rate was maintained at 300 nL/min. The electrospray voltage of 2 kV versus the inlet of the mass spectrometer was used.

The mass spectrometer was run under data dependent acquisition mode, and automatically switched under MS and MS/MS mode. MS1 mass resolution was set as 70K with m/z 300–1800 and MS/MS resolution was set as 17.5K under HCD mode. The dynamic exclusion time was set as 10 s.

2.10. Western blotting

Equal amount of total proteins of each sample was loaded and resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Under 200A, 90min, proteins were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), which were blocked in 5% non-fat milk/1x Tris Buffered Saline (TBS)-Tween20 immediately for 1 h. The membranes were incubated overnight at 4 °C with the following primary antibodies: fibrinogen gamma chain (Proteintech, 15841-1-AP, Wuhan, CHN), complement component 3 (C3) (Proteintech, 66157-1-Ig, Wuhan, CHN), transferrin (TF) (Proteintech, 66171-1-Ig, Wuhan, CHN) and Beta Actin (Proteintech, 60008-1-Ig, Wuhan, CHN). After washed with 1x TBS/Tween20, the membranes were incubated with second antibody for 1 h at room temperature. Protein bands were visualized using Tanon 5200 chemiluminescent imaging system (Shanghai).

2.11. Data analysis

Tandem mass spectra were processed by PEAKS Studio version 8.5 (Bioinfor Inc. CA). PEAKS DB was set up to search the Uniprot-rat database (ver.201708, 29978 entries) assuming the digestion enzyme Trypsin. PEAKS DB was searched with a fragment ion mass tolerance of 0.05 Da and a parent ion tolerance of 10 ppm. Carbamidomethylation was specified as a fixed modification. Oxidation (M), Deamidation (NQ), Acetylation (Protein N-term), were specified as variable modifications. Peptides were filter by 1% FDR and 1 unique peptide. ANOVA was used for peptide and protein abundance calculation. Normalization was performed on averaging the abundance of all

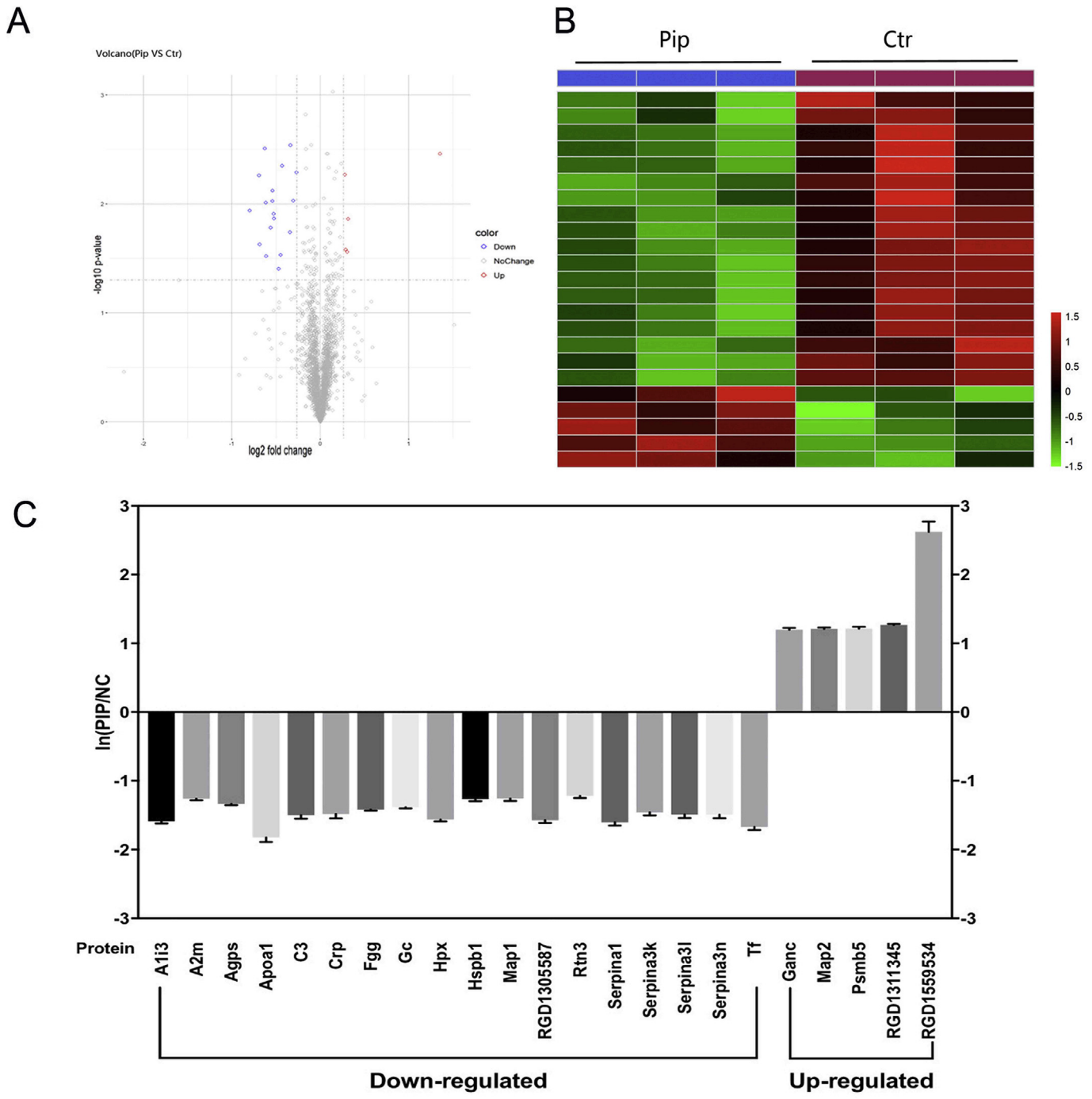

Fig. 5. DEPs analysis between the PIP group and control group (n = 3 per group). **(A)** Volcano plot of significant DEPs between the PIP and control group. Upregulated genes are shown in red, while downregulated genes are shown in blue ($p < 0.05$; absolute fold change > 1.2; unique peptides ≥ 1). **(B)** Hierarchical clustering of 23 proteins that were significantly changed (peptide threshold 1.0% FDR, 1 unique peptide). **(C)** Specific names and fold-change of the DEPs.

peptides. Medians were used for averaging. Different expressed proteins were filtered if their fold change were over 1.2 fold and contained 1 unique peptides with P (ANOVA test) below 0.05. The statistical analysis was performed with the SPSS for Windows 21.0 software package. The images were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA). Student's t-tests were used for comparisons between two groups. Difference with P values < 0.05 were considered significant.

3. Results

3.1. Piperine reduces ischemic brain injury

To evaluate the function of piperine in ischemic brain injury, rats were subjected to focal cerebral ischemia (Fig. 1). Their brains were collected for histological analysis. TTC and HE staining were performed to examine the neuroprotective effects of piperine (Fig. 2A and C). TTC stains normal brain tissue red, while stains the ischemic tissue white. Compared to the sham group, TTC staining in the control group revealed a significant volume of infarction (36.38%). Piperine treatment

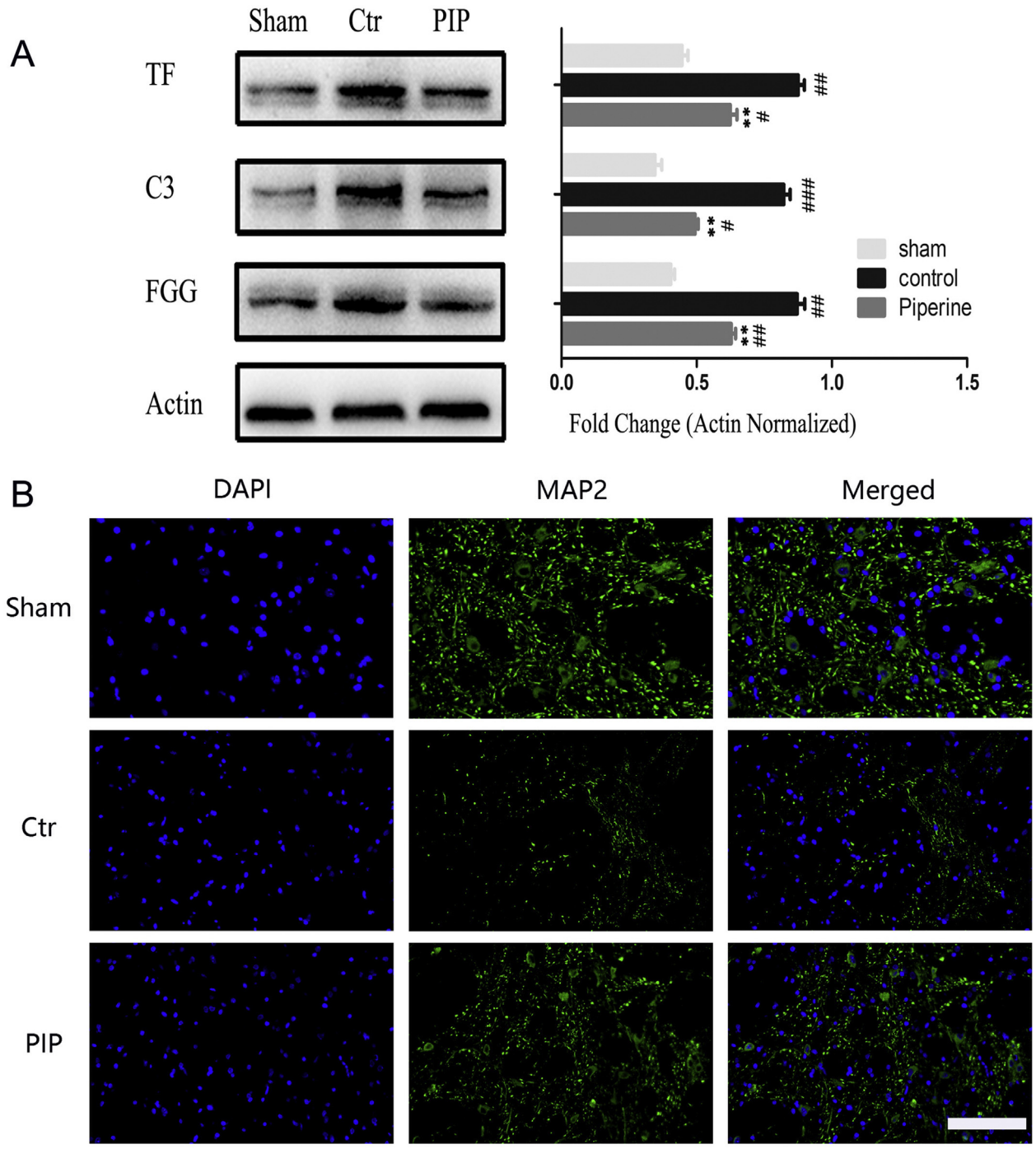

Fig. 6. Validation by western blotting and immunofluorescence. (A) Relative expression levels of TF, FGG and C3 between the PIP group and the control group in the cerebral ischemic penumbral tissue. Bar graphs show the densitometric analysis of the abundance of two DEPs in the two groups. The expression levels of TF, C3 and FGG were lower in the PIP group (**$p < 0.01$, vs. the control group; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$, vs. the sham group; $n = 5$ per group). **(B)** PIP pretreatment increased MAP2 expression ($n = 3$ per group). Scale bar, 100 μm.

significantly reduced infarct volume (22.1%; Fig. 2B). HE staining of sections of ipsilateral penumbral cortex showed less neuronal loss in the piperine group (17.38%) compared with the control group (27.84%; Fig. 2C). Furthermore, these results were consistent with TUNEL staining for cell death in the brain tissue (Fig. 3). Together, these findings suggested that piperine had neuroprotective effects in cerebral I/R injury.

3.2. Quantitative proteomics of the cerebral ischemic penumbra

Next, we used iTRAQ proteomics to provide insights into the molecular mechanisms underlying the neuroprotective action of piperine in the cerebral ischemic penumbra. A total of 30,762 unique and highly confident peptides were detected (confidence 95%, at less 2 unique peptides matched and FDR < 1%), and 3687 proteins were identified. Among the 3687 proteins, there were 23 significantly differentially expressed proteins (DEPs) (Fig. 5C) in the ischemic penumbra compared with the control group. Among the 23 proteins, 18 were

Table 1
The 10 main KEEG pathways and differentially expressed proteins.

Pathway Description	Pathway ID	Input number	P-Value	Corrected P-Value	Matching.ID
Complement and coagulation cascades	rno04610	3	5.9E-05	0.001123795	P02680 A0A0G2JY31 P06238
Vitamin digestion and absorption	rno04977	1	0.02302	0.082622038	P04639
Galactose metabolism	rno00052	1	0.0238	0.082622038	D4A7G5
Ether lipid metabolism	rno00565	1	0.0324	0.082622038	Q9EQR2
African trypanosomiasis	rno05143	1	0.0324	0.082622038	P04639
Fat digestion and absorption	rno04975	1	0.03395	0.082622038	P04639
Proteasome	rno03050	1	0.04014	0.082622038	P28075
Mineral absorption	rno04978	1	0.04246	0.082622038	P12346
Starch and sucrose metabolism	rno00500	1	0.04323	0.082622038	D4A7G5
VEGF signaling pathway	rno04370	1	0.04783	0.082622038	G3V913

The 10 main KEEG pathways and differentially expressed proteins.

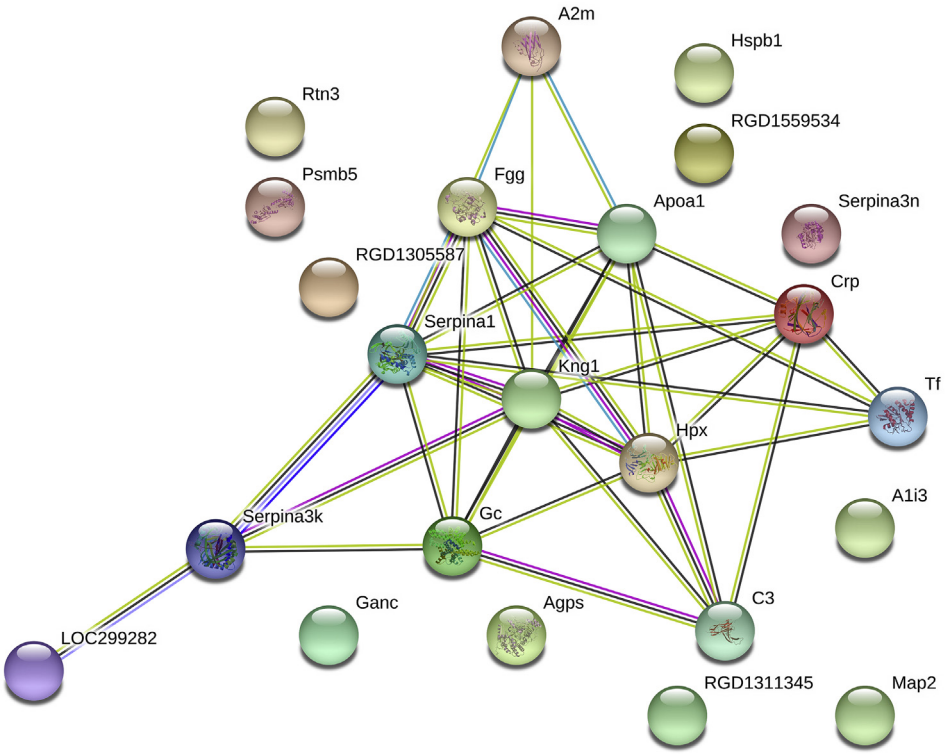

Fig. 7. Protein–protein interaction networks of DEPs. Each node represents a protein. Empty nodes represent proteins of unknown 3D structure. Filled nodes represent proteins for which the 3D structure is known or predicted. The light blue lines represent database evidence. The purple lines represent experimental evidence. The yellow lines represent text mining evidence. The black lines represent co-expression evidence (n = 3 per group).

downregulated (< 0.83-fold), and five were upregulated (> 1.2-fold) (Fig. 5A and C).

3.3. Validation by western blotting and immunofluorescence

To confirm the iTRAQ proteomic data, we examined the expression of transferrin (TF), fibrinogen gamma chain (FGG), and complement component 3 (C3) in protein isolates of the ischemic penumbra by western blotting. Compared with the control group, there was a significant decrease in TF, FGG, and C3 expression in the piperine-treated group (Fig. 6A). Additionally, microtubule-associated protein 2 (MAP2) immunofluorescence was performed (Fig. 6B). The results were consistent with the iTRAQ proteomics data.

3.4. Gene ontology analysis of functional enrichment

The DEPs were categorized into three main gene ontology (GO) classification categories (biological process, cellular component, and molecular function), and significantly altered functions of the DEPs were calculated using the Fisher's exact test in Blast2GO. The top 10 GO terms in each category are shown in Fig. 4. The 'regulation of response to stress' and 'regulation of response to external stimulus' terms were the most abundant biological processes, both of which contained heat shock protein family B member 1, C3, kininogen 2,FGG, and apolipoprotein A1 (ApoA1). The 'acute inflammatory response' was the most relevant biological process, which contained the serpin family A member 1 (SerpinA1), C3, kininogen 2, and alpha-2-macroglobulin (A2M) (Fig. 4). The 'enzyme regulator activity' and 'molecular function regulator' were the most abundant molecular function enrichments, and both contained 10 DEPs. In addition, both 'extracellular region' and 'extracellular region part' in the cellular component enrichment contained 17 DEPs.

3.5. DEPs in the kyoto encyclopedia of genes and genomes pathways (KEEG) and protein interaction networks

To further analyze the abundance profiles of significantly changed proteins between the piperine and control groups, we performed hierarchical clustering analysis using heatmaps for 23 DEPs (Fig. 5B). Unfortunately, there were no upregulated or downregulated clusters. The DEPs were then used to search against the KEGG database using KOBAS (http://kobas.cbi.pku.edu.cn/), which identified a total of 19 KEGG pathways involving nine DEPs (Table 1). Of these, the 'complement and coagulation cascades (rno04610)' was the most relevant pathway

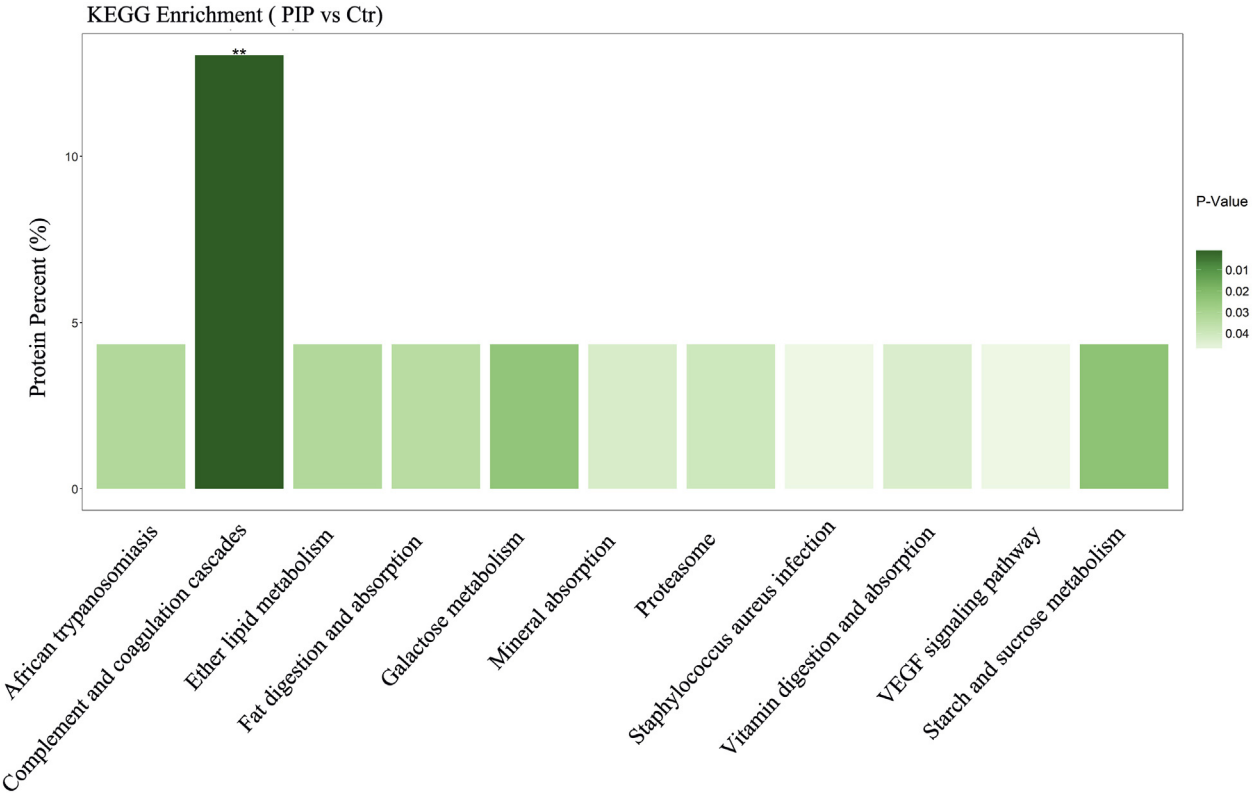

Fig. 8. KEEG pathways enrichment bar plot of DEPs. The 'complement and coagulation cascades' was the most relevant pathway (**$p < 0.01$, $n = 3$ per group).

(Fig. 8). Next, we used STRING v10 (https://string-db.org/) to assess the protein–protein interaction (PPI) networks of the DEPs. A total of 36 interaction links between 23 proteins were found with the PPI Networks (Fig. 7), 11 of which had no interactions with other proteins. However, kininogen1, serpinA1, FGG, ApoA1, hemopexin, vitamin D binding protein and C-reactive protein exhibited substantial interconnectivity, and had the greatest capacity to regulate the PPI networks.

4. Discussion

Previous studies have already mentioned that piperine was able to improve neurobehavioral deficits and reduce inflammation in cerebral ischemic stroke through the repression of cyclooxygenase-2, nitric oxide synthase-2, and nuclear factor kappa B (Vaibhav et al., 2012). In the present study, we wanted to use iTRAQ technology to excavate proteomic changes comprehensively in the penumbral tissue following piperine treatment in rats subjected to cerebral I/R injury, and our date suggested that the neuroprotective effects of piperine may relevant to complement and coagulation cascades. Definitely, we identified 23 novel DEPs, including 18 that were downregulated and five that were upregulated. Furthermore, using KEGG pathway analysis, we found that piperine treatment was associated with a significant decrease in the expression of A2M, FGG and serpinA1 in the ischemic penumbra, which are involved in the complement and coagulation cascades. The complement system is a key mediator of innate immunity (Bajic et al., 2015), while excessive or prolonged complement activation can result in tissue damage in rheumatoid arthritis, multiple sclerosis, myasthenia gravis, and I/R injury (Carroll and Sim, 2011). Additionally, we found that piperine effected nutrient metabolism (Kim et al., 2017).

A key finding of the present study was that C3 expression was downregulated by piperine treatment. C3 is a critical molecule in complement activation, and plays a central role in the classical, lectin, and alternative pathways (Banda et al., 2014). Previous studies have reported that C3 expression is increased in the brain following I/R injury (Di Napoli, 2001; Nishino et al., 1994). Moreover, siRNA-mediated C3-knockdown was also reported to prevent neuronal apoptosis, reduce cerebral infarction volume, and improve functional recovery after I/R injury (Wang et al., 2018). Furthermore, C3 played a key role in complement-mediated inflammatory tissue injury following cerebral ischemia (Mocco et al., 2006). The blood–brain barrier is a major obstacle for drugs, including C3 inhibitors, targeting the central nervous system (Yanagida et al., 2017). In the present study, we found that oral piperine reduced C3 expression following I/R injury (Fig. 5C). Indeed, piperine has been reported to cross the blood–brain barrier (Liu et al., 2013). These results suggested that piperine might inhibit C3, and thereby suppressed brain inflammation following cerebral ischemia.

The process of blood coagulation involves a series of proenzyme-to-serine protease conversions, and the formation of thrombin, an enzyme responsible for the conversion of soluble fibrinogen to an insoluble fibrin clot (Rau et al., 2007). Landi et al. (1987) evaluated coagulation and platelet function in 70 patients with recent cerebral infarction or hemorrhage versus 45 age-matched controls, and reported that hypercoagulability is an important prognostic factor in stroke (Cote et al., 2000). Similarly, coagulation and fibrinolytic cascade markers are related to the recanalization rate after thrombolytic therapy (Castellanos and Serena, 2007). Interestingly, in the present study, piperine reduced the expression levels of serpinA1, FGG and A2M, which play important roles in the coagulation pathway (Fig. 5C).

SerpinA1 has been used as an early risk marker for predicting cognitive impairment in Parkinson's disease dementia (Halbgebauer et al., 2016), and was considered to be a biomarker in the prognosis of colorectal cancer and breast cancer (Chan et al., 2015; Kwon et al., 2015). Our current findings suggest that serpinA1 played a key role in cerebral ischemia as well.

A2M is a broad-spectrum protease inhibitor that is important in inflammation by functioning as a carrier protein for interleukin-6 and interleukin-1β (Feinman, 1994). A2M enhances human leukocyte

functions and innate immune responses (Federici et al., 2015), and acts as a promising biomarker for ischemic stroke (Nezu et al., 2013).

Fibrinogen is comprised of two sets of three polypeptide chains termed A alpha, B beta and γ polypeptide chains (linked by disulphide bonds), which are encoded by the fibrinogen alpha, fibrinogen beta and FGG genes, respectively (Mosesson et al., 2001). The FGG/fibrinogen alpha haplotypes are independently associated with ischemic stroke (Jood et al., 2008), while the FGG haplotype 3 can reduce the risk of ischemic stroke (Cheung et al., 2008), These observations are consistent with our current findings.

The complement and coagulation systems are important in host defense and hemostasis, respectively. Coagulation and complement are two distinct, but closely linked systems. For example, coagulation factor XII can activate complement C1, which leads to initiation of the classical complement pathway (Ghebrehiwet et al., 1983). Complement can also activate the coagulation cascade directly or indirectly (Gulla et al., 2010). Disruption of the complement and coagulation cascade is involved in numerous clinical conditions, including sepsis, systemic lupus erythematosus, inflammatory complications, and I/R injury (Oikonomopoulou et al., 2012). And the potential mechanisms may be that piperine reduces the inflammatory response in cerebral I/R injury firstly by decreasing the key molecule C3 in alternative complement pathway, then affecting coagulation system and reduceing the expression of A2M, FGG, SerpinA1, ultimately leads to protection on the brain.

Finally, there's another interesting point, piperine can enhance the absorption of a variety of substances (Kim et al., 2017), which is in line with our results showing that piperine treatment is related to vitamin digestion and absorption, galactose metabolism, ether lipid metabolism, fat digestion and absorption, and starch and sucrose metabolism (Fig. 8). However, it is difficult to explain the upregulation of ApoA1 expression following piperine treatment in the present study. Piperine can alleviate hepatic steatosis and insulin resistance in rats fed a high-fat diet (Choi et al., 2013). Similarly, in mice fed a high-fat diet (a model of type 2 diabetes), ApoA1 expression is upregulated by piperine (Yoshimura et al., 2013). Thus, it is possible that piperine protects against hepatic steatosis by increasing ApoA1, although further studies are required to confirm this finding.

5. Conclusion

Our findings suggest that piperine protects against cerebral ischemic injury by regulating the complement and coagulation cascades. We identified 23 proteins that were changed by piperine treatment. Further studies are required to assess the roles of these proteins in the neuroprotective actions of piperine following cerebral ischemia.

Conflicts of interest

The authors declare that there is no conflict of interest associated with this study.

Acknowledgements

This work was supported by National Natural Science Foundation of China (8157051331 and 8177051550) to Jincao Chen and The Fund of Collaborative Innovation Center for Brain Science to Q.W.

Reference

Akyildiz, H.Y., Karabacak, A., Akyuz, M., Sozuer, E., Akcan, A., 2013. Effects of piperine in experimental intestinal ischemia reperfusion model in rats. Ulus Travma Acil Cerrahi Derg 19, 387–391.

Anissian, D., Ghasemi-Kasman, M., Khalili-Fomeshi, M., Akbari, A., Hashemian, M., Kazemi, S., Moghadamnia, A.A., 2018. Piperine-loaded chitosan-STPP nanoparticles reduce neuronal loss and astrocytes activation in chemical kindling model of epilepsy. Int. J. Biol. Macromol. 107, 973–983.

Ashwal, S., Tone, B., Tian, H.R., Cole, D.J., Pearce, W.J., 1998. Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. Stroke 29, 1037–1046 discussion 1047.

Bajic, G., Degn, S.E., Thiel, S., Andersen, G.R., 2015. Complement activation, regulation, and molecular basis for complement-related diseases. EMBO J. 34, 2735–2757.

Banda, N.K., Mehta, G., Chao, Y., Wang, G., Inturi, S., Fossati-Jimack, L., Botto, M., Wu, L., Moghimi, S.M., Simberg, D., 2014. Mechanisms of complement activation by dextran-coated superparamagnetic iron oxide (SPIO) nanoworms in mouse versus human serum. Part. Fibre Toxicol. 11, 64.

Bronner, L.L., Kanter, D.S., Manson, J.E., 1995. Primary prevention of stroke. N. Engl. J. Med. 333, 1392–1400.

Carroll, M.V., Sim, R.B., 2011. Complement in health and disease. Adv. Drug Deliv. Rev. 63, 965–975.

Castellanos, M., Serena, J., 2007. Applicability of biomarkers in ischemic stroke. Cerebrovasc. Dis. 24 (Suppl. 1), 7–15.

Chan, H.J., Li, H., Liu, Z., Yuan, Y.C., Mortimer, J., Chen, S., 2015. SERPINA1 is a direct estrogen receptor target gene and a predictor of survival in breast cancer patients. Oncotarget 6, 25815–25827.

Cheung, E.Y., Uitte, D.W.S., Vos, H.L., Leebeek, F.W., Dippel, D.W., Bertina, R.M., de Maat, M.P., 2008. Fibrinogen gamma' in ischemic stroke: a case-control study. Stroke 39, 1033–1035.

Choi, S., Choi, Y., Choi, Y., Kim, S., Jang, J., Park, T., 2013. Piperine reverses high fat diet-induced hepatic steatosis and insulin resistance in mice. Food Chem. 141, 3627–3635.

Cote, R., Wolfson, C., Solymoss, S., Mackey, A., Leclerc, J.R., Simard, D., Rouah, F., Bourque, F., Leger, B., 2000. Hemostatic markers in patients at risk of cerebral ischemia. Stroke 31, 1856–1862.

Da, C.G., Felipe, C.F., Scorza, F.A., Da, C.M., Tavares, A.F., Menezes, M.L., de Andrade, G.M., Leal, L.K., Brito, G.A., Da, G.N.-M.M., Cavalheiro, E.A., de Barros, V.G., 2013. Piperine decreases pilocarpine-induced convulsions by GABAergic mechanisms. Pharmacol. Biochem. Behav. 104, 144–153.

Dhivya, V., Priya, L.B., Chirayil, H.T., Sathiskumar, S., Huang, C.Y., Padma, V.V., 2017. Piperine modulates isoproterenol induced myocardial ischemia through antioxidant and anti-dyslipidemic effect in male Wistar rats. Biomed. Pharmacother. 87, 705–713.

Di Napoli, M., 2001. Systemic complement activation in ischemic stroke. Stroke 32, 1443–1448.

Donnan, G.A., Fisher, M., Macleod, M., Davis, S.M., 2008. Stroke. Lancet 371, 1612–1623.

Federici, C.D., Pavlov, A.M., Norling, L.V., Gobbetti, T., Brunelleschi, S., Le Fauder, P., Cenac, N., Sukhorukov, G.B., Perretti, M., 2015. Alpha-2-macroglobulin loaded microcapsules enhance human leukocyte functions and innate immune response. J. Contr. Release 217, 284–292.

Feigin, V.L., Forouzanfar, M.H., Krishnamurthi, R., Mensah, G.A., Connor, M., Bennett, D.A., Moran, A.E., Sacco, R.L., Anderson, L., Truelsen, T., O'Donnell, M., Venketasubramanian, N., Barker-Collo, S., Lawes, C.M., Wang, W., Shinohara, Y., Witt, E., Ezzati, M., Naghavi, M., Murray, C., Global Burden of Diseases, I., Risk Factors, S., the, G.B.D.S.E.G, 2014. Global and regional burden of stroke during 1990-2010: findings from the global burden of disease study 2010. Lancet 383, 245–254.

Feinman, R.D., 1994. The proteinase-binding reaction of alpha 2M. Ann. N. Y. Acad. Sci. 737, 245–266.

Ghebrehiwet, B., Randazzo, B.P., Dunn, J.T., Silverberg, M., Kaplan, A.P., 1983. Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. J. Clin. Invest. 71, 1450–1456.

Greenshields, A.L., Doucette, C.D., Sutton, K.M., Madera, L., Annan, H., Yaffe, P.B., Knickle, A.F., Dong, Z., Hoskin, D.W., 2015. Piperine inhibits the growth and motility of triple-negative breast cancer cells. Cancer Lett. 357, 129–140.

Gulla, K.C., Gupta, K., Krarup, A., Gal, P., Schwaeble, W.J., Sim, R.B., O'Connor, C.D., Hajela, K., 2010. Activation of mannan-binding lectin-associated serine proteases leads to generation of a fibrin clot. Immunology 129, 482–495.

Halbgebauer, S., Nagl, M., Klafki, H., Haussmann, U., Steinacker, P., Oeckl, P., Kassubek, J., Pinkhardt, E., Ludolph, A.C., Soininen, H., Herukka, S.K., Wiltfang, J., Otto, M., 2016. Modified serpinA1 as risk marker for Parkinson's disease dementia: analysis of baseline data. Sci. Rep. 6, 26145.

Jood, K., Danielson, J., Ladenvall, C., Blomstrand, C., Jern, C., 2008. Fibrinogen gene variation and ischemic stroke. J. Thromb. Haemostasis 6, 897–904.

Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., National Centre for the Replacement, R., Reduction of Amimals in, R, 2011. Animal research: reporting in vivo experiments–the ARRIVE guidelines. J. Cerebr. Blood Flow Metabol. 31, 991–993.

Kim, J., Lee, K.P., Lee, D.W., Lim, K., 2017. Piperine enhances carbohydrate/fat metabolism in skeletal muscle during acute exercise in mice. Nutr. Metab. 14, 43.

Kwon, C.H., Park, H.J., Choi, J.H., Lee, J.R., Kim, H.K., Jo, H.J., Kim, H.S., Oh, N., Song, G.A., Park, D.Y., 2015. Snail and serpinA1 promote tumor progression and predict prognosis in colorectal cancer. Oncotarget 6, 20312–20326.

Lakhan, S.E., Kirchgessner, A., Hofer, M., 2009. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J. Transl. Med. 7, 97.

Landi, G., D'Angelo, A., Boccardi, E., Candelise, L., Mannucci, P.M., Nobile, O.E., Morabito, A., 1987. Hypercoagulability in acute stroke: prognostic significance. Neurology 37, 1667–1671.

Liu, H., Luo, R., Chen, X., Liu, J., Bi, Y., Zheng, L., Wu, X., 2013. Tissue distribution profiles of three antiparkinsonian alkaloids from Piper longum L. in rats determined by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 928, 78–82.

Lu, Y., Liu, J., Li, H., Gu, L., 2016. Piperine ameliorates lipopolysaccharide-induced acute lung injury via modulating NF-kappaB signaling pathways. Inflammation 39, 303–308.

Margaill, I., Plotkine, M., Lerouet, D., 2005. Antioxidant strategies in the treatment of stroke. Free Radic. Biol. Med. 39, 429–443.
McGrath, J.C., Lilley, E., 2015. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br. J. Pharmacol. 172, 3189–3193.
Meairs, S., Alonso, A., Hennerici, M.G., 2012. Progress in sonothrombolysis for the treatment of stroke. Stroke 43, 1706–1710.
Mocco, J., Mack, W.J., Ducruet, A.F., Sosunov, S.A., Sughrue, M.E., Hassid, B.G., Nair, M.N., Laufer, I., Komotar, R.J., Claire, M., Holland, H., Pinsky, D.J., Connolly, E.J., 2006. Complement component C3 mediates inflammatory injury following focal cerebral ischemia. Circ. Res. 99, 209–217.
Mosesson, M.W., Siebenlist, K.R., Meh, D.A., 2001. The structure and biological features of fibrinogen and fibrin. Ann. N. Y. Acad. Sci. 936, 11–30.
Nezu, T., Hosomi, N., Aoki, S., Deguchi, K., Masugata, H., Ichihara, N., Ohyama, H., Ohtsuki, T., Kohno, M., Matsumoto, M., 2013. Alpha2-macroglobulin as a promising biomarker for cerebral small vessel disease in acute ischemic stroke patients. J. Neurol. 260, 2642–2649.
Nishino, H., Czurko, A., Fukuda, A., Hashitani, T., Hida, H., Karadi, Z., Lenard, L., 1994. Pathophysiological process after transient ischemia of the middle cerebral artery in the rat. Brain Res. Bull. 35, 51–56.
Oikonomopoulou, K., Ricklin, D., Ward, P.A., Lambris, J.D., 2012. Interactions between coagulation and complement–their role in inflammation. Semin. Immunopathol. 34, 151–165.
Rau, J.C., Beaulieu, L.M., Huntington, J.A., Church, F.C., 2007. Serpins in thrombosis, hemostasis and fibrinolysis. J. Thromb. Haemostasis 5 (Suppl. 1), 102–115.
Szallasi, A., 2005. Piperine: researchers discover new flavor in an ancient spice. Trends Pharmacol. Sci. 26, 437–439.
Vaibhav, K., Shrivastava, P., Javed, H., Khan, A., Ahmed, M.E., Tabassum, R., Khan, M.M., Khuwaja, G., Islam, F., Siddiqui, M.S., Safhi, M.M., Islam, F., 2012. Piperine suppresses cerebral ischemia-reperfusion-induced inflammation through the repression of COX-2, NOS-2, and NF-kappaB in middle cerebral artery occlusion rat model. Mol. Cell. Biochem. 367, 73–84.
Vijayakumar, R.S., Surya, D., Nalini, N., 2004. Antioxidant efficacy of black pepper (Piper nigrum L.) and piperine in rats with high fat diet induced oxidative stress. Redox Rep. 9, 105–110.
Wang, Y., Li, S.Y., Shen, S., Wang, J., 2018. Protecting neurons from cerebral ischemia/reperfusion injury via nanoparticle-mediated delivery of an siRNA to inhibit microglial neurotoxicity. Biomaterials 161, 95–105.
Yanagida, K., Liu, C.H., Faraco, G., Galvani, S., Smith, H.K., Burg, N., Anrather, J., Sanchez, T., Iadecola, C., Hla, T., 2017. Size-selective opening of the blood-brain barrier by targeting endothelial sphingosine 1-phosphate receptor 1. Proc. Natl. Acad. Sci. U. S. A. 114, 4531–4536.
Yoshimura, Y., Nishii, S., Zaima, N., Moriyama, T., Kawamura, Y., 2013. Ellagic acid improves hepatic steatosis and serum lipid composition through reduction of serum resistin levels and transcriptional activation of hepatic ppara in obese, diabetic KK-A(y) mice. Biochem. Biophys. Res. Commun. 434, 486–491.
Zhang, Z.F., Chen, J., Han, X., Zhang, Y., Liao, H.B., Lei, R.X., Zhuang, Y., Wang, Z.F., Li, Z., Chen, J.C., Liao, W.J., Zhou, H.B., Liu, F., Wan, Q., 2017. Bisperoxovandium (pyridin-2-squaramide) targets both PTEN and ERK1/2 to confer neuroprotection. Br. J. Pharmacol. 174, 641–656.