

Original Research

# ***Panax notoginseng* Saponins Attenuate Cerebral Ischemia-Reperfusion Injury via Mitophagy-Induced Inhibition of NLRP3 Inflammasome in Rats**

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## **Abstract**

**Background:** The mitophagy/NLRP3 inflammasome pathway is a promising therapeutic target for cerebral ischemia-reperfusion (I/R). *Panax notoginseng* (Burkill) F.H. Chen, one of the most valuable components of traditional Chinese medicine, and *Panax notoginseng* saponins (PNS), the main active ingredients of *P. notoginseng*, are patent medicines commonly used to treat cardio- and cerebrovascular diseases. However, their effects on the mitophagy and the NLRP3 inflammasome activation in I/R remain unclear. Therefore, in this study, we investigated how PNS might affect the mitophagy/NLRP3 inflammasome pathway in I/R. **Methods:** Cerebral I/R injury was induced by middle cerebral-artery occlusion, and expression levels of NLRP3 inflammasome signaling pathway-associated proteins were detected by western blot. We tested I/R injury using a neurological-deficit score, infarct volume, and hematoxylin and eosin staining, after which we detected both mitophagy- and NLRP3 inflammasome-related proteins in PNS-treated rats to determine whether PNS could attenuate I/R injury and the possible mechanisms involved. **Results:** Our results showed that cerebral I/R could induce activation of the NLRP3 inflammasome, aggravating brain injury, whereas PNS effectively alleviated cerebral I/R injury in rats by inhibiting the NLRP3 inflammasome and promoting mitophagy via the PINK1/Parkin pathway. Moreover, mitophagy inhibited the NLRP3 inflammasome and mediated the anti-injury effects of PNS. **Conclusions:** In conclusion, PNS could promote mitophagy via the PINK1/Parkin pathway by inhibiting activation of the NLRP3 inflammasome, alleviating cerebral I/R injury in rats.

**Keywords:** *Panax notoginseng* saponins; NLRP3 inflammasome; mitophagy; cerebral ischemia-reperfusion; PINK1; Parkin

## **1. Introduction**

Stroke is one of the main causes of death and disability in humans, the most common type being ischemic [1]. Currently, the guiding principle for the treatment of ischemic stroke is to restore blood perfusion to ischemic tissue over time [2]. In some cases, recovering tissue blood flow after ischemia cannot effectively restore tissue function but exacerbates its damage, producing an ischemia-reperfusion (I/R) injury [2]. Cerebral I/R injury is the important link in the pathogenesis of ischemic stroke, and its prevention is the key to the current treatment of stroke [2,3]. Furthermore, cerebral I/R can cause a series of changes at cellular and molecular levels, and the pathogenic mechanisms include mitochondrial-energy metabolism impairment, the toxicity of excitatory amino acids, ion balance disorder, oxidative stress, inflammatory responses, mitophagy, apoptosis, and blood-brain barrier (BBB) destruction [2,4–6]. Specifically, the inflammatory response plays an important role in the pathological process of cerebral I/R injury [7].

Inflammasomes are central to the inflammatory response. The nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3

(NLRP3) inflammasome, the most thoroughly studied inflammasome, mediates the incidence and progression of many neurodegenerative diseases [3,8]. When stimulated by intracellular danger signals, NLRP3, apoptosis-associated speck-like protein containing a Caspase activation and recruitment domain (ASC) and pro-Caspase-1 assemble into a multiprotein complex, termed the NLRP3 inflammasome. This complex then converts pro-Caspase-1 into Caspase-1, which allows activated Caspase-1 to cleave pro-interleukin-1 $\beta$  (pro-IL-1 $\beta$ ) and pro-IL-18 into mature IL-1 $\beta$  and IL-18, respectively. Finally, IL-1 $\beta$  and IL-18 are secreted into the extracellular matrix, mediating the inflammatory response [9]. Mitophagy, a process for removing senescent or damaged mitochondria, is an important mechanism in mitochondrial quality control. Recent studies have revealed that mitophagy inhibits activation of the NLRP3 inflammasome in cerebral ischemia, while promoting it inhibits NLRP3 inflammasome activation [10–13]. Additionally, recent studies have shown that mitophagy improves viral pneumonia by inhibiting activation of the NLRP3 inflammasome [14].



*Panax notoginseng* (Burkill) F.H.Chen, a highly valued Chinese medicine for treating cardiovascular (CV) and cerebrovascular diseases, is the dried root and rhizome of this species belonging to the Araliaceae family. *P. notoginseng* is a functional food that is recorded in the ancient book “Compendium of Materia Medica”. Not only does it possess pharmacological activity in tissues including those of the nervous, CV, and immune systems [15], *P. notoginseng* is also used to make medicinal preparations such as decoctions, tablets, powders, and medicinal liquors [16]. Moreover, *Panax notoginseng* saponins (PNS) are the main bioactive ingredients of *P. notoginseng*, which contains diverse monomer saponins; PNS preparations, such as Xuesaitong injection, have been widely used to prevent and treat CV and cerebrovascular diseases [17]. Previous studies, including ours, have shown that PNS has multiple pharmacological activities during ischemic brain injuries, such as anti-inflammatory, antioxidant, and antithrombotic activities, and demonstrates the ability to inhibit apoptosis of neuronal cells and protect the BBB [18–20]. This evidence suggests that PNS could play a protective role in cerebral I/R through multiple targets. However, further study is needed to confirm whether mitophagy and the NLRP3 inflammasome are involved in the anti-cerebral-I/R injury effects of PNS and whether the mitophagy/NLRP3-inflammasome pathway mediates the mechanism in the protective effects of PNS. Therefore, in the current study, we further investigated whether PNS reduced I/R injury from the perspectives of the NLRP3 inflammasome and mitophagy.

## 2. Materials and Methods

### 2.1 Chemicals

We purchased PNS from Chengdu Manster Biotechnology Co., Ltd. (DST200706-054; Chengdu, China). The major effective constituents of PNS compounds included 6.96% (v/v) Notoginsenoside R1, 34.29% (v/v) Ginsenoside Rg1, 2.65% (v/v) Ginsenoside Re, 41.34% (v/v) Ginsenoside Rb1, and 10.19% (v/v) Ginsenoside Rd [21]. We also purchased an autophagy inhibitor (3-methyladenine [3-MA]; M9281; Sigma-Aldrich, St. Louis, MO, USA), Caspase-1 inhibitor (Ac-TYR-VAL-ALA-ASP-C MK [Ac-YVAD-CMK]; SML0429; Sigma-Aldrich), NLRP3 inhibitor (MCC950; S7809; Selleck Chemicals, Houston, TX, USA), mitophagy inhibitor (mitochondrial-division inhibitor 1 [Mdivi-1]; S7162; Selleck), goat anti-rabbit secondary antibody (AP132P; Merck Millipore, Darmstadt, Germany), and goat anti-mouse secondary antibody (AP124P; Merck Millipore, Darmstadt, Germany).

### 2.2 Animals

We purchased healthy adult male Sprague Dawley rats weighing 200–220 g (specific-pathogen-free [SPF] grade) from the Experimental Animal Center of HUCM (Animal Certificate No. SYXK, Xiang, 2018-0002). The rats were

fed under SPF conditions for 1 week before experiments, with tap water and normal chow supplied ad libitum. The breeding room was provided with natural light and maintained at a temperature of 20–25 °C. All protocols followed the ARRIVE guidelines in terms of study design, sample size, randomization, outcome measures, data analysis, experimental procedures, and reporting of results. This study was approved by the Animal Ethics Committee of Hunan University of Chinese Medicine (HUCM; Changsha, China; Approval No. LLBH-202103290002), in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). We declare that all methods were performed in accordance with the relevant guidelines and regulations.

### 2.3 Middle Cerebral-Artery Occlusion (MCAO) Models

We generated an MCAO model as described previously [21]. Rats were anesthetized with isoflurane (1.5%) in 30%/70% oxygen/nitrous oxide. Their body temperature was maintained at 37 °C ± 0.5 °C using a heating pad throughout the surgical procedure and afterward, until the rats recovered from the anesthesia. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed via a neck incision and then isolated. We subsequently ligated the distal portion of the ECA using surgical thread and then disconnected it and its branches proximal to the ligation site using electrocoagulation. We then made a slipknot to ligate the distal part of the ICA and clamped the distal ICA and CCA with arterial clamps. A small orifice was carefully made in the ECA stump. Then, occlusion wire (2636A2; Beijing Cinontech Co., Ltd., Beijing, China) was introduced into the ECA and directed into the ICA via the bifurcation of the CCA. Next, we opened the artery clip that clamped the distal ICA and inserted the occlusion wire into the intracranial portion of the ICA.

The length of the line embolism inserted into the artery was 18 ± 2 mm distal from the CCA bifurcation. When the filament was inserted into the anterior cerebral artery (ACA), its tip reached the origin of the middle cerebral artery; the insertion was stopped when resistance increased. We then fastened the ICA to prevent bleeding and movement and sutured the neck skin. After 2 h of blood flow blockage and followed by 24 h of reperfusion. The sham group underwent operations involving isolation of the CCA, ECA, and ICA without occlusion wire insertion. We then carried out subsequent experiments.

### 2.4 Experimental Groups and Procedures

We randomly divided the rats into the following treatment subgroups: sham, model, low-dose PNS (PNS-L), medium-dose PNS (PNS-M), high-dose PNS (PNS-H), PNS + 3-MA, PNS + Mdivi-1, Ac-YVAD-CMK, and MCC950 group. The rats were given microinjections of 3-

MA (40 nmol) at a concentration of 5  $\mu$ L per lateral ventricle [22,23]. Mdivi-1 (0.15 mg/mL) was administered by i.p. injection at 10 mL/kg [24], and Ac-YVAD-CMK (0.5 mg/mL) and MCC950 (2.5 mg/mL) were administered at doses of 10 mL/kg [25]. The PNS dose was calculated based on a previous study [26]: low dose, 40 mg/kg; medium dose, 80 mg/kg; and high dose, 160 mg/kg. PNS was administered three times via gavage at the abovementioned concentrations for the respective PNS groups. Optimal administration times were 50, 26, and 2 h before the model establishment [27]. The sham and model groups were given equal volumes of physiological saline, whereas the remaining groups were injected using a microliter syringe (Hamilton Co., Reno, NV, USA) at the onset of reperfusion.

### 2.5 Neurological-Deficit Score (NDS)

Following reperfusion for 24 h, we evaluated rats in each group to determine the NDS via the Longa method [21]: 0 = no neurological deficit; 1 = the left forelimb could not be extended when the rat lifted its tail; 2 = circling to the contralateral side when walking; 3 = tilting to the contralateral side when walking; 4 = no spontaneous motor activity, or loss of consciousness.

### 2.6 Measurement of Cerebral-Infarction Volume

After reperfusion for 24 h, rats were euthanized by intracardial injection of 500 mg/kg 10% potassium chloride solution; their brains were removed, placed on ice, and frozen for 15 min at  $-20^{\circ}\text{C}$ . We evenly cut each brain into five 2-mm serial coronal sections. Then, the slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) phosphate buffer (BCBP3272V; Sigma-Aldrich, Saint Louis, MO, USA), immersed in a dark, constant-temperature bath of  $37^{\circ}\text{C}$  for 15 min, and stirred gently every 5 min. After staining, we fixed the brain sections in 4% paraformaldehyde for a minimum of 24 h. The infarct regions eventually turned white, and the unaffected regions turned red. The brain slices were then photographed and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Additionally, brains were divided into the ipsilateral hemisphere (ischemic side) and contralateral hemisphere. The following formula was used to calculate cerebral infarction volume, as described previously [28]: percentage of infarct volume = (total contralateral hemisphere volume - non-infarcted ipsilateral hemisphere volume) / (total contralateral hemisphere volume)  $\times 100\%$ .

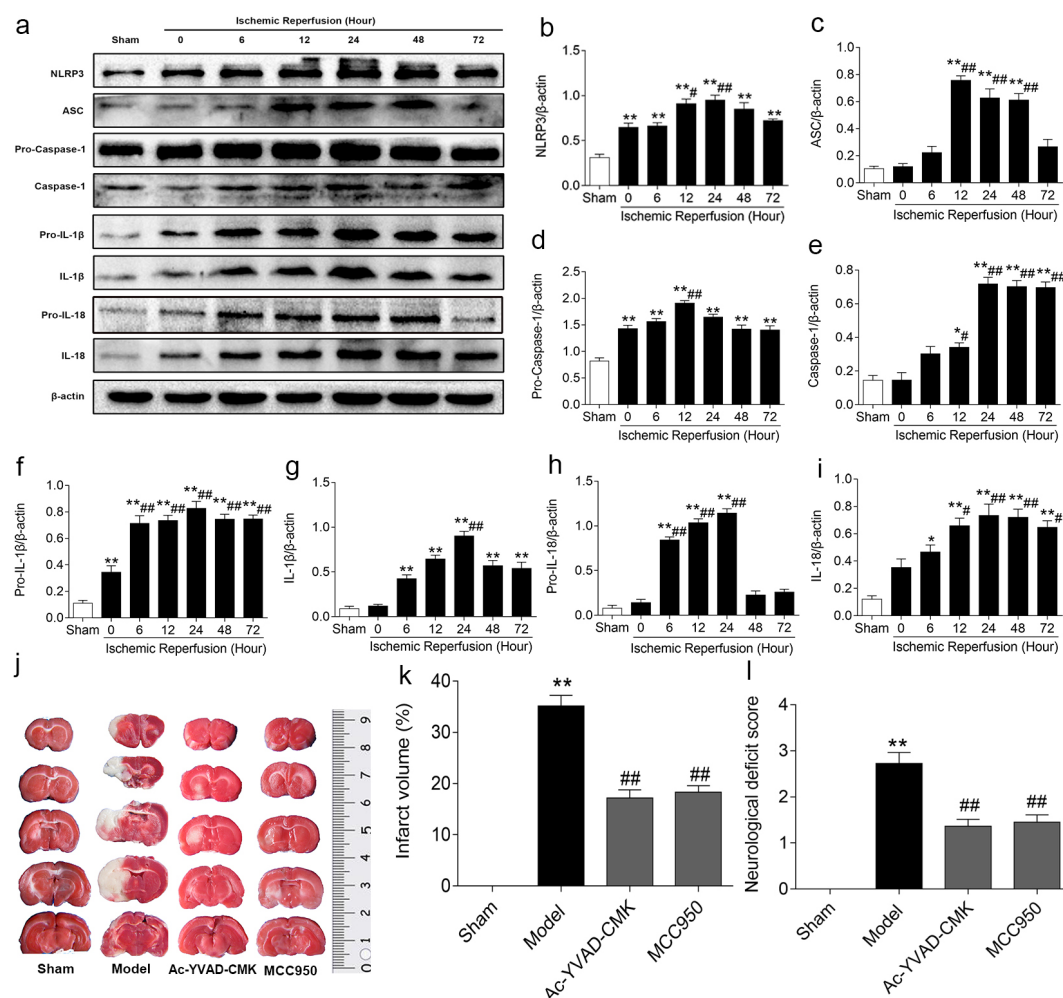
### 2.7 Hematoxylin and Eosin Staining to Observe Pathological Changes in the Cerebral Cortex

Following reperfusion for 24 h, rats ( $n = 8$ ) were anesthetized and euthanized via intracardial injection of 500 mg/kg 10% potassium chloride solution and then killed using rapid decapitation. We then fixed the brains in 4%

paraformaldehyde and sliced them into sections with 3-mm thickness, cutting coronally through the optic cross-plane. The slices were dehydrated using gradient alcohol, made transparent with xylene, embedded in paraffin, and finally cut into 3- $\mu$ m-thin sections, all of which were fixed on glass slides for hematoxylin and eosin (H&E) staining. Then, we observed the H&E-stained sections under a Zeiss AxioScope 5 microscope (Zeiss, Oberkochen, Germany) and calculated the rate of cell damage. Additionally, five non-overlapping visual fields in the ischemic cortex were randomly selected for observation of damaged cells. Such cells were primarily characterized by vacuolar degeneration, eosinophilic degeneration, nuclear contraction, and nuclear dissolution. Finally, we counted the total number of cells and number of damaged cells in each high-magnification field. The rate of cell damage was calculated as described previously: [28] (number of damaged cells/total number of cells)  $\times 100\%$ .

### 2.8 Western Blot (WB)

We used WB to detect NLRP3 inflammasome levels and mitophagy-related proteins. After reperfusion at 24 h, in accordance with the literature [29], we weighed and homogenized the cerebral cortex of the ischemic penumbra and then added it to 10 volumes of pyrolysis liquid (P0013; Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China), a protease inhibitor cocktail (B14002; Bimake, Houston, TX, USA), and phosphatase inhibitors (B15002; Bimake, Houston, TX, USA). The lysate was then centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and the supernatant was removed as total protein. According to the manufacturer's instructions, we used an isolation kit (89801; Thermo Fisher Scientific, Waltham, MA, USA) to extract cytosolic and mitochondrial proteins, which were then separated via gradient electrophoresis on 8–15% sodium dodecyl sulfate gels and transferred to polyvinylidene difluoride (PVDF) membranes (ISEQ00010; Merck Millipore, Darmstadt, Germany). The PVDF membranes were then washed and incubated with the following diluted primary antibodies at  $4^{\circ}\text{C}$  overnight: NLRP3 (1:1000; NBP2-12446; Novus Biologicals, Littleton, CO, USA), ASC (1:500; NBP1-45453; Novus), Caspase-1 (1:500; NBP1-45433; Novus Biologicals, Littleton, CO, USA), IL-1 $\beta$  (1:1000; ab9722; Abcam, Cambridge, UK), IL-18 (1:1000; ab191860; Abcam, Cambridge, UK), pro-Caspase-1 (1:500; 22915-1-AP; Proteintech, Chicago, IL, USA), pro-IL-1 $\beta$  (1:200; 16806-1-AP; Proteintech, Chicago, IL, USA), pro-IL-18 (1:500; 10663-1-AP; Proteintech, Chicago, IL, USA), light chain 3B (LC3B; 1:1000; 2775S; Cell Signaling Technology [CST], Danvers, MA, USA), P62 (1:1000; 23214s; CST, Danvers, MA, USA), cytochrome C oxidase subunit 4I1 (COX IV 1, 1:1000; 4850s; CST, Danvers, MA, USA), translocase of outer mitochondrial-membrane 40 homolog (TOMM20; 1:1000; 42406s; CST, Danvers, MA, USA), PINK1 (1:2000; sc-517353; Santa Cruz Biotechnology,



**Fig. 1. NLRP3 inflammasome was activated, and suppressing it reduced injury during cerebral I/R in rats.** (a) WB bands of NLRP3 inflammasome-related proteins in different groups. (b–i) Comparisons of relative protein expression levels among groups (n = 5). (j) TTC-stained brain slices; pale areas are infarcted (n = 8). \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. the sham group; #  $p < 0.05$ , ###  $p < 0.01$  vs. the 0-h reperfusion group. (k) Statistical bar chart of determined cerebral-infarct volumes, created using Image-Pro Plus software (n = 8). (l) Statistical bar chart of evaluated NDS (n = 11). \*\*  $p < 0.01$  vs. sham group; ##  $p < 0.01$  vs. model group.

Dallas, TX, USA), Parkin (1:1000, 4211s, CST, Danvers, MA, USA), and  $\beta$ -actin (1:5000; A2228; Sigma-Aldrich, Shanghai, China). After washing, we added horseradish peroxidase-conjugated goat anti-rabbit (1:10,000; AP132P; EMD Millipore, Billerica, USA) or goat anti-mouse immunoglobulin G (1:10,000; AP124P; EMD Millipore, Billerica, USA) secondary antibodies, diluted at 1:10,000, and incubated the PVDF membranes for 1 h at 20–25 °C. Finally, the membranes were washed, developed, and fixed, and images were observed and analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). We used  $\beta$  and COX4I1 as internal references for total-brain and mitochondrial proteins, respectively.

## 2.9 Processing of Results and Statistical Analysis

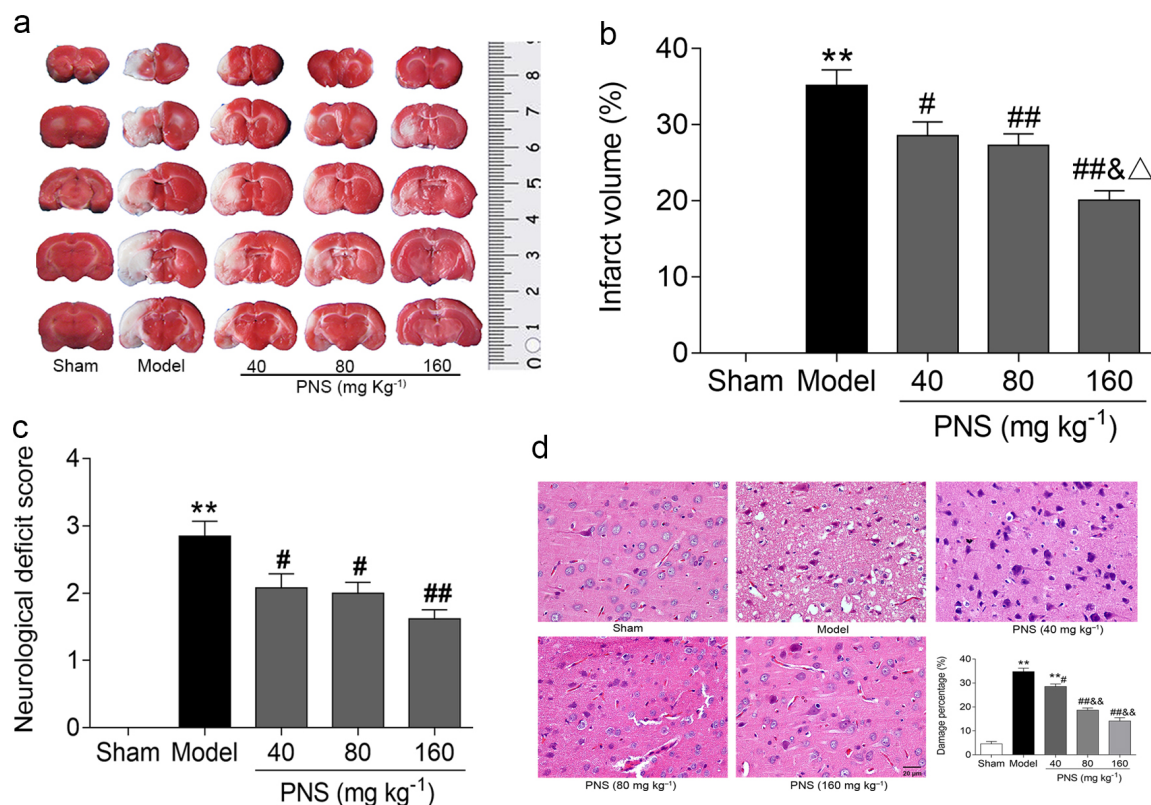
We analyzed the collected data using IBM SPSS version 22.0 (IBM Corp., Armonk, NY, USA). The results are

expressed as mean  $\pm$  standard deviation. For comparisons among multiple groups, a one-way analysis of variance was applied. Pairwise comparisons were conducted using the least-significant-difference method. Differences were considered statistically significant with  $p < 0.05$ .

## 3. Results

### 3.1 NLRP3 Inflammasome Activated in Cerebral I/R, and Its Inhibition Could Reduce Damage in Rats

We detected NLRP3 inflammasome-related protein expression using WB. The results showed that proteins related to NLRP3 inflammasome were significantly elevated in the model group, as compared with the sham group. Expression of NLRP3, Caspase-1, pro-IL-1 $\beta$ , IL-1 $\beta$ , pro-IL-18, and IL-18 reached the highest levels after 24 h of reperfusion (Fig. 1a,b,e–i) whereas the expression of ASC and pro-Caspase-1 reached such levels after 12 h (Fig. 1a,c,d).



**Fig. 2. PNS reduced cerebral I/R damage in rats.** (a) TTC-stained brain slices from the sham, model, and PNS (PNS-L, -M, and -H) groups; pale areas are areas of infarction (n = 8). (b) Statistical chart of determined cerebral-infarct volumes in each group (n = 8), created using Image-Pro Plus software. (c) Statistical bar chart of evaluated NDS in each group (n = 13). (d) Pathological changes in the cerebral cortex and statistical map of the cell injury rate in each group (n = 8). \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. sham group; #  $p < 0.05$  and ##  $p < 0.01$  vs. model group; &  $p < 0.05$  and &&  $p < 0.01$  vs. PNS-L group; △  $p < 0.05$  vs. PNS-M group.

Therefore, we selected 24 h after reperfusion as the optimal time point for the subsequent experiments.

To determine the role of NLRP3 inflammasome activation in cerebral I/R, MCAO rats received i.p. injections of Ac-YVAD-CMK and MCC950, after which we ascertained their infarction volumes and NDS. The results showed that infarction volumes were larger (Fig. 1j,k), and NDS was higher (Fig. 1l), in the model group than in the sham group. Conversely, infarction volumes and NDS were significantly decreased in both Ac-YVAD-CMK and MCC950 groups versus the model group (Fig. 1j,k,l). These findings showed that cerebral I/R could activate both the NLRP3 inflammasome and Caspase-1 and that inhibiting these could reduce cerebral I/R injury in rats.

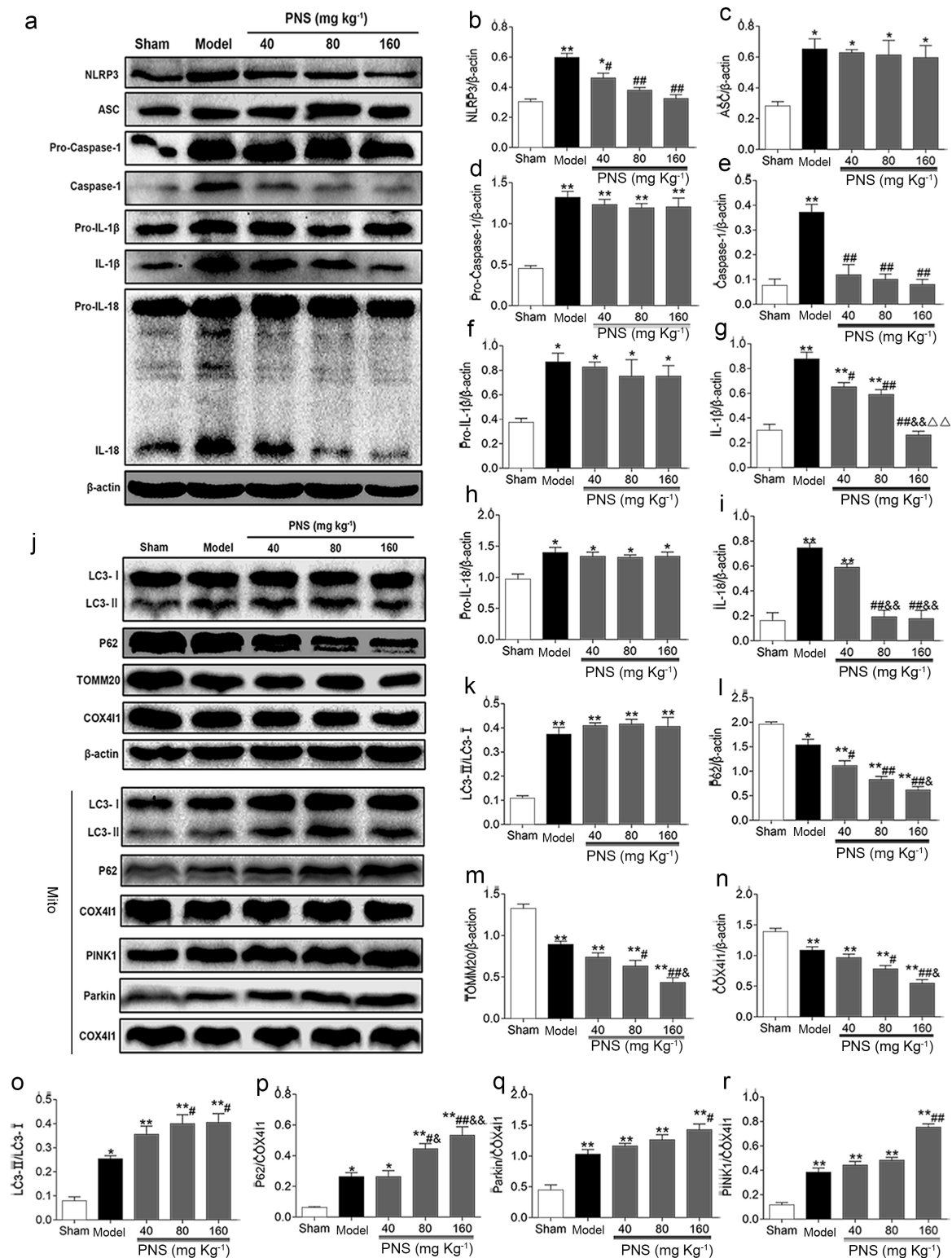
### 3.2 PNS Alleviated Cerebral I/R Injury in Rats

To reveal the protective effects of PNS on I/R injury, we recorded the infarction size, NDS, and cell damage rate after PNS treatment. The results of TTC staining showed that the infarct volume was larger in the model group than in the sham group whereas the administration of PNS reduced infarct volume in MCAO rats in a dose-dependent manner (Fig. 2a,b). Neurological function in the sham group was

normal (NDS = 0) whereas the model group displayed clear neurological deficits and had a noticeably higher NDS than the sham group (Fig. 2c). Additionally, the NDS and rate of cell damage in the PNS groups were significantly reduced compared with the model group (Fig. 2c,d). From H&E staining, we also found a reduced degree of cell damage that was directly proportional to PNS dose (Fig. 2d). Thus, the conclusion can be drawn that PNS could effectively reduce cerebral I/R injury in rats.

### 3.3 PNS Restrained NLRP3 Inflammasome Activation and Activated Mitophagy via the PINK1/Parkin Pathway in Rats

We used WB to assess changes in the NLRP3 inflammasome and its downstream proteins in rats treated with or without PNS to explore the function of PNS on NLRP3 inflammasome activation. Compared with the sham group, the expressions of NLRP3, ASC, pro-Caspase, Caspase, pro-IL-1 $\beta$ , pro-IL-18, IL-1 $\beta$ , and IL-18 were significantly increased in the model group (Fig. 3a-i). Additionally, there were significant reductions in NLRP3, Caspase-1, IL-1 $\beta$ , and IL-18 expression levels but no significant difference in ASC, pro-Caspase-1, pro-IL-1 $\beta$ , or pro-IL-18 lev-



**Fig. 3.** PNS inhibited NLRP3 inflammasome activation and activated mitophagy via the PINK1/Parkin pathway in rats. (a,j) WB analysis of relative proteins in the sham, model, and PNS (PNS-L, -M, and -H) groups (n = 5). (b-i, k-r). Densitometry scanning of band densities was used to quantify expression of proteins in each group using Quantity One software (n = 5). \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. sham group; #  $p < 0.05$  and ##  $p < 0.01$  vs. model group; &  $p < 0.05$  and &&  $p < 0.01$  vs. PNS-L group;  $\Delta$   $p < 0.05$  and  $\Delta\Delta$   $p < 0.01$  vs. PNS-M group.

els after PNS treatment, with IL-18 in the PNS-M group and IL-1 $\beta$  and IL-18 in the PNS-H group showing the most obvious changes (Fig. 3a–i). This suggested that PNS could inhibit activation of the NLRP3 inflammasome and its downstream molecules.

To discern the role of mitochondrial autophagy in PNS-mediated neuroprotection, we evaluated changes in protein markers of mitochondrial autophagy via WB. The results indicated that mitochondrial p62 protein, as well as LC3-II/LC3-I ratio of both total-brain and mitochondrial proteins, increased significantly in MCAO rat brain tissues; expression of total-brain P62 and mitochondrial COX4I1 and TOMM20 exhibited the opposite changes (Fig. 3j–p). Furthermore, there was a significant reduction in cerebral P62 level, with insignificant changes in the ratio of cerebral LC3-II/LC3-I after PNS administration. Moreover, in the PNS-M and PNS-H groups, expression levels of COX4I1 and TOMM20 proteins were significantly decreased compared with the vehicle group, and the LC3-II/LC3-I ratio and p62 protein level in mitochondria were also increased (Fig. 3j–p). Additionally, with decreasing expression of total-brain P62, levels of mitochondrial P62, COX4I1, and TOMM20 were concurrently increased in the PNS-H group compared with the PNS-L group (Fig. 3j,m,n,p).

We then examined the effect of PNS on PINK1 and Parkin expression levels. The WB results showed significantly increased mitochondrial PINK1 and Parkin in both the model and PNS-H groups compared with the sham group (Fig. 3j,q,r). These results showed that PNS inhibited activation of NLRP3 inflammasome and activated *PINK1/Parkin*-mediated mitophagy during cerebral I/R.

### 3.4 PNS-mediated Mitophagy Suppressed NLRP3 Inflammasome Activation and Reduced Cerebral I/R Injury in Rats

We administered autophagy inhibitor 3-MA or the mitochondrial-autophagy inhibitor Mdivi-1 to rats just after MCAO to confirm the effect of PNS-mediated mitophagy on NLRP3 inflammasome activation. As shown in Fig. 4, expression levels of cerebral NLRP3, Caspase-1, IL-1 $\beta$ , and IL-18 proteins (Fig. 4a–e) along with P62 and the LC3-II/LC3-I ratio of mitochondrial proteins were significantly higher (Fig. 4a,h,i) and TOMM20 and COX4I1 were lower in the model group than in the sham group (Fig. 4f,g). However, only NLRP3, Caspase-1, IL-1 $\beta$ , and IL-18 levels were reversed; the other proteins mentioned above exhibited further significant changes over their original levels after medium-dose PNS treatment (Fig. 4a–i). Moreover, 3-MA or Mdivi-1 treatment significantly reversed the above protein changes in the PNS + 3-MA and PNS + Mdivi-1 groups (Fig. 4a–i), suggesting that suppression of mitophagy could reverse the inhibitory effect of PNS on activation of the NLRP3 inflammasome.

To further identify the involvement of mitophagy in the protective effect of PNS, we detected cerebral I/R in-

jury after PNS co-administration with 3-MA or Mdivi-1 in each group. The results showed that cerebral-infarct volume and NDS were significantly elevated in the vehicle group compared with the sham group (Fig. 4j–l). In addition, these two indicators were significantly decreased by medium-dose PNS but increased again after the subsequent addition of 3-MA or Mdivi-1 (Fig. 4j–l). Finally, we also observed similar results in cell damage rates via H&E staining (Fig. 4m). These results suggested that mitophagy mediated the inhibitory effect of PNS on activation of the NLRP3 inflammasome and had anti-brain injury effects in rats with cerebral I/R.

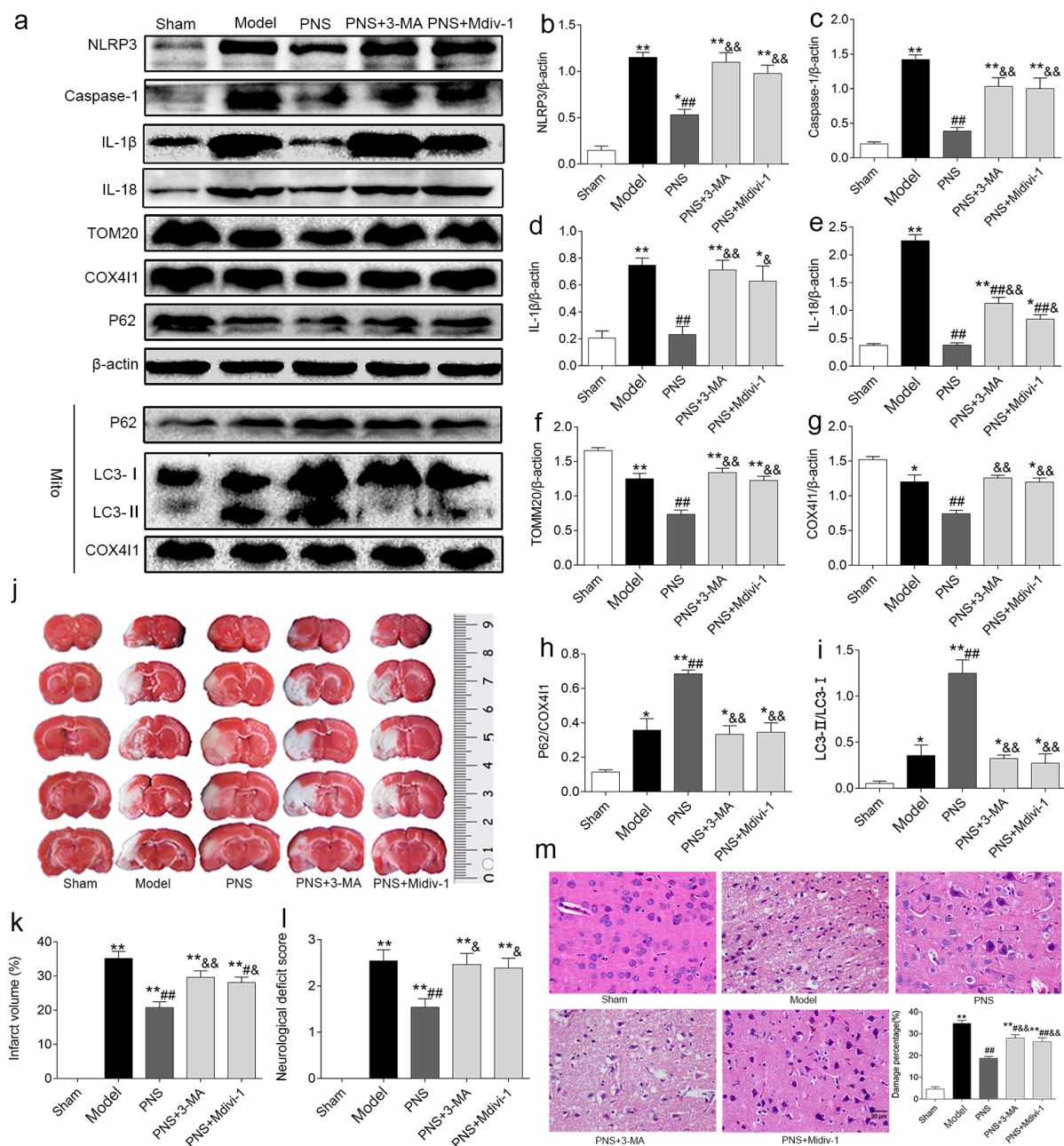
## 4. Discussion

We demonstrated that NLRP3-related protein expression reached its highest levels after 12 or 24 h of reperfusion, which showed that cerebral I/R induced both priming and activation of the NLRP3 inflammasome. Additionally, our results showed that inhibition of the NLRP3 inflammasome by Ac-YVAD-CMK and MCC950 ameliorated cerebral I/R injury in rats. Moreover, these results were consistent with findings from previous studies [30–34]. We found that the activation of the NLRP3 inflammasome and Caspase-1 aggravated cerebral I/R injury in rats after MCAO and its inhibition could alleviate cerebral I/R injury. Several studies have revealed the promotion of mitophagy-suppressed activation of the NLRP3 inflammasome in cerebral I/R [10–13].

PNS exert protective effects in cerebral ischemia through multifactorial pathways [10,20,35], which has also been confirmed in animal experiments, in vitro cell experiments, and clinical data [18,35]. However, the mechanism that PNS regulates NLRP3 inflammasome activation via mitophagy in cerebral I/R needs further research. This was therefore our focus in the current study.

We first demonstrated that PNS attenuated cerebral I/R injury in MCAO rats by decreasing the cerebral-infarct volume, NDS, and cell damage rate. Additionally, our results showed the activation of NLRP3 inflammasome and a significant decrease of Caspase-1, IL-1 $\beta$ , and IL-18 after PNS treatment. This suggested that PNS inhibited activation of the NLRP3 inflammasome, which could be an important mechanism to protect against cerebral I/R injury.

Evidence produced by other researchers has provided insight into the idea that mitophagy can be activated and plays a protective role in cerebral ischemia-reperfusion injury [7,8]. Inhibiting mitophagy can reduce cerebral ischemia-reperfusion injury [36], but such differences may be attributed to varying times of ischemia, reperfusion, or cell types. However, in the present constructed brains of male SD rats, 2-h arterial occlusion and 24-h reperfusion promoted mitophagy and inhibited the activation of inflammasome, which attenuated cerebral ischemia-reperfusion injury. Therefore, we clarified whether PNS regulates mitophagy during cerebral I/R. Our further investigation re-



**Fig. 4.** PNS-mediated mitophagy suppressed NLRP3 inflammasome activation and alleviated cerebral I/R injury in rats. (a) WB analysis of relative protein levels in the sham, model, PNS-M, PNS + 3-MA, and PNS + Mdivi-1 groups (n = 5). (b–i) Densitometry scanning of band densities was used to quantify the expression of proteins in each group (n = 5). (j) TTC-stained brain slices from the sham, model, PNS-M, PNS + 3-MA, and PNS + Mdivi-1 groups; pale areas are areas of infarction. (k) Statistical bar chart of determined cerebral-infarct volumes in each group (n = 8), created using Image-Pro Plus software. (l) Statistical bar chart of NDS evaluated according to the Longa method in each group (n = 13). (m) Pathological changes in the cerebral cortex and statistical map of the cell injury rate in each group (n = 8). \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. sham group; #  $p < 0.05$  and ##  $p < 0.01$  vs. model group; &  $p < 0.05$  and &&  $p < 0.01$  vs. PNS-M group.

vealed that in terms of total proteins after PNS treatment, LC3-II protein levels demonstrated no significant change whereas P62 protein levels decreased. However, levels of COX411 and TOMM20, as well as those of mitochondrial LC3-II and P62, increased significantly. This suggested

that PNS could selectively promote mitophagy rather than autophagy and that the relevant mechanism might be related to PNS promoting P62 translocation to mitochondria. This speculation aligned with the findings of a previous report [37]. Furthermore, it has been reported that PNS activates

mitophagy to protect the kidneys in cisplatin nephrotoxicity [38]. Multiple signaling pathways are involved in regulating mitophagy, including Parkin-dependent and Parkin-independent pathways, such as BNIP3/NIX and FUNDC1. In these regulatory pathways, Pink1/Parkin has been most thoroughly studied. After mitochondrial damage, the ability to degrade PINK1 is weakened. PINK1 accumulates on the mitochondrial outer membrane, phosphorylates Parkin, and recruits Parkin from cytoplasm to mitochondria. The activated Parkin is activated by the signal adaptor protein p62, which recognizes and binds SQSTM1. p62 can recruit ubiquitinated substances to autophagosomes by binding to LC3, eventually leading to mitochondrial degradation by lysosomes [39]. After cerebral ischemia-reperfusion, fluorescence observation of the penumbral area of the rat cortex reveals PINK1 accumulation on the mitochondrial outer membrane, Parkin translocated to mitochondria, and elevated autophagy-related proteins [40], consistent with our experimental results. Moreover, we demonstrated that PNS could further elevate PINK1 and Parkin protein levels in the cerebral mitochondria of MCAO rats, meaning that mitophagy activated by PNS was promoted through the PINK1/Parkin pathway.

In addition, when PNS were administered, NLRP3, Caspase-1, IL-1 $\beta$ , and IL-18 levels were significantly decreased. The cerebral infarct volume, NDS, and cell injury rate followed the same trend. However, 3-MA and Mdivi-1 could substantially reverse these changes, suggesting that PNS-activated mitophagy inhibited NLRP3 inflammasome activation and reduced cerebral I/R injury (Fig. 5).

## 5. Conclusions

This study extended previous observations that NLRP3 inflammasome activation exists in cerebral I/R and aggravates cerebral I/R injury. We concluded that PNS could promote mitophagy via the PINK1/Parkin pathway, inhibit NLRP3 inflammasome activation, and reduce cerebral I/R injury.

## Author Contributions

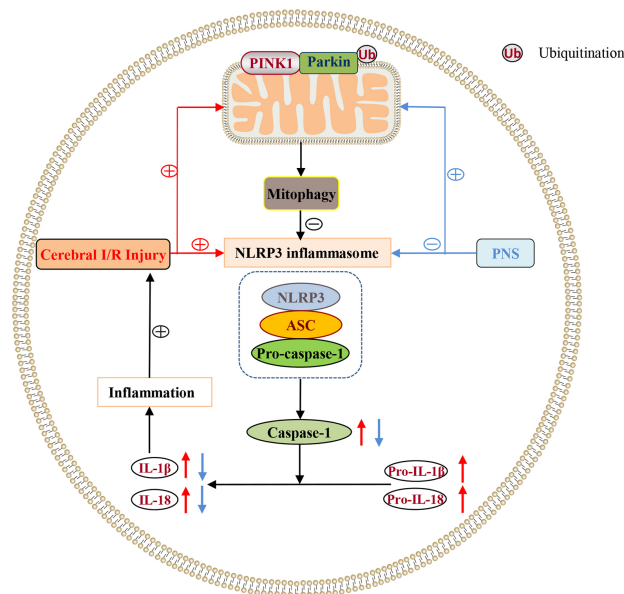
BT and QX designed the research study; QX, ZK, CL and BT performed the research; CL analyzed the data; QX, ZK and CL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

## Ethics Approval and Consent to Participate

Ethical approval for the study was obtained from the Hunan University of Chinese Medicine (HUCM) Animal Ethics Committee (Changsha, China; Approval Number: LL2021042803).

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Not applicable.



**Fig. 5. Mechanism of PNS against cerebral I/R injury mediated by the mitophagy/NLRP3 inflammasome pathway.**

PINK1 accumulated in the outer membrane of mitochondria and recruited Parkin from the cytoplasm to transfer it to the outer membranes of mitochondria. Parkin ubiquitinated mitochondrial-surface substances and mediated mitophagy, which further inhibited activation of the NLRP3 inflammasome. The first part of this experiment demonstrated that mitophagy was activated via the PINK1/Parkin pathway during cerebral I/R, which could promote priming and activation of the NLRP3 inflammasome, aggravating inflammation and, therefore, injury. In the second part of the experiment, PNS was shown to activate mitophagy via the PINK1/Parkin pathway, thus inhibiting NLRP3 inflammasome activation and alleviating cerebral I/R injury. However, the mechanism of how PNS promotes mitophagy and what role is played by the PINK1/Parkin pathway in this mechanism needs further clarification, which are limitations of this study. Further studies will be carried out on other pathways, such as ferroptosis. Revealing the anti-CIRI mechanism of PNS based on the interaction between various pathways will be an important direction of future research.

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## Conflict of Interest

The authors declare no conflict of interest.

## References

- [1] Kim J, Thayabaranathan T, Donnan GA, Howard G, Howard VJ, Rothwell PM, *et al.* Global Stroke Statistics 2019. *International Journal of Stroke*. 2020; 15: 819–838.
- [2] Wu M, Yiang G, Liao W, Tsai A, Cheng Y, Cheng P, *et al.* Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *Cellular Physiology and Biochemistry*. 2018; 46: 1650–1667.
- [3] Prabhakaran S, Ruff I, Bernstein RA. Acute Stroke Intervention. *JAMA*. 2015; 313: 1451.
- [4] Malone K, Amu S, Moore AC, Waeber C. The immune system and stroke: from current targets to future therapy. *Immunology and Cell Biology*. 2019; 97: 5–16.
- [5] Wang P, Shao B, Deng Z, Chen S, Yue Z, Miao C. Autophagy in ischemic stroke. *Progress in Neurobiology*. 2018; 163–164: 98–117.
- [6] Jiang X, Andjelkovic AV, Zhu L, Yang T, Bennett MVL, Chen J, *et al.* Blood-brain barrier dysfunction and recovery after ischemic stroke. *Progress in Neurobiology*. 2018; 163–164: 144–171.
- [7] Mizuma A, Yenari MA. Anti-Inflammatory Targets for the Treatment of Reperfusion Injury in Stroke. *Frontiers in Neurology*. 2017; 8: 467.
- [8] Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. Targeting the NLRP3 inflammasome in inflammatory diseases. *Nature Reviews Drug Discovery*. 2018; 17: 588–606.
- [9] Spel L, Martinon F. Inflammasomes contributing to inflammation in arthritis. *Immunological Reviews*. 2020; 294: 48–62.
- [10] Shao N, Yu X, Ma X, Lin W, Hao M, Kuang H. Exenatide Delays the Progression of Nonalcoholic Fatty Liver Disease in C57BL/6 Mice, which may Involve Inhibition of the NLRP3 Inflammasome through the Mitophagy Pathway. *Gastroenterology Research and Practice*. 2018; 2018: 1–9.
- [11] Mai C, Wu M, Wang C, Su Z, Cheng Y, Zhang X. Palmatine attenuated dextran sulfate sodium (DSS)-induced colitis via promoting mitophagy-mediated NLRP3 inflammasome inactivation. *Molecular Immunology*. 2019; 105: 76–85.
- [12] Xu Y, Wang J, Xu W, Ding F, Ding W. Prohibitin 2-mediated mitophagy attenuates renal tubular epithelial cells injury by regulating mitochondrial dysfunction and NLRP3 inflammasome activation. *American Journal of Physiology-Renal Physiology*. 2019; 316: F396–F407.
- [13] Ren J, Pei Z, Chen X, Berg MJ, Matrougui K, Zhang Q, *et al.* Inhibition of CYP2E1 attenuates myocardial dysfunction in a murine model of insulin resistance through NLRP3-mediated regulation of mitophagy. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*. 2019; 1865: 206–217.
- [14] Liu H, You L, Wu J, Zhao M, Guo R, Zhang H, *et al.* Berberine suppresses influenza virus-triggered NLRP3 inflammasome activation in macrophages by inducing mitophagy and decreasing mitochondrial ROS. *Journal of Leukocyte Biology*. 2020; 108: 253–266.
- [15] Tong Q, Zhu PC, Zhuang Z, Deng LH, Wang ZH, Zeng H, *et al.* Notoginsenoside R1 for Organs Ischemia/Reperfusion Injury: A Preclinical Systematic Review. *Frontiers in Pharmacology*. 2019; 10: 1204.
- [16] Peiran L, Ying L, Mingzhuo Z, Ye Y, Xiuming C. The development of a Panax notoginseng medicinal liquor processing technology using the response surface method and a study of its antioxidant activity and its effects on mouse melanoma B16 cells. *Food & Function*. 2017; 8: 4251–4264.
- [17] Zhang X, Wu J, Zhang B. Xuesaitong injection as one adjuvant treatment of acute cerebral infarction: a systematic review and meta-analysis. *BMC Complementary and Alternative Medicine*. 2015; 15: 36.
- [18] Hu S, Wu Y, Zhao B, Hu H, Zhu B, Sun Z, *et al.* Panax notoginseng Saponins Protect Cerebral Microvascular Endothelial Cells against Oxygen-Glucose Deprivation/Reperfusion-Induced Barrier Dysfunction via Activation of PI3K/Akt/Nrf2 Antioxidant Signaling Pathway. *Molecules*. 2018; 23: 2781.
- [19] Shi X, Yu W, Liu L, Liu W, Zhang X, Yang T, *et al.* Panax notoginseng saponins administration modulates pro-/anti-inflammatory factor expression and improves neurologic outcome following permanent MCAO in rats. *Metabolic Brain Disease*. 2017; 32: 221–233.
- [20] Liu L, Zhu L, Zou Y, Liu W, Zhang X, Wei X, *et al.* Panax notoginseng saponins promotes stroke recovery by influencing expression of Nogo-A, NgR and p75NGF, *in vitro* and *in vivo*. *Biological & Pharmaceutical Bulletin*. 2014; 37: 560–568.
- [21] Tang B, She X, Deng CQ. Effect of the combination of astragaloside IV and Panax notoginseng saponins on pyroptosis and necroptosis in rat models of cerebral ischemia-reperfusion. *Experimental and Therapeutic Medicine*. 2021; 22: 1123.
- [22] Li Q, Zhang T, Wang J, Zhang Z, Zhai Y, Yang G, *et al.* Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke. *Biochemical and Biophysical Research Communications*. 2014; 444: 182–188.
- [23] Yu J, Li X, Matei N, McBride D, Tang J, Yan M, *et al.* Ezetimibe, a NPC1L1 inhibitor, attenuates neuronal apoptosis through AMPK dependent autophagy activation after MCAO in rats. *Experimental Neurology*. 2018; 307: 12–23.
- [24] Zhang X, Yan H, Yuan Y, Gao J, Shen Z, Cheng Y, *et al.* Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy*. 2013; 9: 1321–1333.
- [25] Li JR, Xu HZ, Nie S, Peng YC, Fan LF, Wang ZJ, *et al.* Fluoxetine-enhanced autophagy ameliorates early brain injury via inhibition of NLRP3 inflammasome activation following subarachnoid hemorrhage in rats. *Journal of Neuroinflammation*. 2017; 14: 186.
- [26] Li JX, Yang XQ, Tang B, Liu XD, Tang YH, Deng CQ, *et al.* [Effect of astragaloside IV combined with Panax notoginseng saponins on cerebral ischemia-reperfusion injury and study of pharmacokinetics in rats]. *Zhongguo Zhong Yao Za Zhi*. 2017; 42: 3786–3794.
- [27] Li H, Deng C, Chen B, Zhang S, Liang Y, Luo X. Total saponins of Panax Notoginseng modulate the expression of caspases and attenuate apoptosis in rats following focal cerebral ischemia-reperfusion. *Journal of Ethnopharmacology*. 2009; 121: 412–418.
- [28] Yuan Y, Zheng Y, Zhang X, Chen Y, Wu X, Wu J, *et al.* BNIP3L/NIX-mediated mitophagy protects against ischemic brain injury independent of PARK2. *Autophagy*. 2017; 13: 1754–1766.
- [29] Li S, Chen L, Zhou X, Li J, Liu J. MiRNA-223-3p and let-7b-3p as potential blood biomarkers associated with the ischemic penumbra in rats. *Acta Neurobiologiae Experimentalis*. 2019; 79: 205–216.
- [30] Fann DY, Lim Y, Cheng Y, Lok K, Chunduri P, Baik S, *et al.* Evidence that NF- $\kappa$ B and MAPK Signaling Promotes NLRP3 Inflammasome Activation in Neurons Following Ischemic Stroke. *Molecular Neurobiology*. 2018; 55: 1082–1096.
- [31] Chen A, Xu Y, Yuan J. Ginkgolide B ameliorates NLRP3 inflammasome activation after hypoxic-ischemic brain injury in the neonatal male rat. *International Journal of Developmental Neuroscience*. 2018; 69: 106–111.
- [32] Zhang S, Jiang L, Che F, Lu Y, Xie Z, Wang H. Arctigenin attenuates ischemic stroke via SIRT1-dependent inhibition of NLRP3 inflammasome. *Biochemical and Biophysical Research Communications*. 2017; 493: 821–826.
- [33] Hou Y, Wang Y, He Q, Li L, Xie H, Zhao Y, *et al.* Nrf2 inhibits NLRP3 inflammasome activation through regulating Trx1/TXNIP complex in cerebral ischemia reperfusion injury.

Behavioural Brain Research. 2018; 336: 32–39.

- [34] Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M. Inhibition of Caspase-1-Like Activity by Ac-Tyr-Val-Ala-Asp-Chloromethyl Ketone Induces Long-Lasting Neuroprotection in Cerebral Ischemia through Apoptosis Reduction and Decrease of Proinflammatory Cytokines. *The Journal of Neuroscience*. 2000; 20: 4398–4404.
- [35] Meng L, Lin J, Huang Q, Liang P, Huang J, Jian C, *et al*. Panax notoginseng Saponins Attenuate Oxygen-Glucose Deprivation/Reoxygenation-Induced Injury in Human SH-SY5Y Cells by Regulating the Expression of Inflammatory Factors through miR-155. *Biological & Pharmaceutical Bulletin*. 2019; 42: 462–467.
- [36] Lan R, Zhang Y, Wu T, Ma Y, Wang B, Zheng H, *et al*. Xiao-Xu-Ming Decoction Reduced Mitophagy Activation and Improved Mitochondrial Function in Cerebral Ischemia and Reperfusion Injury. *Behavioural Neurology*. 2018; 2018: 1–12.
- [37] Shen Z, Zheng Y, Wu J, Chen Y, Wu X, Zhou Y, *et al*. PARK2-dependent mitophagy induced by acidic postconditioning protects against focal cerebral ischemia and extends the reperfusion window. *Autophagy*. 2017; 13: 473–485.
- [38] Liang X, Yang Y, Huang Z, Zhou J, Li Y, Zhong X. Panax notoginseng saponins mitigate cisplatin induced nephrotoxicity by inducing mitophagy via HIF-1 $\alpha$ . *Oncotarget*. 2017; 8: 102989–103003.
- [39] Eiyama A, Okamoto K. PINK1/Parkin-mediated mitophagy in mammalian cells. *Current Opinion in Cell Biology*. 2015; 33: 95–101.
- [40] Lan R, Wu J, Wu T, Ma Y, Wang B, Zheng H, *et al*. Mitophagy is activated in brain damage induced by cerebral ischemia and reperfusion via the PINK1/Parkin/p62 signalling pathway. *Brain Research Bulletin*. 2018; 142: 63–77.