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P-hydroxybenzyl alcohol ameliorates neuronal cerebral ischemia-reperfusion injury by activating mitochondrial autophagy through SIRT1

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Abstract. Mitochondrial autophagy serves a key role in clearing damaged mitochondria. P-hydroxybenzyl alcohol (pHBA) can improve neuronal injury induced by cerebral ischemia-reperfusion (I/R). However, the mechanism of pHBA improving I/R damage through the mitochondrial pathway remains unclear. A rat model of middle cerebral artery occlusion and reperfusion (MCAO/R) was used in the present study. The rats were treated with sirtuin 1 (SIRT1) inhibitor EX527 and pHBA for 7 days, followed by reperfusion. At 24 h after reperfusion, the infarct size was calculated and the severity of nerve damage was evaluated. Hematoxylin and eosin and Nissl staining revealed cellular changes in the ischemic penumbra. Changes in mitochondrial structure were observed using electron microscopy. Mitochondrial function was evaluated by detecting mitochondrial membrane potential (MMP), mitochondrial permeability transition pore (mPTP) and ATP levels using commercially available kits. In addition, the ischemic penumbra tissues were used for immunofluorescence staining for p62 and LC3 proteins. The expression of SIRT1 and mitochondrial autophagy-related proteins, PTEN-induced kinase 1 (PINK1) and Parkin, were detected by western blotting. Finally, apoptosis was analyzed by TUNEL staining and the expression of apoptosis-related proteins (Bax, Bcl-2 and Caspase-3) by western blotting. The results suggested that postoperative pHBA treatment may reduce the size of cerebral infarction and damage to the nervous system, and may improve cell damage in the ischemic penumbra of MCAO/R rats. Compared with rats in the untreated MCAO/R group, the mitochondrial structure of the pHBA-treated group was improved, the levels of MMP and ATP were increased, and the degree of opening of mPTP was decreased. Simultaneously, immunofluorescence and western blotting results showed that compared with the MCAO/R group, the number of LC3- and TUNEL-positive cells increased, the number of p62-positive cells decreased, SIRT1 and autophagy protein (PINK1, Parkin and LC3 II/I) expression levels increased and p62 expression decreased in the pHBA group. However, these improvements were blocked by treatment with EX527. In summary, results from the present study suggested that pHBA may improve neuronal injury in the ischemic penumbra of MCAO/R rats through SIRT1-activated mitochondrial autophagy and mitochondrial-mediated neuronal apoptosis.

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Introduction

Ischemic stroke caused by cerebral artery embolism is characterized by high morbidity (158/100,000 each year) (1), mortality (11.6% of total deaths) (2), and it accounts for ~80% of all strokes (3). At present, restoring blood flow and oxygen in the ischemic area is the primary clinical treatment for patients with ischemic stroke (4). However, the process of restoring blood flow can lead to severe secondary damage or cerebral ischemia-reperfusion (I/R) injury (CIRI) (5), including aggravating a series of pathological reactions, such as oxidative stress, neuroinflammation and apoptosis, which inhibits treatment of ischemic stroke (6). Therefore, improving CIRI is a feasible way to treat ischemic stroke. It is reported that the death of neurons is predominantly caused by hypoxia and insufficient supply of glucose, which is closely related to mitochondrial damage (7). Several studies have shown that protecting mitochondrial function can improve disorders in energy metabolism *in vitro* and inhibit apoptosis, which serves an essential role in the treatment of oxygen-/glucose-deprivation/reoxygenation in cell models and in middle cerebral artery occlusion and reperfusion (MCAO/R) model rats *in vivo* (8-10). The results suggest that maintaining the health of mitochondria can improve CIRI and is of great significance for treating ischemic stroke.

In recent years, it has been discovered that during the treatment of cerebral ischemia, damaged mitochondria can be eliminated by promotion of mitophagy, thus maintaining a healthy mitochondrial network (11). Mitochondrial autophagy

is an early defense and protection process; promoting mitochondrial autophagy causes dysfunctional or damaged mitochondria to recruit mitochondrial autophagy proteins to the mitochondrial membrane (12). PTEN-induced kinase 1 (PINK1)/Parkin is a classical mitochondrial autophagy pathway. Activation of the PINK1/Parkin pathway can promote autophagy vesicle formation, degrade damaged mitochondria, maintain intracellular homeostasis, improve mitochondrial function and protect neurons from CIRI (13). Recent studies have shown that when the PINK1/Parkin pathway is activated, it promotes mitochondrial autophagy (14,15). However, knockout of the PINK1 or Parkin gene inhibited the activation of the PINK1/Parkin pathway, hindered mitochondrial clearance and weakened the neuroprotective effect of drugs. Notably, the histone deacetylase sirtuin 1 (SIRT1) is an essential regulator of mitochondrial autophagy; it performs a vital role in the neuroprotection of cerebral ischemia (16). Previous studies have found that activating SIRT1 expression can significantly improve hippocampal neuronal damage, reduce hippocampal neuronal apoptosis and improve cognitive impairment in MCAO/R mice (17,18). However, the relationship between SIRT1 and mitochondrial autophagy during cerebral I/R is unclear.

Gastrodia elata is an herbal medicine commonly used in Asian countries to treat depression, epilepsy and other neurological diseases (19). Our previous study demonstrated that a component of *G. elata*, p-hydroxybenzaldehyde, can inhibit apoptosis and improve brain injury in MCAO/R rats (20). In addition, another component, p-hydroxybenzyl alcohol (pHBA), was found to have pharmacological effects such as anti-cerebral ischemic injury, anti-platelet aggregation and anti-inflammation (21). Previous studies found that pHBA can reduce the permeability of the blood-brain barrier during cerebral ischemia, inhibit the expression of aquaporins, reduce brain edema induced by cerebral ischemia and reduce the inflammatory response of acute cerebral ischemic injury in rats (22,23). Other studies have found that pHBA can improve cerebral ischemic injury by reducing apoptosis (24), inhibiting oxidative stress and excitotoxicity, and reducing neuronal death in the hippocampal CA1 region (25). Therefore, pHBA has a potential therapeutic effect on cerebral ischemia, but its protective effect on CIRI through SIRT1-mediated mitophagy has yet to be elucidated. In the present study, SIRT1 inhibitors were used to investigate whether pHBA-mediated mitochondrial autophagy inhibits neuronal apoptosis through SIRT1 to protect neurons in the ischemic penumbra of MCAO/R rats.

Materials and methods

Animals. Male Sprague-Dawley rats (n=48; age, 5-8 weeks; weight, 250-280 g) were purchased from the Hunan Slack Jingda Experimental Animal Co., Ltd., China. All rats were raised in a specific pathogen-free room, with a temperature of 23±2˚C, relative humidity of 40-60%, a 12-h light-dark cycle and free access to food and water. The Animal Ethics Committee of Yunnan University of Chinese Medicine (Kunming, China) approved the animal experiment (approval no. R-062021088), and the experiments followed the guidelines of the National Institutes of Health Care and Use of Laboratory Animals (26). Before the investigation, the animals were randomly divided into sham operation group (Sham), MCAO/R model group (MCAO/R), pHBA group (MCAO/R + pHBA) and EX527 group (MCAO/R + pHBA + EX527), with 12 rats in each group.

Drug administration. According to our previous studies (22,23), the effective dose of pHBA (cat. no. AF21030253; Chengdu Alfa Biotechnology Co., Ltd.) was determined to be 20 mg/kg. In the pHBA and EX527 groups, pHBA was administered by gavage once a day, for 7 days. On the 7th day, the MCAO/R model was prepared after intragastric administration of pHBA for 30 min. In the EX527 group, during the intragastric administration of pHBA, 5 mg/kg of the SIRT1 inhibitor EX527 (cat. no. 110221220509; Beyotime Biotech, Inc.) was injected into the subarachnoid space, on four occasions, once every other day. The control and MCAO groups were given equal volumes of saline.

MCAO/R model and neurological deficiency score. The rat MCAO/R model was established by operation according to previous methods (27,28). Briefly, rats were anesthetized with 5% isoflurane inhalation and maintained with 3% isoflurane (29). The right common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were exposed by an incision along the midline of the neck. A 0.36±0.02 mm diameter, rounded poly nylon monofilament (cat. no. 2636-50A4; Beijing Cinontech Co., Ltd.) was introduced into the ECA and slowly inserted into the ICA to block the start of the middle cerebral artery. After 2 h of occlusion, the nylon thread was gently removed to restore blood flow. The body temperature of all rats was maintained at 37˚C throughout the operation. The rats were evaluated by nerve score after 24 h of reperfusion. Only the arteries were separated in the Sham operation group, and no thread emboli were introduced. The neurological deficit score was given a 0-4 rating (Table I) (27).

Measurement of cerebral infarct area. The rats were anesthetized by inhalation of 5% isoflurane and sacrificed by cervical dislocation. The whole brain (n=3) was removed and frozen at -20˚C for 20 min. Each brain was cut into 5 slices, each ~2 mm thick, and stained with 2% 2,3,5-triphenyltetrazolium hydrochloride (TTC; cat. no. A610558; Sangon Biotech) at 37˚C for 20 min, before being fixed with 4% paraformaldehyde at room temperature 24 h. Normal, healthy tissue was stained red and ischemic tissue was stained white. The experimental results were analyzed by ImageJ v1.52a (National Institutes of Health).

Hematoxylin and eosin (H&E). The ischemic hemisphere tissues were prepared for H&E staining, and an H&E staining kit was used for histomorphological analysis (cat. no. KGA224; Key GEN Biotech). The brain tissue was fixed overnight at room temperature in 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). Briefly, the prepared paraffin-embedded sections of brain tissue (n=3) were baked at 60˚C for 3 h before being stained with hematoxylin for 2 min. Tissues were differentiated with 1% hydrochloric acid alcohol for several seconds, rinsed for 5 min with water and stained with eosin dye for 1 min before the residual dye solution was washed off. The sections were dehydrated and dried with an ascending ethanol gradient, then cleared with xylene to make and sealed

Table I. Neurological deficit score in rats.

Score	Animal behavioral characteristics
0	Regular activity; no neurological impairment.
1	When lifting the tail, the left forelimb was adducted and could not be completely extended.
2	The body rotated to the left when crawling.
3	The body tilted to the left when crawling.
4	The levels of consciousness decreased; unable to walk on their own.

with neutral glue. The pathological changes of neurons in the ischemic hemisphere were observed using an IXplore inverted phase contrast microscope (Olympus Corporation; OM Digital Solutions Corporation) at x400 magnification, and the images were analyzed by ImageJ.

Nissl staining. Brain paraffin sections (n=3) were routinely dewaxed at room temperature with xylene I and xylene II, each 10 min, and then gradient alcohol dehydration (100, 10 0, 95, 90, 80, 70 and 50%, each 5 min) and incubated in Nissl staining solution (cat. no. G1430; Beijing Solarbio Science & Technology Co., Ltd.) for 40 min at 50-60˚C. Sections were washed with deionized water, differentiated with Nissl differentiation solution, dehydrated with anhydrous ethanol, cleared with xylene and sealed with neutral gum. The pathological changes of neurons in the ischemic hemisphere were observed at x400 magnification using an IX83 inverted phase contrast microscope (Olympus Corporation; OM Digital Solutions Corporation). Images were analyzed by ImageJ.

Transmission electron microscopy (TEM). At 24 h after reperfusion, the rats were euthanized (n=3) and the ischemic brain tissue was isolated for TEM to observe the changes in mitochondrial ultrastructure. The fresh tissues were cut into small pieces of 1 mm^3 and soaked in precooled glutaraldehyde at 4˚C for 4 h. Sections were then dehydrated with a gradient of ethanol and acetone, as follows: 50% alcohol for 10 min; 70% alcohol for 10 min; 80% acetone for 10 min, twice; 90% acetone for 10 min, twice; anhydrous acetone for 10 min, twice. After dehydration, the slices were embedded in epoxy resin and polymerized in an incubator at 60˚C for 48 h. Ultra-thin sections of 80 nm were prepared and stained with uranium acetate and lead citrate (cat. nos. GZ02625 and GA1070, respectively; Beijing Zhongjing Keyi Technology Co., Ltd.). Tissues were stained at room temperature for 15 min. The images were observed and photographed using a JEM-1400flash transmission electron microscope (JEOL, Ltd.).

Detection of ATP levels, mitochondrial permeability transition pores (mPTP) and mitochondrial membrane potential (MMP). The levels of ATP, mPTP and MMP were detected by first grinding frozen brain tissue from the ischemic hemisphere (n=6); 100 mg of tissue was used for each test. ATP levels in brain tissue were detected using an ATP Detection kit (cat. no. A095-1-1; Nanjing Jiancheng Biological Engineering Research Institute). A Mitochondrial Extraction kit (cat. no. SM0020; Beijing Solarbio Science & Technology Co., Ltd.) was used to isolate mitochondria from brain tissue. BCA Protein Assay kits (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.) were used to detect mitochondrial concentration. The mitochondrial swelling method was used to determine the degree of opening of mPTP (cat. no. GMS10101; Shanghai Genmed Gene Medicine Technology Co., Ltd.). Briefly, after the mitochondria were extracted, the concentration of brain mitochondrial protein was adjusted to 10 mg/ml by the BCA method, and 20 μl mitochondrial samples were added to a 96-well plate. A total of 170 μl GERMED buffer (Reagent A) was added to each well, and the initial reading of 0 min was obtained. After that, 10 μl GERMED expansion solution (Reagent B) was added immediately at room temperature, and the changes of 10 min absorbance were dynamically recorded by the VariosKan Flash Multi-detection Microplate Reader (Thermo Fisher Scientific, Inc.). The actual absorption readings were calculated as, optical density $(OD)_{0\ min}$-$OD_{10\ min}$; if the actual absorption reading is high, this indicates high mitochondrial expansion. The VariosKan Flash Multi-detection Microplate Reader was used to detect the level of MMP (cat. no. GMS10013.1; Shanghai Genmed Gene Medicine Technology Co., Ltd.), according to the manufacturer's protocol.

Immunofluorescence. Ischemic hemispheres of rats were selected for immunofluorescence detection (n=3). The paraffin-embedded sections (10 μm each) were blocked for 10 min at 37˚ containing 5% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology). Primary antibodies anti-LC3 (1:200; cat. no. 4108; Cell Signaling Technology, Inc.) and p62 (1:250; cat. no. 18420-1-AP; Proteintech Group, Inc.) were incubated overnight at 4˚C. Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; cat. no. ab150077; Abcam) and rabbit anti-mouse (1:1,000; cat. no. ab150125; Abcam) secondary antibodies were incubated at 37˚C for 1 h. The nuclei were stained with DAPI for 5 min at room temperature in the dark, and then the slides were sealed with 90% glycerol. The sections were observed and photographed under x400 magnification with a Zeiss LSM laser-scanning confocal microscope (Carl Zeiss AG).

Western blotting. Frozen brain tissues (n=3) were lysed using ice-cold RIPA buffer (PSMF:RIPA lysate, 1:100; cat. no. 051021210825; Beyotime Institute of Biotechnology) for 20 min. The lysate was centrifuged at 12,000 g for 5 min at 4˚C. Total protein was quantified using the BCA method. Proteins (80 μg) were separated by SDS-PAGE on an 8% gel. The separated proteins were subsequently transferred onto a PVDF membrane (Bio-Rad Laboratories, Ltd.) and was blocked in 5% skim milk at room temperature for 2 h. The membrane was incubated overnight with primary antibodies against SIRT1 (1:1,000; cat. no. ab110304; Abcam), PINK1 (1:500; cat. no. sc-517353; Santa Cruz Biotechnology), Parkin (1:1,000; cat. no. 4211; Cell Signaling Technology), LC3 A/B (1:1,000; cat. no. 4108; Cell Signaling Technology), p62 (polyclonal; 1:500; cat. no. 18420-1-AP; Proteintech) and β-Actin (1:1,000; cat. no. ab8226; Abcam) at 4˚C. HRP-conjugated goat anti-rabbit IgG H&L (1:10,000; cat. no. ab6721) and rabbit anti-mouse IgG H&L (1:10,000; cat. no. ab6728;

Abcam) secondary antibodies were added and the membranes a were incubated for 1 h at room temperature. The protein bands were visualized by ECL using SuperSignal™ West Atto Ultimate Sensitivity Substrate (cat. no. A38555; Thermo Fisher Scientific, Inc.), and images of the bands were captured using a Tanon 6600 Luminescent Imaging Workstation (Tanon Science and Technology Co., Ltd.) and analyzed using ImageJ software.

TUNEL assay. The ischemic hemispheres of rats (n=3) were stained with TUNEL Detection Kit (n=3; cat. no. C1088; Beyotime Institute of Biotechnology). The paraffin sections were routinely dewaxed, as follows: After baking at 60˚ for 60 min, the sections were incubated twice with xylene at room temperature for 10 min each time, and rehydrated in a descending ethanol series (100, 95, 80 and 75%; 5 min each). Subsequently, 20 μg/ml protease K (without DNase) was added, and the tissues were incubated at 37˚C for 15-30 min. Sections were washed three times with PBS before 50 μl of TUNEL detection solution was added to each sample and incubated at 37˚C for 1 h. The sections were then washed three times with PBS, and the nuclei were counterstained with DAPI for 5 min at room temperature. The sections were observed and images captured using a Zeiss LSM laser confocal microscope (Carl Zeiss AG) at x400 magnification. ImageJ software was used to calculated the fluorescence intensity of positive cells in three non-overlapping visual fields.

Statistical analysis. GraphPad Prism software (version 9.0.0; Dotmatics) was used for statistical analysis. For normal distribution, the Kolmogorov-Smirnov test was used. If the data were normally distributed, and if the variances were homogeneous, they were compared using ANOVA followed by Bonferroni's multiple comparison post hoc test. If the variances were unequal, Welch's ANOVA test was used followed by Dunnett's T3 multiple comparison tests. All values are presented as mean ± SEM. $P<0.05$ was used to indicate a statistically significant difference.

Results

pHBA improves cerebral infarction area, neurological function and pathological injury after MCAO/R injury. An overview of the design of the present study is shown in Fig. 1A. The chemical structure of pHBA is a phenolic compound consisting of a benzene ring and two hydroxyl groups (Fig. 1B). Our previous study examined a pHBA derivative, p-hydroxybenzaldehyde, which comprised a benzene ring and an aldehyde group with a hydroxyl group (20). Following drug administration, MCAO modelling and reperfusion, the neurological deficit score of rats was evaluated. It was found that the neurological deficit score of the MCAO/R group was significantly higher compared with that of the Sham group (Fig. 1C); neurological deficit scores were decreased after pHBA treatment compared with untreated MCAO/R. The rats were euthanized and the cerebral infarction area was measured. The TTC staining results showed that the area of cerebral infarction in the MCAO/R group was significantly larger compared with the Sham group (Fig. 1D and E). Compared with MCAO/R group, the cerebral infarction area in the pHBA group was significantly smaller. In addition, H&E and Nissl staining revealed that the neurons in the Sham operation group showed clear nuclei and Nissl bodies (Fig. 1F and G, respectively). H&E staining demonstrated that the nerve cells in the Sham group were arranged neatly and the morphological structure was normal, whereas the fiber arrangement in the MCAO/R model group was disordered, and the nuclei were pyknotic. The morphology and structure of nerve cells in the pHBA-treated group were more neatly arranged compared with those in the model group, and the cell bodies were transparent. In the EX527 co-treatment group, fibers were displaced, cell distribution was disordered and the cell body was blurred. Nissl staining showed that the structure of Nissl corpuscles was clear and arranged neatly in the Sham operation group. Cell damage in the MCAO/R group was characterized by nuclear pyknosis and disordered arrangement of Nissl bodies. Compared with the MCAO/R group, the injury of nerve cells in the pHBA group was alleviated, and the shape and size of Nissl bodies were uniform. Compared with the pHBA group, the arrangement of Nissl bodies in the EX527 group was disordered, and some of the nuclear pyknosis was deeply stained. These data suggested that pHBA may improve infarct size, neurological function and neuronal damage after CIRI, potentially through the SIRT1 pathway.

pHBA ameliorates mitochondrial microstructure damage and dysfunction after MCAO/R injury. To determine whether pHBA can improve the mitochondrial dysfunction caused by MCAO/R injury, the microstructure of mitochondria in the ischemic penumbra was observed by TEM. The results showed that the mitochondria swelled and the cristae disappeared in the MCAO/R group compared with the Sham group (Fig. 2A). Compared with the MCAO/R group, pHBA could reduce mitochondrial swelling and cristae disappearance (Fig. 2A). Compared with the pHBA group, EX527 aggravated mitochondrial swelling and cristae disappearance (Fig. 2A). In addition, the brain tissues of ischemic hemispheres were isolated, and the levels of mitochondrial ATP, mPTP and MMP were analyzed. The data showed that, compared with the Sham group (ATP, 40.64±2.28 μm/g protein; mPTP, 0.016±0.001 OD; MMP, 5.58±0.36 relative fluorescence) (Fig. 2B-D, respectively), the level of ATP (14.84±1.29 μm/g protein) and MMP (1.74±0.18 relative fluorescence) in the MCAO/R group decreased significantly, whereas the openness of mPTP (0.033±0.002 OD) increased. Treatment with pHBA reversed the effect of MCAO/R on ATP (29.67±1.14 μm/g protein), mPTP (0.018±0.001 OD) and MMP (4.08±0.40 relative fluorescence). However, these improvements were inhibited by EX527 co-treatment. These results suggested that part of the protective effect of pHBA on MCAO/R damage may be to improve mitochondrial function through the SIRT1 pathway.

pHBA activates mitochondrial autophagy in neurons through SIRT1. To verify whether pHBA activates mitochondrial autophagy through the SIRT1 pathway, immunofluorescence staining was performed. The data showed that the percentage of LC3-positive cells in the MCAO/R group was significantly lower (6.93±0.70%) compared with that in the Sham group (26.50±1.52%) (Fig. 3A-D), whereas the rate of p62-positive cells was significantly higher in the MCAO/R group (43.81±1.32 vs. 58.37±2.44%). Compared with the MCAO/R group, pHBA

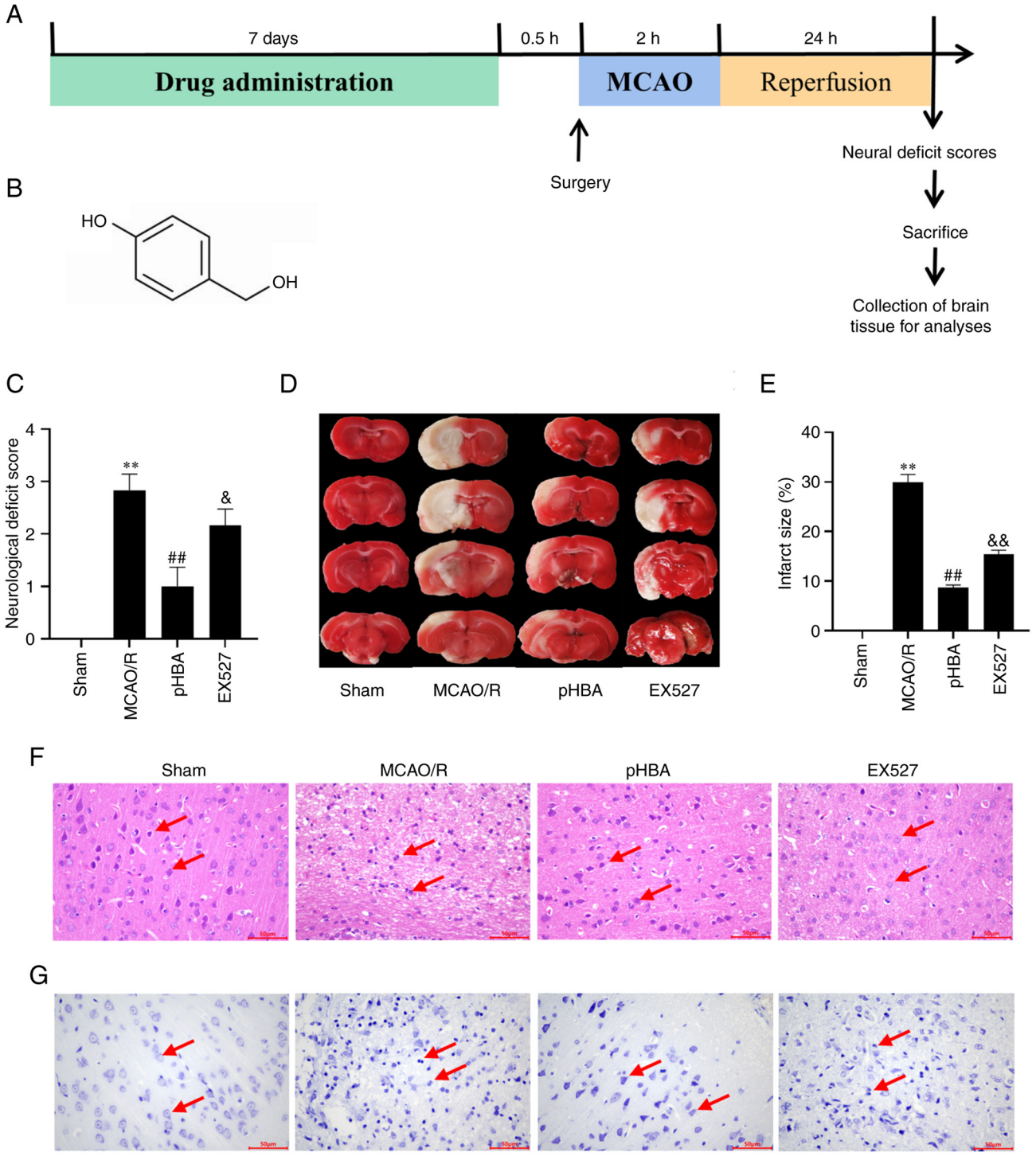

Figure 1. pHBA treatment reduces infarct size, neurological deficit and neuronal damage after cerebral I/R. (A) Timeline schematic of MCAO/R surgery-induced cerebral I/R. Here, 0.5 h indicates that the model will be established following 0.5 h of pHBA intragastric administration on the 7th day. (B) Chemical structure of pHBA. (C) Neurological deficit score of each group (n=12). At 24 h after reperfusion, (D) brain sections from rats in each group (n=3) were stained with TTC, and (E) quantitative analysis was conducted to determine the infarct size. (F) Representative H&E stained tissue sections from each group (n=6); arrows indicate nerve cells. Scale bar, 50 μm. (G) Representative Nissl stained tissue sections from each group (n=6); the arrows indicate Nissl bodies. Scale bar, 50 μm. All data are presented as the mean ± SEM. **P<0.01 vs. Sham; $^{\#\#}$P<0.01 vs. MCAO/R; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. pHBA. EX527, sirtuin inhibitor; I/R, ischemia-reperfusion; MCAO/R, middle cerebral artery occlusion and reperfusion; pHBA, p-hydroxybenzyl alcohol; TTC, 2,3,5-triphenyltetrazolium hydrochloride.

significantly increased the percentage of LC3-positive cells (53.16±2.50%) and decreased p62-positive cells (8.65±0.76%); however, inhibiting the expression of SIRT1 reversed these effects. Western blotting results showed that pHBA significantly increased the relative protein expression levels of SIRT1, PINK1, Parkin and LC3 II/I, and significantly decreased the relative expression of p62 (Fig. 3E-J); these effects were inhibited after treatment with the EX527 SIRT1 inhibitor. These results suggested that the regulation of mitochondrial autophagy by pHBA may be related to SIRT1.

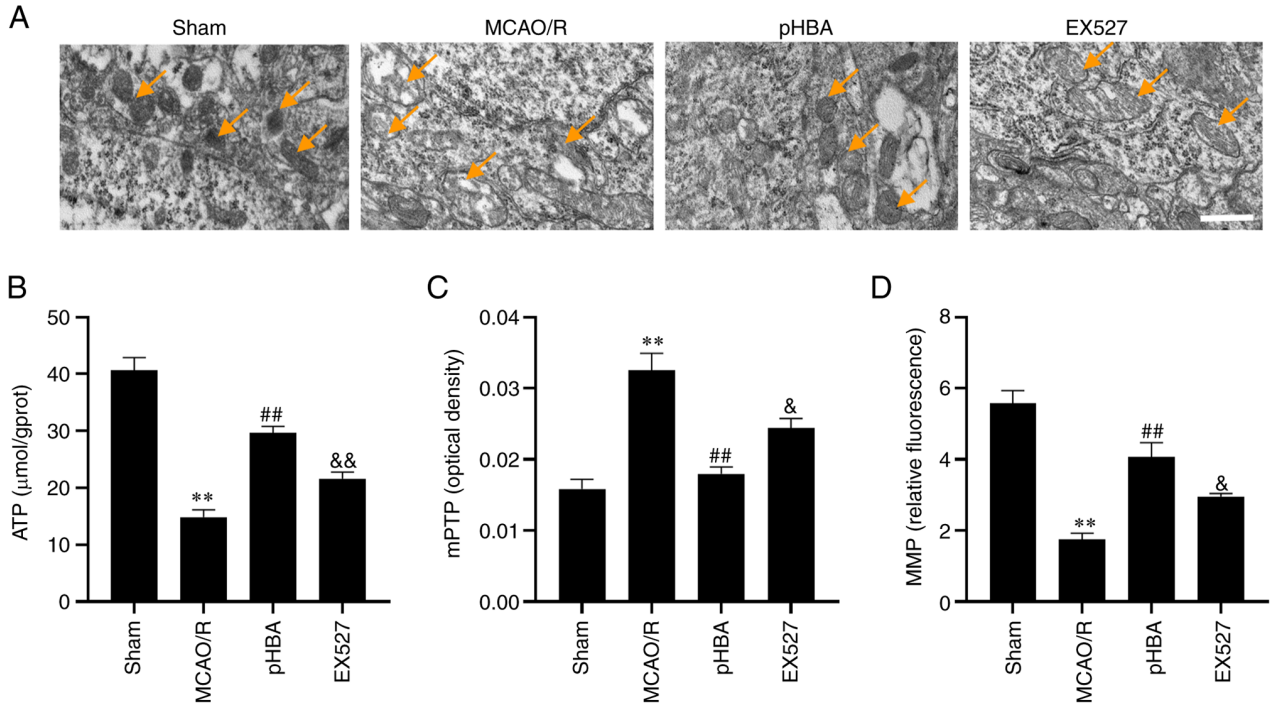

Figure 2. pHBA improves microstructure damage and dysfunction of mitochondria following cerebral ischemia-reperfusion. (A) Representative transmission electron microscopy images of tissue sections from rats each group (n=3) showing mitochondria (arrows) in the ischemic penumbra affected tissues. In the MCAO/R group, the shape of the mitochondria (arrows) not clear, swelling occurred and the cristae disappeared. In the pHBA group, the morphology of the mitochondria indicated by the arrow was clear, and the structure was complete. In the EX527 group, the mitochondria indicated by the arrow were swollen, and the cristae were broken. Scale bar, 500 nm. (B) ATP level, (C) degree of opening of mPTP and (D) MMP in rats from each group (n=6). All data are presented as the mean ± SEM. **P<0.01 vs. Sham; $^{\#\#}$P<0.01 vs. MCAO/R; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. pHBA. EX527, sirtuin inhibitor; MCAO/R, middle cerebral artery occlusion and reperfusion; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; pHBA, p-hydroxybenzyl alcohol.

pHBA inhibits neuronal apoptosis induced by MCAO/R injury by activating SIRT1. The effect of SIRT1 on neuronal apoptosis was examined by TUNEL. The results showed that the number of TUNEL-positive cells increased significantly in the MCAO/R group, and this was decreased significantly after administration of pHBA (Fig. 4A and B); however the ameliorative effect of pHBA was reversed after co-treatment with EX527. In addition, western blotting results showed that the protein expression of Bax and cleaved caspase-3 protein in the MCAO/R group was higher compared with that in the Sham group, whereas the expression level of Bcl-2 was lower in the MCAO/R group (Fig. 4C-G). Compared with the MCAO/R group, pHBA treatment could inhibit the expression of Bax and cleaved caspase-3 protein in MCAO/R injury and significantly increase the expression level of Bcl-2. Notably, these effects were blocked by the SIRT1 inhibitor. These results suggested that pHBA may inhibit apoptosis through SIRT1.

Discussion

Our previous study showed that p-hydroxybenzaldehyde, a component of *G. elata*, can protect MCAO/R rats from oxidation and apoptosis (20). Compared with the MCAO/R group, p-hydroxybenzaldehyde treatment reduced mitochondrial ROS and malondialdehyde contents, reduced mPTP opening, and decreased Bax and caspase-3 protein expression levels. In addition, ATP content, cytochrome *c* oxidase and total superoxide dismutase activities, as well as protein expression of Bcl2 all increased (20). Similarly, results from the present study demonstrated that pHBA treatment increased ATP level, mPTP opening and Bcl2 expression, whereas mPTP opening and Bax and cleaved caspase-3 expression levels were decreased compared with the untreated MCAO/R model group. In addition, this study found that pHBA may protect CIRI in MCAO/R model rats by autophagy and apoptosis induced by mitochondrial pathway. The results suggested that pHBA treatment may significantly reduce the neurological deficit score and cerebral infarction and significantly improve cell damage, such as nuclear condensation and reduction of Nissl bodies caused by MCAO/R injury. It was also discovered that pHBA may activate mitochondrial autophagy following brain injury. In addition, pHBA treatment promoted the expression of SIRT1 after MCAO/R, which was inhibited after EX527 treatment. Notably, pHBA increased the expression of the anti-apoptotic protein Bcl-2 and decreased the expression of the pro-apoptotic protein Bax in MCAO/R model rats, suggesting that pHBA may inhibit neuronal apoptosis. Therefore, these results indicate that pHBA may have a neuroprotective effect on MCAO/R rats by activating the SIRT1/mitochondrial autophagy pathway and inhibiting neuronal apoptosis.

Mitochondrial injury is a marker of ischemic stroke. During cerebral I/R, the structure and function of mitochondria are damaged, which can aggravate pathological processes, such as oxidative stress, calcium overload and inflammatory (27). However, these pathological processes also aggravate mitochondrial damage, forming a negative chain of events (14). Mitochondrial damage is mainly characterized by a decrease of MMP and an impairment of the ability to produce ATP. At

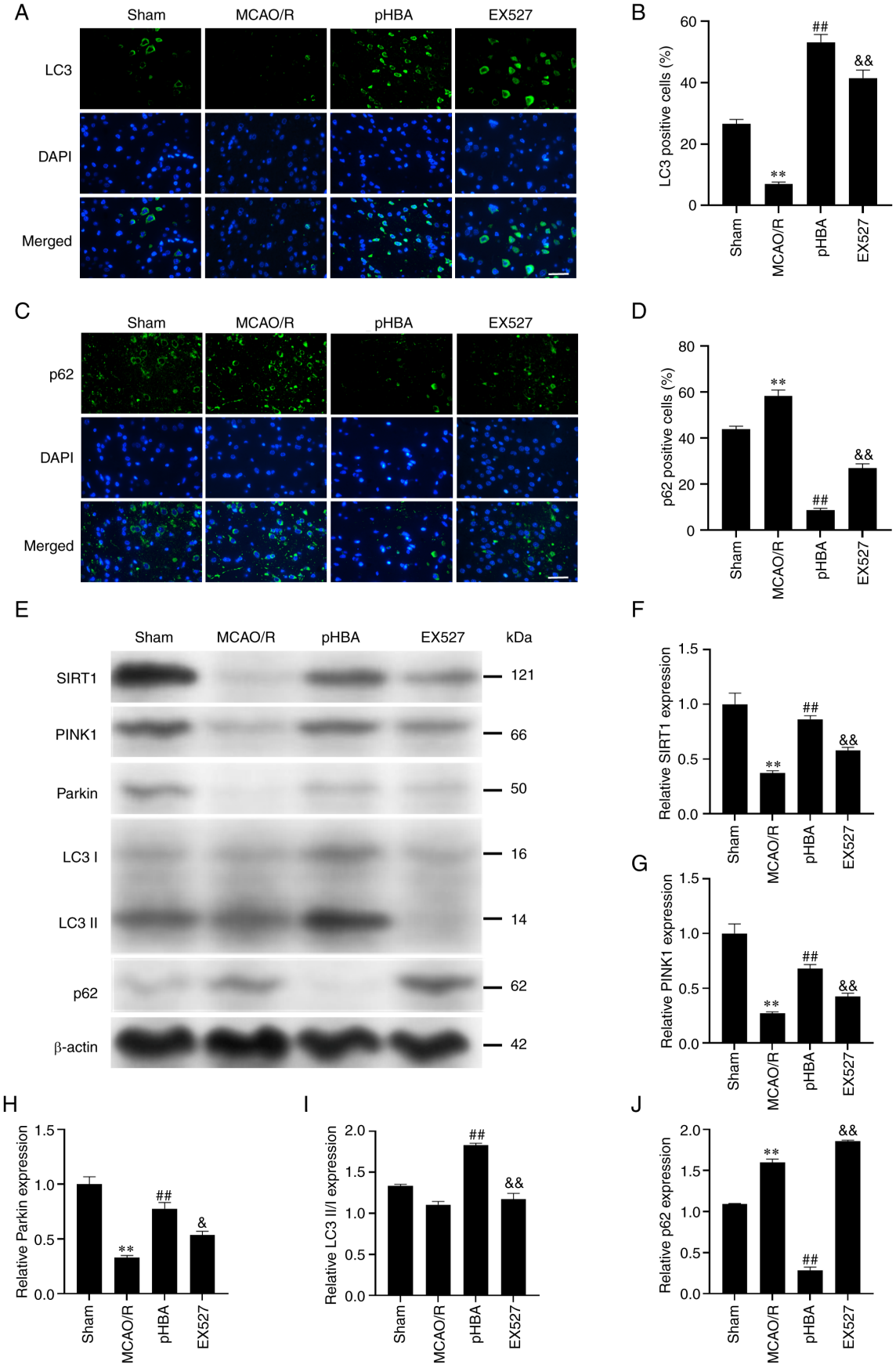

Figure 3. pHBA activates neuronal mitochondrial autophagy through SIRT1. (A) Representative images of LC3 fluorescence staining (green) on the ischemic tissue sections of each group (n=3); nuclei were stained with DAPI (blue). Scale bar, 50 μm. (B) Percentage of LC3-positive cells across each group. (C) Representative images of p62 fluorescence staining (green) on the ischemic tissue sections of each group (n=3); nuclei were stained with DAPI (blue). Scale bar, 50 μm. (D) Percentage of p62-positive cells across each group. (E) Representative western blotting images and semi-quantitative analysis of (F) SIRT1, (G) PINK1, (H) Parkin, (I) LC3 II/I and (J) p62 in each group. All data are presented as the mean ± SEM.**P<0.01 vs. Sham; $^{\#\#}$P<0.01 vs. MCAO/R; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. pHBA. pHBA, p-hydroxybenzyl alcohol; SIRT1, sirtuin 1; PINK1, PTEN-induced kinase 1

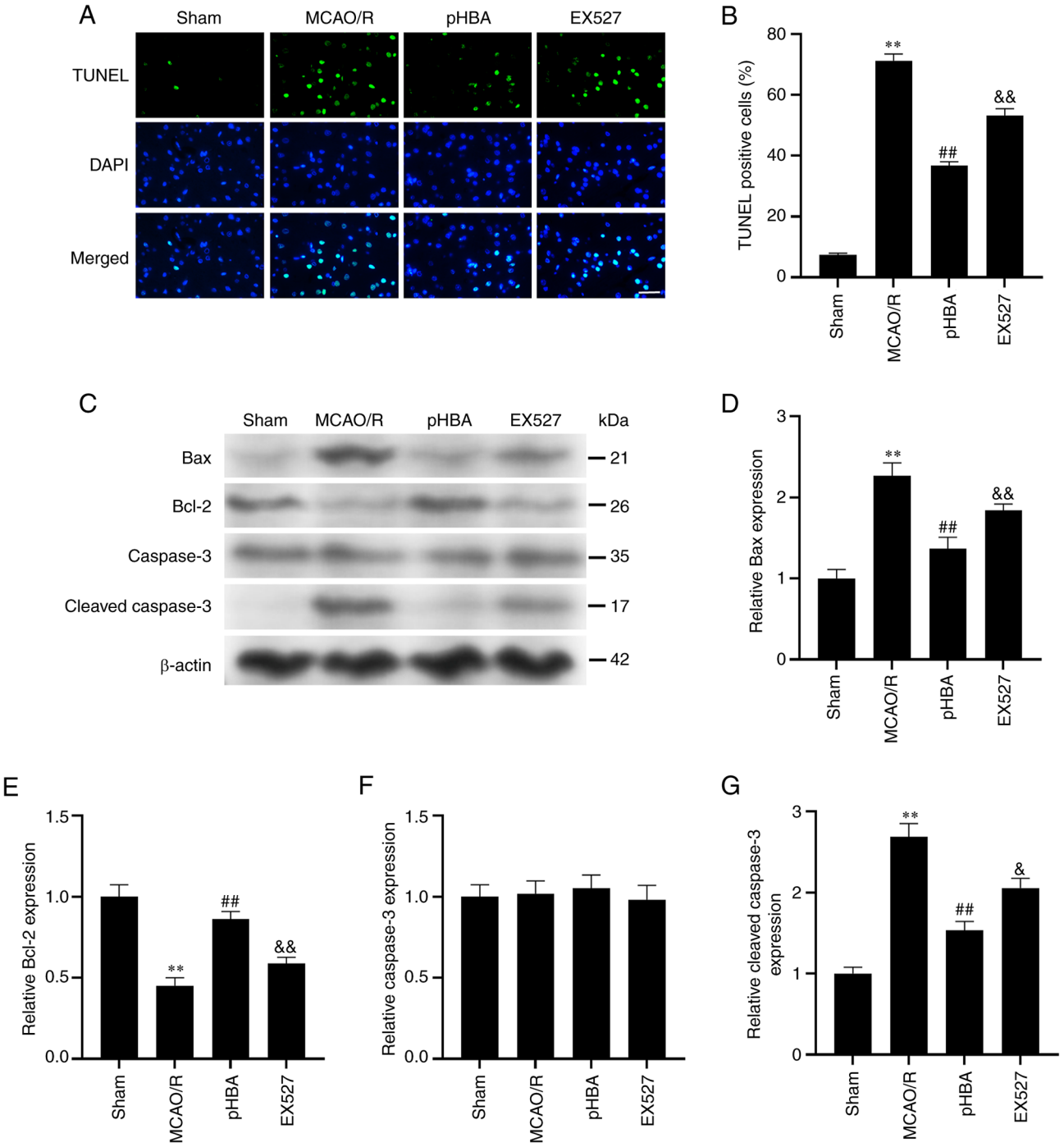

Figure 4. pHBA inhibits MCAO/R injury-induced neuronal apoptosis through sirtuin 1. (A) Representative TUNEL staining images (green) from each group (n=3); nuclei were stained with DAPI (blue). Scale bar, 50 μm. (B) Quantitative analysis of apoptosis determined by TUNEL staining. (C) Representative western blotting images and semi-quantitative analysis of (D) Bax, (E) Bcl-2, (F) caspase-3 and (G) cleaved caspase-3 in each group (n=3). All data are presented as the mean ± SEM. **P<0.01 vs. Sham; $^{\#\#}$P<0.01 vs. MCAO/R; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. MCAO/R, middle cerebral artery occlusion and reperfusion; pHBA, p-hydroxybenzyl alcohol.

the same time, these pathological factors (ATP and MMP) will also open mPTP and release apoptosis factors and accelerate nerve cell death (30). Huang *et al* (31) found that brain microvascular endothelial cells injury induced by hypoxia-glucose deprivation and reoxygenation (OGD/R) led to the opening of mPTP. Nonetheless, hydroxysafflor yellow A treatment not only inhibited the opening of mPTP, but also reduced the release of apoptotic factors. Wang *et al* (32) found that influenza A virus PB1-F2 protein treatment could increase levels of MMP and ATP, and prevent damage to mitochondrial function caused by CIRI. Consistent with these data, the present study found that pHBA treatment improved the ultrastructure and morphology of mitochondria in the ischemic penumbra of MCAO/R rats. It also reduced the opening of mitochondrial mPTP in ischemic hemispheres and increased the levels of MMP and ATP, reversing damage to mitochondrial function. It is suggested that pHBA may improve mitochondrial dysfunction after CIRI.

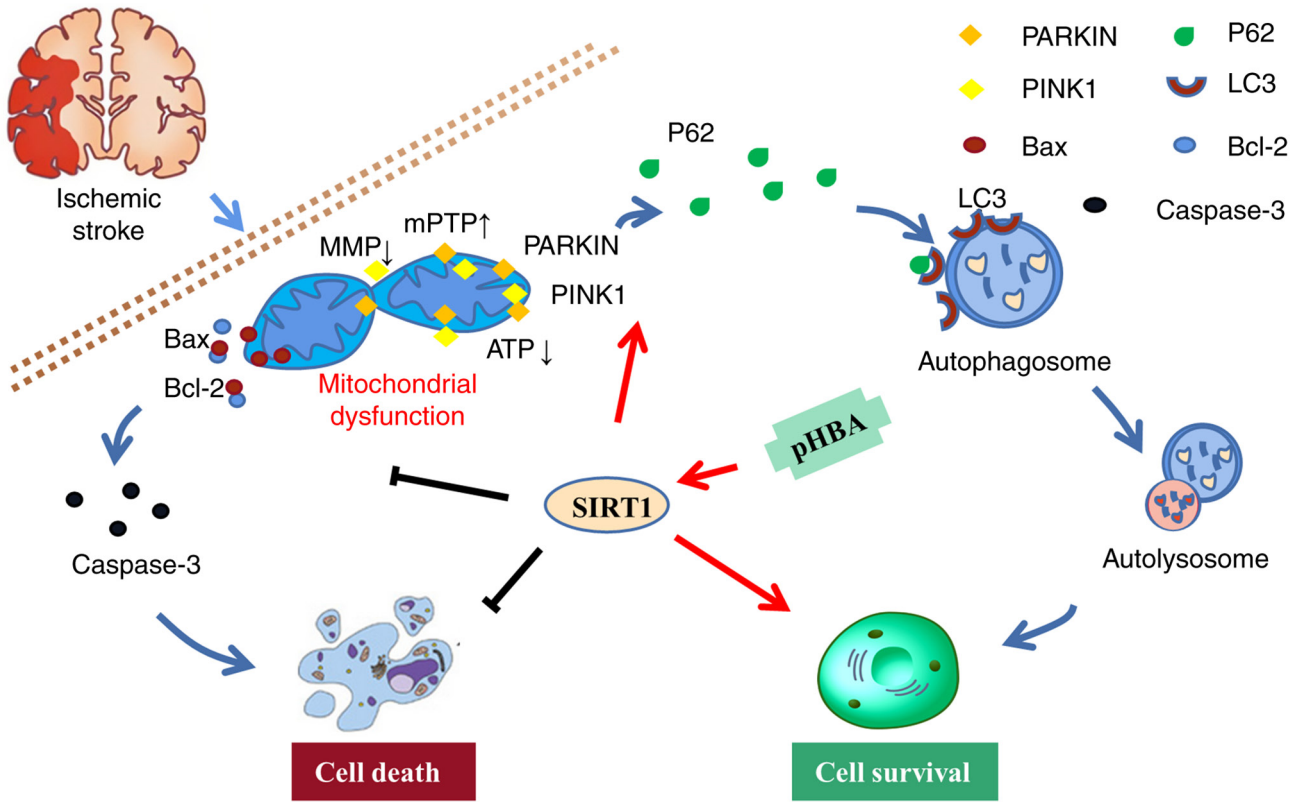

Figure 5. Illustration of the ameliorating effects of pHBA on MCAO/R-induced brain injury by activating mitophagy and inhibiting mitochondria-mediated apoptosis through the SIRT1 pathway. Mitochondrial damage was induced in rats following MCAO/R surgery. SIRT1 can promote the mitochondrial recruitment of PINK1 and Parkin, further promote the binding of p62 and LC3, contribute to the formation of autophagosomes, participate in mitochondrial autophagy, clear the damaged mitochondria induced by MCAO/R, and improve Cell survival. In addition, damaged mitochondria cause the pro-apoptotic protein Bax to move to the mitochondrial membrane, which triggers the activation of caspase-3 and promotes apoptosis. pHBA may serve a neuroprotective role by activating SIRT1 to promote mitochondrial autophagy and inhibit apoptosis. The black line represents inhibition, and the red arrow represents promotion. MCAO/R, middle cerebral artery occlusion and reperfusion; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; pHBA, p-hydroxybenzyl alcohol; PINK1, PTEN-induced kinase 1; SIRT1, sirtuin 1.

Mitochondrial autophagy is the primary way to identify and remove damaged mitochondria, to maintain the mitochondrial network's stability (4), and to recycle decomposed cellular components to maintain cellular homeostasis (14). When mitochondrial autophagy occurs, PINK1 in the cytoplasm accumulates on the outer membrane of damaged mitochondria and recruits Parkin to initiate mitochondrial autophagy (32,33). Subsequently, it promotes the binding of p62 and LC3 to activate autophagosomes, resulting in the degradation of damaged mitochondria by lysosomes, which supports the use of p62 and LC3 proteins as indicators to verify autophagy activation. In the present study, it was found that p62 decreased and LC3 increased after pHBA treatment. Similarly, western blotting showed that pHBA treatment promoted the expression of mitochondrial autophagy-related proteins PINK1, Parkin and LC3 II/I, and decreased the expression of p62. It is suggested that pHBA may activate mitochondrial autophagy after cerebral I/R.

Neuronal apoptosis is the main cause of brain injury after intracerebral hemorrhage, which can lead to brain infarction and loss of nerve function (34). Mitochondrial-mediated apoptosis is one of the main pathways of apoptosis. When cells are injured, the expression levels of anti-apoptotic protein Bcl-2 decreases, and the expression of the pro-apoptotic protein Bax increases; Bax accumulates on the mitochondrial membrane and reduces MMP (35). Additionally, Bax opens mPTP, releases apoptotic signals and activates caspase-3 to trigger apoptosis (36). It is suggested that the function of mitochondria can affect apoptosis, and it has been reported that improving mitochondrial function can reduce apoptosis (7,37). In the present study, mitochondrial autophagy was shown to maintain a healthy mitochondrial network, which can enhance the level of mitochondrial MMP and ATP, and reduce the opening of mPTP, indicating that mitochondrial autophagy can affect apoptosis. Several studies have found that promoting mitochondrial autophagy can reduce mitochondrial-mediated neuronal apoptosis (13,38,39). Similarly, in the present study, pHBA treatment significantly reduced the number of TUNEL-positive cells and the protein expression of Bax and cleaved caspase-3, and upregulated the expression of Bcl2. These findings suggested that the protective effect of pHBA on CIRI is related to the inhibition of mitochondrial-mediated apoptosis.

SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase involved in various biological processes, including metabolism, apoptosis, aging, oxidative stress, energy metabolism and inflammation (40,41). Studies have shown that SIRT1 is involved in the regulation of energy metabolism, autophagy, apoptosis and oxidation following ischemic stroke (42-44). In addition, SIRT1 also serves a

crucial role in eliminating damaged mitochondria through mitochondrial autophagy. A number of studies have found that the PINK1/Parkin signaling pathway can be activated by SIRT1 (45). For example, Huang *et al* (46) found that SIRT1 can induce mitochondrial autophagy by activating the PINK1/Parkin signaling pathway, reducing brain CIRI damage and OGD/R human neuroblastoma cell damage. In addition, Shao *et al* (47) found that SIRT1-mediated PINK1/Parkin-dependent mitochondrial autophagy participates in the neuroprotective effect of mouse hippocampal HT22 OGD/R model cells on mitochondrial dysfunction. In the present study, pHBA treatment upregulated the expression of SIRT1, and SIRT1 may be an upstream autophagy protein. The results also showed that the use of SIRT1 inhibitor, EX527, repressed the improvement effects of pHBA and decreased the expression of SIRT1 protein. Inhibition of mitochondrial autophagy aggravated mitochondrial dysfunction, reduced the expression of anti-apoptotic factors and increased the expression of pro-apoptotic factors. In addition, EX527 co-treatment negatively affected neurological function and cerebral infarction area, and inhibited the improvement of organelles such as neuronal nuclei and Nissl bodies. Therefore, the present study concluded that the protective effect of pHBA on CIRI may be related to the promotion of mitochondrial autophagy and the inhibition of mitochondrial-mediated apoptosis by the SIRT1 pathway.

In conclusion, the present study found that pHBA has a neuroprotective effect against CIRI, potentially elicited by activation of the SIRT1/mitochondrial autophagy pathway and the inhibition of apoptosis in ischemic penumbra, as illustrated in Fig. 5. Currently, pHBA can be artificially synthesized (48), and using commercial pHBA in the experiment will not harm *G. elata* species. These findings suggested that the natural component pHBA may be a promising option for treating CIRI, and the SIRT1/mitophagy pathway may be a target for future treatment. However, there are some limitations in the present study, including the lack of detection of additional autophagy markers (ATG5, ATG7 and ATG12) and the lack of relevant cell experiments to analyze the protein or mRNA expression levels of other apoptosis-related proteins, such as cytochrome *c* or cleaved caspase-9. In addition, the absence of observation of autophagosome and autolysosome formation using electron microscopy is also a limitation of this study and will form part of our next research direction.

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Availability of data and materials

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

Authors' contributions

XD, PC and XY designed the experiments. YL, LY and XY performed the experiments. The results were analyzed by XY. LY wrote the manuscript, and XD edited the manuscript. XD, PC and LY confirm the authenticity of all the raw data. All the authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of Yunnan University of Chinese Medicine (Kunming, China; approval no. R-062021088).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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