

Rhodiola rosea glycosides modulate ER β /BNIP3-mediated mitochondrial autophagy in cerebral ischemic neuroprotection

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Abstract

Objectives: Objective To investigate the neuroprotective effect and mechanism of *Rhodiola rosea* glycosides(SAL) on cerebral ischemia/reperfusion injury (CIRI) rats through the mitochondrial autophagy pathway mediated by estrogen receptor β (ER β)/B-cell lymphoma-2/adenovirus E1B interacting protein 3 (BNIP3).

Methods: 135 SD rats were divided into OVX and model groups (OVX+MCAO/R), estradiol control group (OVX+MCAO/R+E2), ER β inhibitor group (OVX+MCAO/R+SAL+PHTPP), autophagy inhibitor group (OVX+MCAO/R+SAL+Mdivi-1), and SAL low, medium, and high dose group (OVX+MCAO/R+SAL). MCAO/R+SAL), the middle cerebral artery embolism (MCAO/R) model was constructed using the wire embolism method, and reperfusion was performed for 24h after 1h of ischaemia. Neurological function scoring was performed after 24h of reperfusion; TTC staining was used to detect the volume of cerebral infarction; water content of brain tissue was determined by wet and dry gravimetry; the permeability of blood-brain barrier was determined by Evans blue (EB) content; the levels of oestrogen (E2), superoxide dismutase (SOD) and malondialdehyde (MDA) were analysed by the kit; neuron pathology was observed on the ischemic side by hematoxylin and eosin (HE) staining; and the damage of neuron in brain tissue by Nissl staining was observed by the kit. Nissl staining was used to observe neuronal damage in brain tissue; transmission electron microscopy (TEM) was used to observe mitochondrial autophagosomes; and Western blotting was used to detect the expression of ER β and autophagy-related proteins, BNIP3, NIX, Beclin-1 and LC3.

Results: There was no statistical difference ($P>0.05$) in Longa score, TTC, brain water content, EB, E2 and oxidative stress level, HE, Nissl's staining and transmission electron microscopy in the Con group compared with the OVX group, indicating that removal of the ovaries had no effect on the subsequent experiments; whereas, there was a statistically significant difference ($P<0.05$) and a decrease in the expression of autophagy-related proteins in the MCAO/R group compared with the E2 and SAL groups, indicating that Mdivi-1 and PHTPP inhibitor groups played a protective role against neural damage compared with the SAL-H group. It indicated that E2 and SAL exerted a protective effect against nerve injury; whereas the two inhibitor groups, Mdivi-1 and PHTPP, were statistically significantly different ($P<0.05$) compared with the SAL-H group, and the inhibitor group reversed the protective effect of SAL and decreased the expression of autophagy-related proteins, suggesting that SAL may protect neuronal cells through ER β -mediated mitochondrial autophagy.

Conclusions: Conclusion SAL may improve neurological function in cerebral ischemic rats by modulating the level of ER β /BNIP3-mediated mitochondrial self, providing a new way for drug development based on SAL combined with ER β as a drug for cerebral ischemia.

Introduction

Strokes, also known as strokes, often result in loss of speech and hemiparesis, and have become the second leading cause of death worldwide. 80% of strokes are ischaemic strokes(Lim et al., 2021; Venketasubramanian, Yoon, Pandian, & Navarro, 2017), caused by embolism or occlusion of cerebral arteries, which can lead to cellular dysfunction and cell death in the ischaemic region of the brain.Clinically, stroke is treated by adopting the method of reperfusion, and the restoration of blood supply by reperfusion can improve the ischemic tissues, but reperfusion can further promote the tissue damage caused by oxidative stress and inflammation, so that the neurons will be damaged secondary to the injury, which is known as cerebral ischemia-reperfusion injury (CIRI)(H. Liu et al., 2021)and it is considered to be the main pathological injury of cerebral ischemia, including oxidative stress, mitochondrial dysfunction, inflammatory response, and damage to the blood-brain barrier(Xu et al., 2023).The challenge of designing neuroprotective strategies for CIRI injury has increased in recent years with the development of endovascular treatments for ischaemic stroke. It has been found that the brain endogenously produces estrogen from cholesterol and that estrogen crosses the blood-brain barrier, resulting in a lower incidence of pre-menopausal strokes in women than in men of the same age group, and a significantly higher incidence of post-menopausal strokes, suggesting that estrogen has a role in CIRI(L. Wang, Wang, Shan, Shu, & Guo, 2023),Therefore, the incidence of stroke in women before menopause is lower than that in men of the same age group, and the incidence of stroke after menopause is significantly higher and the same as that in men, which suggests that oestrogen has a good protective effect on the neurological system damaged by CIRI.

Mitochondria are important target organelles in the reperfusion injury phase, and normal neuronal cells are protected by removing damaged mitochondria through fission and autophagy(X. Zhang et al., 2013).Mitochondrial autophagy is a selective form of macroautophagy, which plays a key role in maintaining the homeostasis of the intracellular environment. It is a process in which mitochondria that have been damaged or have abnormal function are specifically encapsulated into autophagosomes and transported to lysosomal fusion to be degraded and removed(Ahsan et al., 2021).Bcl-2/adenovirus E1B 19-kDa interacting protein 3, BNIP3 is a mitochondrial outer membrane protein commonly used to respond to mitochondrial autophagy levels, which can mediate the(Nan et al., 2023; Ney, 2015)protective effect after upregulation, thus affecting neurological injury caused by cerebral ischaemia-reperfusion.Recent studies have shown that (Lei, Yang, Lu, Zhang, & Li, 2021)activation of mitochondrial autophagy in the CIRI stage reduces the size of cerebral infarction and protects neurons from damage.

SALidroside is an active ingredient derived from the traditional Chinese medicine *Rhodiola* family(X. Liu et al., 2018).SALidroside is widely used in European countries and China for its anti-aging, autophagy promotion and neuroprotective effects, and it is mostly used to inhibit apoptosis, anti-oxidative stress and reduce inflammatory response, but the studies on the mechanisms of regulating autophagy, promoting neural remodelling and protecting the blood-brain barrier have been insufficient(Gu et al., 2020; Pu et al., 2020; Zeng et al., 2022).However, the research on the mechanisms of regulating autophagy, promoting neural remodelling and protecting the blood-brain barrier is slightly insufficient. Our group takes the characteristics of SAL, such as fast absorption and clearance in the body, as well as the fact that SAL enters the bloodstream mainly in the form of glycoside tyrosol after oral administration, as the basis for

our study (Magani et al., 2020). Based on the rapid absorption and clearance of SAL in the body, and the fact that SAL mainly enters the bloodstream in the form of glycogen tyrosol after oral administration, our group investigated whether SAL could protect against neurological damage by activating mitochondrial autophagy.

The studies on the mechanism of anti-ischemic injury of *rhodiola rosea* glycosides have become more and more in-depth, but the studies from the estrogen receptor pathway have not yet been seen, and the activation of the estrogen receptor pathway to exert therapeutic effects are mostly focused on gynecological oncology studies. The present study is different from previous estrogen receptor studies in that it explores the neuroprotective effects of SAL on cerebral ischemic nerves after activation of estrogen receptor, and provides a new perspective on the possibility of SAL as a new ligand for estrogen receptor. This paper verifies the role and mechanism of SAL in alleviating cerebral ischemic injury by modulating the ER β /BNIP3-mediated mitochondrial autophagy pathway and exerting neuroprotective effects, suggesting that SAL, ER β may prove to be a potential therapeutic agent for ischemic stroke.

Materials and Methods

Experimental Animals

One hundred and twenty SFP-grade female SD rats, weighing (230–260) g, were purchased from the Animal Experiment Centre of Xinjiang Medical University (SCSK (Xin) 2020-003). Rats were housed in individual cages on a 12-h light/dark cycle at constant room temperature with free access to food and water. Every effort was made to minimise the number of rats used and the suffering of the animals.

Drugs and Reagents

Rhodioloside (purity > 99%, CAS:10338-51-9, wkq, China); PHTPP (CAS:ab145148, Abcam, UK); Mdivi-1 (S276713, Selleck, USA); Estradiol (B24215, Shanghai Yuanye, China); Thread bolus (A5-243450, CINONTECH, China); Electron microscopy fixative (G1102, Servicebio, China); TTC (298-96-4, Servicebio, China); CINONTECH, China); electron microscopy fixative (G1102, Servicebio, China); TTC (298-96-4, Solarbio, China); Evans blue (E2129, Sigma, Germany); E2/SOD/MDA (A001-3-2, A006-2-1, A005-2-1, Nanjing Jianjian Bioengineering Institute, China); BCA protein quantification kit (P0012, Beyotime, USA); ER β (ab149467, Abcam, USA); LC3II/I (81004-1-RR, Proteintech, China); BNIP3 (ab109362 Abcam, USA); NIX (12986-1-AP, Proteintech, China); goat anti-rabbit secondary antibody (ab150077, abcam, USA).

Instruments

Transmission electron microscope (HT7800, hitachi, Japan); Ultrathin sectioning machine (UC7, Leica, Germany); Enzyme labelling instrument (Multiskan GO, Thermo, USA); Gel electrophoresis instrument (PowerPac, Bio-Rad, USA); High-speed centrifuge (3-18KS, Sigma, Germany); Grinding instrument (KZ-111-FP, Servicebio, China); orthostatic microscope (224–8533, NIKON, Japan).

Animal Grouping and Drug Administration

A total of 135 rats were randomly divided into 9 groups, 15 rats were taken as normal group(Con), 120 rats were divided into ovary removal group(OVX), model group (OVX + MCAO/R),estradiol control group (OVX + MCAO/R + E2), ERβ inhibitor group (OVX + MCAO/R + SAL-H + PHTPP), autophagy inhibitor group (OVX + MCAO/R + SAL-H + Mdivi-1),and SAL low-, medium-, and high-dose groups (OVX + MCAO/R + SAL).The Con group was left untreated; equal volumes of saline were given intraperitoneally for both OVX and OVX + MCAO/R; oestradiol was given to OVX + MCAO/R + E2(0.8 mg/kg), OVX + MCAO/R + SAL + PHTPP(4mg/kg); OVX + MCAO/R + SAL + Mdivi-1(3mg/kg),and OVX + MCAO/R + SAL (5mg/kg, 10mg/kg, and 20mg/kg) were given once/d for 6 days.

MCAO/R Surgery

The rat MCAO/R injury model was prepared with reference to the literature (Jin et al., 2022)and other methods with modification. After fasting for 12 h, rats were anaesthetised, fixed in the supine position, and a longitudinal incision was made along the middle part of the neck, separating the tissues to expose the right common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA), and the vagus nerves on the vascular surface were completely stripped. The CCA and proximal ECA were ligated, and a wire bolus was inserted from the CCA toward the ICA, stopping by slowly pushing 18–20 mm and ligating the CCA.Throughout the procedure, the temperature was maintained at about 25°C. 24h after reperfusion, rats were sacrificed.

Neurological Function Scores

The Longa score was used(Longa, Weinstein, Carlson, & Cummins, 1989)to detect the degree of neurological deficits, the experiment took rats scored 0–3 and rats were scored by researchers who were unaware of the grouping. Detailed criteria are shown in the table below.

Score	Neurological Function Scoring Scale
0	Rats with no neurological deficit
1	Rats unable to fully extend left paw (mild neurological impairment)
2	Rats turning to the opposite side (moderate neurological impairment)
3	Rat leans to the left (severe neurological impairment)
4	Rats unable to walk, no longer conscious (excluded)

TTC Staining

Cerebral infarct volume was determined using 2% 2,3,5-triphenyl tetrazolium chloride (TTC) staining. Intact brain tissue was removed from rats after anaesthesia, frozen for 30 min in -20°C, subjected to 2-mm serial coronal sections, placed in staining solution and photographed after staining.

Measurement of Cerebral Infarction Volume

The water content of brain tissue of rats was determined by wet and dry weight method. Brain tissues were removed and weighed wet weight (W). After baking at 110°C constant temperature oven for 48h, the dry weight (D) was weighed. Brain water content (%) = $[(W-D)/W] \times 100\%$.

Determination of the BBB Permeability by EB

BBB integrity was assessed by Evans blue extravasation. 2% Evans blue was injected into the femoral vein starting 24h after reperfusion, washed with saline via left ventricular perfusion 1h later, weighed, and each hemispherical homogenate was placed in 60% trichloroacetic acid and centrifuged (4000g, 40 groups). Using spectrophotometry, the absorbance of the supernatant at 630 nm was determined

Measurement of Oxidative Stress Levels

To determine the changes in estrogen and oxidative stress levels after surgery, ischaemic lateral brain tissue was taken and centrifuged at 4°C for 15 min and the supernatant was collected. E2, SOD and MDA expression levels in brain homogenates were assayed according to the kit manufacturer's instructions.

Hematoxylin and Eosin (H&E) Staining of the Rat Brain Tissue

Routine paraffin-embedded sections were stained with hematoxylin for 5 min and eosin stain for 5 min, and dehydrated, transparent, neutral gum sealed sections. Observe the pathomorphological changes of brain sections under the microscope.

Nissl Staining

Paraffin sections, after dewaxing and rehydration, stained with Nissl's stain for 1h, and sealed with neutral gum. The morphology of nerve cells in brain tissue was observed under the microscope.

Transmission Electron Microscopy

1mm squares were taken to the fixative solution, while 1% osmium fixation for 2h, acetone gradient dehydration and embedding were performed sequentially. After double staining with uranyl acetate and lead citrate, mitochondrial autophagosomes were observed under electron microscope.

Western Blotting Analysis

Total proteins of each group were extracted, and after electrophoresis, the proteins were transferred to PVDF membrane and closed for 2 h. After washing the membrane, the proteins were incubated with diluted antibodies ER β , BNIP3, NIX, and LC3-II (1:1000), and shaking bed at 4°C overnight, and the secondary antibody (1:10,000) for 1 h. The colour was developed by ECL exposure solution (1:1), and the images of them were obtained by protein imaging system, and the images were calculated by ImageJ software. The grey scale value of protein expression was compared with the grey scale value of the internal reference bands to obtain the relative protein expression.

Statistical Analysis

Statistical processing was performed using SPSS 25.0 and GraphPad 9. One-way ANOVA was used to analyse the differences between groups, and two-way comparisons were made using the LSD-t test, with $P < 0.05$ when the differences were statistically significant.

Results

SAL protects against CIRI-induced brain damage in rats

The experimental procedure was performed as shown in Fig. 1A. After MCAO/R, in order to investigate whether SAL has a protective effect on CIRI in rats, we performed Longa score, TTC, and brain water content measurements, as well as the determination of BBB permeability and the levels of E2 and oxidative stress 24h after reperfusion. The neurological function score (Fig. 1B) can visually reflect the degree of injury of ischemia-reperfusion in rats, and the results showed that the higher the score, the more severe the neurological damage in rats. The results showed that the higher the score was, the more serious the nerve damage was in rats (Y. Zhang et al., 2020). Apparently OVX does not affect the experimental results, and there was no difference between the Con and OVX groups, while the MCAO/R group scores were significantly higher than the rest of the group, indicating successful modelling and a more pronounced neurobehavioural state, which was alleviated after the injection of E2 and SAL. When TTC staining was performed (Fig. 1C), normal tissues were stained red, while infarcted tissues were white, and the volume of cerebral infarction was significantly increased in the MCAO/R group compared with the E2 group and the SAL group. Notably (NOTABLY), the therapeutic effect increased with the increase in the administered dose of SAL, suggesting that the E2 group and the SAL-H group were able to effectively reduce the volume of cerebral infarction. Comparing the cerebral water content of rats in each group (Fig. 1D), the cerebral water content was significantly higher in the MCAO/R group compared with the OVX group, and the administered group exerted a certain protective effect against cerebral oedema compared with the MCAO/R group. CIRI is accompanied by an increase in BBB permeability, which further leads to the aggravation of CIRI, and EB dye is commonly used (Hou et al., 2023) to assess BBB permeability (Fig. 1E), BBB function was significantly impaired and EB content was increased in the MCAO/R group compared with the Con group, whereas pretreatment in the E2 group and the SAL-H group protected the BBB in rats with reduced permeability. Next, we assessed the contents of E2, SOD, and MDA by evaluating the contents of E2, in which after removing the ovaries, the contents of E2 decreased significantly in all groups, indicating that the surgery of removing the ovaries from the rats was successful; whereas, after injecting the E2 group and the SAL group, the contents of E2 increased significantly in the brain of the CIRI rats (Fig. 1F), indicating that the SAL had a significant activating effect on E2; in which SOD and MDA are oxidative stress important biomarkers of oxidative stress (Y. Y. Sun et al., 2023), Its level indirectly reflected the degree of lipid peroxidation. SOD was reduced (Fig. 1G) and MDA was elevated (Fig. 1H) in the MCAO/R group, and CIRI induced cellular oxidative stress, which could be significantly reversed by injection of E2 and SAL, both of which indicated that E2 and SAL could inhibit the activation by oxidative stress in the process of CIRI.

SAL reduces neuronal damage in rats after CIRI

In order to further inflammation the neuroprotective effect of SAL, the sections were stained with HE, Nissl staining (x400) to assess the neuronal damage. In HE staining (Fig. 2A), the neuronal cells of Con group and OVX group were neatly arranged, structurally intact without congestion and edema and obvious pathological changes, and the nuclei of the cells had clear edges and were neatly arranged, whereas in the brain tissues of the MCAO/R group, the hemorrhagic hematoma, neuronal swelling, nuclei consolidation and vacuolike changes were observed; the above conditions were significantly altered by pretreatment with E2 and SAL. Nissl vesicles caused basophilic granules within the neurons. In the MCAO/R group, the neuronal cells were swollen, and the nuclei of the cells were solidified and vacuolated; the above conditions were significantly altered in rats pretreated with E2 and SAL, and the basophilic granules in neurons were caused by Nissl vesicles. (Koliatsos et al., 1990), When the neurons functioned normally with complete and clear structural fixation and abundant Nissl bodies, the staining results showed that (Fig. 2B), the number of Nissl vesicles in the flat layer of the brain in the Con and OVX groups was high and the shape was complete; whereas in the MCAO/R group, the number of Nissl vesicles was reduced, and there was a clear aggregation of vacuole-like alterations; the staining of the Nissl bodies in the E2 group and the SAL group was getting deeper, and the phenomenon of the loss of the Nissl vesicles had significantly improved, and the neuron was uniformly distributed, with the tissue restoration situation being obviously superior to that in the MCAO/R group.

SAL attenuated rat CIRI by inhibiting mitochondrial autophagy

To investigate the role of mitochondrial autophagy in the MCAO/R model, we administered the autophagy inhibitor Mdivi-1 before modeling, and the results showed that inhibition of mitochondrial autophagy induced significant neurological deficits (Fig. 3A), worsening of cerebral infarct volume (Fig. 3B), cerebral edema (Fig. 3C), and a significant increase in EB permeability in MCAO/R rats (Fig. 3D), E2 (Fig. 3E), SOD content was significantly decreased (Fig. 3F) and MDA (Fig. 3G) was elevated in the Mdivi-1 group compared with the SAL-H group. HE and Nissl staining (x400) was performed to observe the changes of Mdivi-1 on neuronal morphology, and it could be clearly seen that Mdivi-1 suppressed the SAL Mdivi-1 inhibited the protective effect of SAL on neuronal cells, and most of the neurons in the tissue were disarranged (Rong et al., 2020), Nuclei were fragmented and morphologically abnormal (Fig. 3H). Nissl vesicles were reduced and lighter in color in the Mdivi-1 group compared with the SAL-H group (Fig. 3I). These results indicated that autophagy inhibitors aggravated the damage to MCAO/R, suggesting that the pathogenesis of cerebral ischemia may be related to mitochondrial autophagy, and when SAL preconditioning then attenuates the CIRI injury, and also suggesting that SAL may play a protective role by activating mitochondrial autophagy.

We also observed the mitochondrial structure of SAL-H and Mdivi-1-treated rat tissues by transmission electron microscopy (TEM), and the microscopic results showed that the SAL-H group attenuated mitochondrial swelling and fission, and the number of autophagic vesicles was increased (Fig. 4A). The Mdivi-1 treatment reversed the protective effect of SAL and induced the accumulation of

autophagosomes, and the morphology was worse, with the occasional autophagic vesicles. Next, we explored the molecular mechanism of mitochondrial autophagy in MCAO/R injury. We used Western blot to analyse the expression of relevant proteins in the mitochondrial autophagy pathway. The results confirmed that the Mdivi-1 group reduced the expression of BNIP3 in the tissues, and compared with the SAL-H group, the Mdivi-1 treatment inhibited the process of autophagy by suppressing the expression of BNIP3, and then inhibited the expression of LC3, which led to inhibition of both upstream and downstream in the pathway mechanism of autophagy (Fig. 4B-F). These data suggest that proper activation of autophagy can help ameliorate brain injury.

ER β alleviates CIRI-induced neuronal damage

To investigate the protective effect of ER β in the MCAO/R model, the rats were pretreated with the ER β inhibitor PHTPP, and the results showed that the injection of PHTPP to rats significantly increased neurological scores (Fig. 5A), cerebral infarct volume (Fig. 5B), and cerebral edema (Fig. 5C) and EB permeability compared with the SAL-H group (Fig. 5D), E2 (Fig. 5E), SOD content was significantly reduced (Fig. 5F) and MDA (Fig. 5G) was elevated in the PHTPP group compared with the SAL-H group. HE and Nissl staining (x400) was used to observe the changes of neuronal morphology caused by PHTPP and it was clearly seen that most of the neurons in the brain tissue appeared to be After ER β inhibition, most of the neurons in the brain tissue were vacuolated and disorganized (Fig. 5H), and the normal morphology of Nissl vesicles disappeared and were unevenly distributed in the PHTPP group compared with the SAL-H group (Fig. 5I). Administration of PHTPP indicated aggravation of MCAO/R injury in rats by inhibiting ER β .

In addition, TEM showed that mitochondria in the PHTPP group showed moderate swelling, matrix thinning and dissolution, and partial breakage of cortical cristae (Fig. 6A). We wondered whether ER β was involved in the BNIP3/NIX autophagy pathway, and Western blot analysis showed that administration of SAL-H activated BNIP3 expression compared with rats in the PHTPP group, whereas PHTPP increased cerebral ischemia/reperfusion injury by decreasing ER β expression and inhibiting mitochondrial autophagy (Fig. 6B-C), indicating that ER β is not only involved in the mitochondrial autophagy pathway, but also located upstream of BNIP3. All this suggests that lowering brain tissue levels and thus attenuating CIRI may be related to a partial protective effect through activation of ER β .

SAL promotes mitochondrial autophagy-related protein expression by increasing ER β

Finally, the structural and molecular mechanisms of autophagy in brain I/R injury by SAL were explored. The ultrastructure of mitochondria was examined using TEM, and as shown in the figure (Fig. 7A), mitochondria in the MCAO/R group appeared to be swollen and cristae were disrupted, whereas treatment in the E2 and SAL groups attenuated these morphological changes in the mitochondria, indicating that activation of autophagy in brain tissue and thus could attenuate the MCAO/R injury in rats. Next, the expression levels of ER β and autophagy-related proteins were investigated (Fig. 7B-G), and the expression of autophagy-related proteins, such as BNIP3, was significantly elevated in the E2 and SAL groups compared with that of the MCAO/R group, suggesting that the E2 and SAL could effectively activate

BNIP3-mediated autophagy of mitochondria, and the expression of BNIP3 was increased in a dose-dependent manner. Therefore, the ER β /BNIP3 pathway is involved in the protective mechanism of SAL in MCAO/R injury.

Discussion

Stroke is responsible for approximately 5.5 million deaths per year, the (H. Shen, Pei, Zhai, Guan, & Wang, 2022). It is second only to cardiovascular disease in mortality. They are mainly classified into ischaemic and haemorrhagic strokes, with ischaemic strokes accounting for 87% of all strokes (Mao et al., 2022). We investigated the effect of SAL on ischaemic stroke induced by the MCAO/R model in ovariectomised rats in this study. The results showed that pretreatment with different doses of E2 and SAL significantly alleviated cerebral I/R injury in rats, and in addition, by inhibiting autophagy and estrogen, it was determined that mechanistically it may be inextricably linked to cerebral ischemia through the ER β /BNIP3-mediated mitochondrial autophagy pathway.

The concept of "autophagy" was first introduced by researchers in 1860 (Ktistakis, 2017) to help cells eliminate unwanted components, phagocytosis continuously binds some components to form a double-membrane structure called autophagosome, which binds to the lysosome to form an autophagic lysosome, which is finally degraded by the lysosome, and the metabolite is discharged out of the cell (Goldsmith, Ordureau, Harper, & Holzbaur, 2022). Mitochondrial autophagy (Sukhorukov et al., 2022) enables neuronal survival through a variety of mechanisms, and the study of the autophagy/lysosomal pathway in stroke has attracted much attention. Nitatori (Nitatori et al., 1995) used transmission electron microscopy to confirm that autophagy occurs in neuronal cells after cerebral ischaemia, and a growing number of experiments have confirmed that cerebral ischaemia is associated with autophagy, and Yu et al. (Yu, Luo, Yang, Chen, & Duan, 2023) shows that Phydroxybenzyl alcohol ameliorates neuronal cerebral ischemia/reperfusion injury by activating mitochondrial autophagy through SIRT1; Neuroprotective Effect of Taohong Siwu Decoction on Cerebral Ischemia/Reperfusion Injury via Mitophagy-NLRP3 Inflammasome Pathway counteracted by Mdivi-1 (Ji et al., 2022); However, the regulation of cell death by autophagy has a dual nature (Agarwal & Muqit, 2022), and it is noteworthy that mitochondrial autophagy exerts its protective role during the reperfusion phase (L. Shen et al., 2021). Consistent with our findings, we demonstrated that mitochondrial autophagy has a protective role in the reperfusion phase of ischemic stroke and that SAL preconditioning-mediated mitochondrial autophagy exerts a protective effect against neurological loss through activation, among others.

Brain tissue is the most sensitive target organ for hypoxia (Molnár, Bereczki, & Fülesdi, 2020). It is easy to be damaged in the hypoxic environment, and the occurrence of ischemic stroke causes tissue edema and local ischemia, which leads to damage of blood vessels and nerve cells in the brain (Alishahi et al., 2019). Currently, most of the drugs used in the treatment of ischaemic stroke have poor efficacy and high incidence of adverse effects (Mendelson & Prabhakaran, 2021). There is an urgent need for effective novel treatment strategies for ischaemic stroke to protect the brain from damage before and during recanalisation, to extend the time window for intervention and to further improve functional

outcomes(Przykaza, 2021).Whereas Chinese medicine has been used clinically for the treatment of cerebrovascular diseases, including ischaemic stroke, for centuries, studies have shown that Chinese medicines have demonstrated concerning advantages in brain protection in ischaemic stroke(Ajoolabady et al., 2021).

Rhodiola rosea, a derivative of the phenolic natural product tyrosine(Magani et al., 2020),has been ted to play a neuroprotective role in cerebral ischemia/reperfusion-induced injuries in the literature(Yin, Ouyang, Lin, Xin, & Ji, 2021)and the mechanism of its protective effect on MCAO has been confirmed by many scholars, but there are few studies on the relationship between cerebrovascular disease and estrogen receptor pathway, and those that have activated estrogen receptor pathway have focused on gynecological oncology research(P. Chen, Li, & Ou-Yang, 2022; Z. L. Zhang et al., 2017).Estrogen receptors include three types of receptors, ER α , ER β and GPER, which form dimers after activation and enter the nucleus to bind DNA and affect transcription.After either transient or permanent ischaemia, oestrogen reduces lesion volume in a dose-dependent manner(Lei et al., 2021),and Scott E(Scott, Zhang, Wang, Vadlamudi, & Brann, 2012)has shown in clinical studies that oestradiol is a neuroprotective hormone that puts postmenopausal women at a lower risk of stroke than menand the American Heart Association suggests that women are more likely to develop the disease after age > 75 years than men of the same age.(Maida, Norrito, Daidone, Tuttolomondo, & Pinto, 2020).Among them, ER β inhibits microglia activation and mediates neuroinflammatory responses to protect against cerebral ischaemic injury. E2 and ER α have been shown to exert a protective effect on cardiomyocyte cell death and autophagy by inhibiting hypoxia-induced BNIP3 levels(B. C. Chen et al., 2018).BNIP3(P. Li et al., 2021; T. Wang & Liu, 2022) and NIX are mitochondrial proteins located in the outer mitochondrial membrane, both with LIR gene sequences, whose transcriptional levels are activated when subjected to hypoxia-ischemia, interacting with autophagy-associated protein LC3 to form autophagosomes to clear damaged mitochondria.

The results of this experiment revealed that pretreatment with E2 and SAL significantly reduced the neurological function score and the infarct area of brain tissue after cerebral ischemia/reperfusion, and the water content of rat brain and brain was evaluated using dry weight and wet weight, and both E2 and SAL reduced the infarct volume, increased the number of Nitrosomes without autophagic vesicle formation, exerted a protective effect on the neuronal cells, lowered the permeability of the blood-brain barrier in the rats, and increased the autophagic protein expression; while by constructing the MCAO/R rat model, the results were opposite, and the protection of autophagy and ER β was attenuated by treatment with the autophagy inhibitor Mdivi-1 as well as the estrogen inhibitor PHTPP, suggesting that SAL, the estrogen receptor ER β , and mitochondrial autophagy all exerted neuroprotection against cerebral ischemia, and could significantly improve the neurological function of the rat brain after cerebral ischemia/reperfusion, and the mechanism may be related to the activation of autophagy, which is similar to other scholars.(P. Li et al., 2021; Yu et al., 2023)

Brain tissue ischemia and hypoxia cause energy metabolism disorders, and Beclin-1 and LC3 proteins are closely related to the occurrence of autophagy (Salminen et al., 2013).Among them, Beclin-1 can induce

other autophagy-related proteins to localise in autophagic vesicles, which is an important molecule in the process of autophagosome formation;LC3(L. Li, Tan, Miao, Lei, & Zhang, 2015)is involved in all stages of autophagy formation and can be used as a marker for detecting autophagic activity. When autophagy occurs, LC3-I is oligomerised to autophagosomes and then converted to LC3-II. Compared with MCAO/R, the expression levels of ER β , BNIP3 and its mediated autophagy-related proteins were significantly up-regulated in the brain tissues of E2 and SAL pretreated rats, and the treatment effect of SAL-H was significantly optimal, contrary to the results of Mdivi-1 and PHTPP, indicating that SAL, estrogen receptor ER β , and autophagy exerted a protective effect against neurological injury, and estrogen receptor was at the upstream of the mitochondrial autophagy pathway. pathway upstream of the mitochondrial autophagy pathway. These results suggest that autophagy plays an important role in alleviating blood-brain barrier damage during hypoxic stress.ER β interferes with the binding of BNIP3 to the mitochondrial membrane and also interferes with expression by downregulating BNIP3 promoter activity. Based on the findings, we suggest that ER β exerts cerebral ischaemia protection by activating the expression of the mitochondrial autophagy protein BNIP3.

Conclusion

In conclusion, the present study demonstrated that SAL attenuates CIRI by regulating the ER β /BNIP3 pathway to induce autophagy, and SAL was reduced in a concentration-dependent manner. Activation of ER β regulates BNIP3-mediated mitochondrial autophagy thereby attenuating cerebral ischemic injury, which may be related to the preventive role of the ER β /BNIP3 signalling pathway against CIRI.

There are still some aspects of this paper that are limited. Although we demonstrated that SAL prevented MCAO/R-induced injury, the drug absorption and metabolism of SAL after stroke need to be investigated in follow-up, and this experiment used 8-week-old female rats, but the incidence of stroke in females is generally after menopause, and elderly menopausal rats can be considered for subsequent inclusion in the experiment.

Declarations

Ethics Statement

This animal study was approved by the Experimental Animal Ethics Committee of Xinjiang Medical University.

Author Contributions

RX, LY, and JL designed the study.RX, YYJ, WHM, and JZ performed the experiments and completed the analyses of all the study data.RX contributed to the interpretation of the results and to the writing of the article.LY and JL reviewed and revised important elements of the article. All authors have read and agreed to publish this manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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References

1. Agarwal, S., & Muqit, M. M. K. (2022). PTEN-induced kinase 1 (PINK1) and Parkin: Unlocking a mitochondrial quality control pathway linked to Parkinson's disease. *Curr Opin Neurobiol*, 72, 111-119. doi:10.1016/j.conb.2021.09.005
2. Ahsan, A., Liu, M., Zheng, Y., Yan, W., Pan, L., Li, Y., . . . Zhang, X. (2021). Natural compounds modulate the autophagy with potential implication of stroke. *Acta Pharm Sin B*, 11(7), 1708-1720. doi:10.1016/j.apsb.2020.10.018
3. Ajoolabady, A., Wang, S., Kroemer, G., Penninger, J. M., Uversky, V. N., Pratico, D., . . . Ren, J. (2021). Targeting autophagy in ischemic stroke: From molecular mechanisms to clinical therapeutics. *Pharmacol Ther*, 225, 107848. doi:10.1016/j.pharmthera.2021.107848
4. Alishahi, M., Ghaedrahmati, F., Kolagar, T. A., Winlow, W., Nikkar, N., Farzaneh, M., & Khoshnam, S. E. (2019). Long non-coding RNAs and cell death following ischemic stroke. *Metab Brain Dis*, 34(5), 1243-1251. doi:10.1007/s11011-019-00423-2
5. Chen, B. C., Weng, Y. J., Shibu, M. A., Han, C. K., Chen, Y. S., Shen, C. Y., . . . Huang, C. Y. (2018). Estrogen and/or Estrogen Receptor α Inhibits BNIP3-Induced Apoptosis and Autophagy in H9c2 Cardiomyoblast Cells. *Int J Mol Sci*, 19(5). doi:10.3390/ijms19051298
6. Chen, P., Li, B., & Ou-Yang, L. (2022). Role of estrogen receptors in health and disease. *Front Endocrinol (Lausanne)*, 13, 839005. doi:10.3389/fendo.2022.839005
7. Goldsmith, J., Ordureau, A., Harper, J. W., & Holzbaur, E. L. F. (2022). Brain-derived autophagosome profiling reveals the engulfment of nucleoid-enriched mitochondrial fragments by basal autophagy in neurons. *Neuron*, 110(6), 967-976.e968. doi:10.1016/j.neuron.2021.12.029
8. Gu, C., Li, L., Huang, Y., Qian, D., Liu, W., Zhang, C., . . . Yin, G. (2020). Salidroside Ameliorates Mitochondria-Dependent Neuronal Apoptosis after Spinal Cord Ischemia-Reperfusion Injury Partially through Inhibiting Oxidative Stress and Promoting Mitophagy. *Oxid Med Cell Longev*, 2020, 3549704. doi:10.1155/2020/3549704

9. Hou, W., Yao, J., Liu, J., Lin, X., Wei, J., Yin, X., . . . He, X. (2023). USP14 inhibition promotes recovery by protecting BBB integrity and attenuating neuroinflammation in MCAO mice. *CNS Neurosci Ther*, 29(11), 3612-3623. doi:10.1111/cns.14292
10. Ji, Z. J., Shi, Y., Li, X., Hou, R., Yang, Y., Liu, Z. Q., . . . Peng, D. Y. (2022). Neuroprotective Effect of Taohong Siwu Decoction on Cerebral Ischemia/Reperfusion Injury via Mitophagy-NLRP3 Inflammasome Pathway. *Front Pharmacol*, 13, 910217. doi:10.3389/fphar.2022.910217
11. Jin, M., Wang, C., Xu, Y., Zhang, Z., Wu, X., Ye, R., . . . Han, D. (2022). Pharmacological effects of salidroside on central nervous system diseases. *Biomed Pharmacother*, 156, 113746. doi:10.1016/j.biopha.2022.113746
12. Koliatsos, V. E., Nauta, H. J., Clatterbuck, R. E., Holtzman, D. M., Mobley, W. C., & Price, D. L. (1990). Mouse nerve growth factor prevents degeneration of axotomized basal forebrain cholinergic neurons in the monkey. *J Neurosci*, 10(12), 3801-3813. doi:10.1523/jneurosci.10-12-03801.1990
13. Ktistakis, N. T. (2017). In praise of M. Anselmier who first used the term "autophagie" in 1859. *Autophagy*, 13(12), 2015-2017. doi:10.1080/15548627.2017.1367473
14. Lei, L., Yang, S., Lu, X., Zhang, Y., & Li, T. (2021). Research Progress on the Mechanism of Mitochondrial Autophagy in Cerebral Stroke. *Front Aging Neurosci*, 13, 698601. doi:10.3389/fnagi.2021.698601
15. Li, L., Tan, J., Miao, Y., Lei, P., & Zhang, Q. (2015). ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. *Cell Mol Neurobiol*, 35(5), 615-621. doi:10.1007/s10571-015-0166-x
16. Li, P., Yao, L. Y., Jiang, Y. J., Wang, D. D., Wang, T., Wu, Y. P., . . . Li, X. T. (2021). Soybean isoflavones protect SH-SY5Y neurons from atrazine-induced toxicity by activating mitophagy through stimulation of the BEX2/BNIP3/NIX pathway. *Ecotoxicol Environ Saf*, 227, 112886. doi:10.1016/j.ecoenv.2021.112886
17. Lim, S., Kim, T. J., Kim, Y. J., Kim, C., Ko, S. B., & Kim, B. S. (2021). Senolytic Therapy for Cerebral Ischemia-Reperfusion Injury. *Int J Mol Sci*, 22(21). doi:10.3390/ijms222111967
18. Liu, H., Li, S., Xu, Y., Wang, X., Ren, R., Zhu, H., & Zhang, S. (2021). Engeletin protects against cerebral ischemia/reperfusion injury by modulating the VEGF/vasohibin and Ang-1/Tie-2 pathways. *Braz J Med Biol Res*, 54(10), e11028. doi:10.1590/1414-431X2020e11028
19. Liu, X., Wen, S., Yan, F., Liu, K., Liu, L., Wang, L., . . . Ji, X. (2018). Salidroside provides neuroprotection by modulating microglial polarization after cerebral ischemia. *J Neuroinflammation*, 15(1), 39. doi:10.1186/s12974-018-1081-0
20. Longa, E. Z., Weinstein, P. R., Carlson, S., & Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*, 20(1), 84-91. doi:10.1161/01.str.20.1.84
21. Magani, S. K. J., Mupparthi, S. D., Gollapalli, B. P., Shukla, D., Tiwari, A. K., Gorantala, J., . . . Tantravahi, S. (2020). Salidroside - Can it be a Multifunctional Drug? *Curr Drug Metab*, 21(7), 512-524. doi:10.2174/1389200221666200610172105
22. Maida, C. D., Norrito, R. L., Daidone, M., Tuttolomondo, A., & Pinto, A. (2020). Neuroinflammatory Mechanisms in Ischemic Stroke: Focus on Cardioembolic Stroke, Background, and Therapeutic

- Approaches. *Int J Mol Sci*, 21(18). doi:10.3390/ijms21186454
23. Mao, Z., Tian, L., Liu, J., Wu, Q., Wang, N., Wang, G., . . . Seto, S. (2022). Ligustilide ameliorates hippocampal neuronal injury after cerebral ischemia reperfusion through activating PINK1/Parkin-dependent mitophagy. *Phytomedicine*, 101, 154111. doi:10.1016/j.phymed.2022.154111
 24. Mendelson, S. J., & Prabhakaran, S. (2021). Diagnosis and Management of Transient Ischemic Attack and Acute Ischemic Stroke: A Review. *Jama*, 325(11), 1088-1098. doi:10.1001/jama.2020.26867
 25. Molnár, C., Bereczki, D., & Fülesdi, B. (2020). [Management of bleeding in neuroanesthesia and neurointensive care]. *Orv Hetil*, 161(37), 1574-1578. doi:10.1556/650.2020.31776
 26. Nan, F., Nan, W., Yu, Z., Wang, H., Cui, X., Jiang, S., . . . Li, Y. (2023). Polygalacin D inhibits the growth of hepatocellular carcinoma cells through BNIP3L-mediated mitophagy and endogenous apoptosis pathways. *Chin J Nat Med*, 21(5), 346-358. doi:10.1016/s1875-5364(23)60452-2
 27. Ney, P. A. (2015). Mitochondrial autophagy: Origins, significance, and role of BNIP3 and NIX. *Biochim Biophys Acta*, 1853(10 Pt B), 2775-2783. doi:10.1016/j.bbamcr.2015.02.022
 28. Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibana, K., . . . Uchiyama, Y. (1995). Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci*, 15(2), 1001-1011. doi:10.1523/jneurosci.15-02-01001.1995
 29. Przykaza, Ł. (2021). Understanding the Connection Between Common Stroke Comorbidities, Their Associated Inflammation, and the Course of the Cerebral Ischemia/Reperfusion Cascade. *Front Immunol*, 12, 782569. doi:10.3389/fimmu.2021.782569
 30. Pu, W. L., Zhang, M. Y., Bai, R. Y., Sun, L. K., Li, W. H., Yu, Y. L., . . . Li, T. X. (2020). Anti-inflammatory effects of *Rhodiola rosea* L.: A review. *Biomed Pharmacother*, 121, 109552. doi:10.1016/j.biopha.2019.109552
 31. Rong, L., Li, Z., Leng, X., Li, H., Ma, Y., Chen, Y., & Song, F. (2020). Salidroside induces apoptosis and protective autophagy in human gastric cancer AGS cells through the PI3K/Akt/mTOR pathway. *Biomed Pharmacother*, 122, 109726. doi:10.1016/j.biopha.2019.109726
 32. Salminen, A., Kaarniranta, K., Kauppinen, A., Ojala, J., Haapasalo, A., Soininen, H., & Hiltunen, M. (2013). Impaired autophagy and APP processing in Alzheimer's disease: The potential role of Beclin 1 interactome. *Prog Neurobiol*, 106-107, 33-54. doi:10.1016/j.pneurobio.2013.06.002
 33. Scott, E., Zhang, Q. G., Wang, R., Vadlamudi, R., & Brann, D. (2012). Estrogen neuroprotection and the critical period hypothesis. *Front Neuroendocrinol*, 33(1), 85-104. doi:10.1016/j.yfrne.2011.10.001
 34. Shen, H., Pei, H., Zhai, L., Guan, Q., & Wang, G. (2022). Salvianolic acid C improves cerebral ischemia reperfusion injury through suppressing microglial cell M1 polarization and promoting cerebral angiogenesis. *Int Immunopharmacol*, 110, 109021. doi:10.1016/j.intimp.2022.109021
 35. Shen, L., Gan, Q., Yang, Y., Reis, C., Zhang, Z., Xu, S., . . . Sun, C. (2021). Mitophagy in Cerebral Ischemia and Ischemia/Reperfusion Injury. *Front Aging Neurosci*, 13, 687246. doi:10.3389/fnagi.2021.687246

36. Sukhorukov, V., Magnaeva, A., Baranich, T., Gofman, A., Voronkov, D., Gulevskaya, T., . . . Illarionov, S. (2022). Brain Neurons during Physiological Aging: Morphological Features, Autophagic and Mitochondrial Contribution. *Int J Mol Sci*, 23(18). doi:10.3390/ijms231810695
37. Sun, X., Wang, D., Zhang, T., Lu, X., Duan, F., Ju, L., . . . Jiang, X. (2020). Eugenol Attenuates Cerebral Ischemia-Reperfusion Injury by Enhancing Autophagy via AMPK-mTOR-P70S6K Pathway. *Front Pharmacol*, 11, 84. doi:10.3389/fphar.2020.00084
38. Sun, Y. Y., Zhu, H. J., Zhao, R. Y., Zhou, S. Y., Wang, M. Q., Yang, Y., & Guo, Z. N. (2023). Remote ischemic conditioning attenuates oxidative stress and inflammation via the Nrf2/HO-1 pathway in MCAO mice. *Redox Biol*, 66, 102852. doi:10.1016/j.redox.2023.102852
39. Venketasubramanian, N., Yoon, B. W., Pandian, J., & Navarro, J. C. (2017). Stroke Epidemiology in South, East, and South-East Asia: A Review. *J Stroke*, 19(3), 286-294. doi:10.5853/jos.2017.00234
40. Wang, L., Wang, J., Shan, Q., Shu, H., & Guo, J. M. (2023). Involvement of baroreflex deficiency in the age-related loss of estrogen efficacy against cerebral ischemia. *Front Aging Neurosci*, 15, 1167170. doi:10.3389/fnagi.2023.1167170
41. Wang, T., & Liu, C. (2022). Role of hypoxia inducible factor 1 α /Bcl-2/adenovirus E1B 19-kDa interacting protein 3 in alleviating effect of interleukin-4 on cerebral ischemia reperfusion injury in mice. *Afr Health Sci*, 22(3), 369-374. doi:10.4314/ahs.v22i3.39
42. Xu, S., Huang, P., Yang, J., Du, H., Wan, H., & He, Y. (2023). Calycosin alleviates cerebral ischemia/reperfusion injury by repressing autophagy via STAT3/FOXO3a signaling pathway. *Phytomedicine*, 115, 154845. doi:10.1016/j.phymed.2023.154845
43. Yin, L., Ouyang, D., Lin, L., Xin, X., & Ji, Y. (2021). Salidroside regulates imbalance of Th17/Treg and promotes ischemic tolerance by targeting STAT-3 in cerebral ischemia-reperfusion injury. *Arch Med Sci*, 17(2), 523-534. doi:10.5114/aoms.2019.85349
44. Yu, X., Luo, Y., Yang, L., Chen, P., & Duan, X. (2023). P-hydroxybenzyl alcohol ameliorates neuronal cerebral ischemia-reperfusion injury by activating mitochondrial autophagy through SIRT1. *Mol Med Rep*, 27(3). doi:10.3892/mmr.2023.12955
45. Zeng, X., Zhang, Y. D., Ma, R. Y., Chen, Y. J., Xiang, X. M., Hou, D. Y., . . . Duan, C. Y. (2022). Activated Drp1 regulates p62-mediated autophagic flux and aggravates inflammation in cerebral ischemia-reperfusion via the ROS-RIP1/RIP3-exosome axis. *Mil Med Res*, 9(1), 25. doi:10.1186/s40779-022-00383-2
46. Zhang, X., Yan, H., Yuan, Y., Gao, J., Shen, Z., Cheng, Y., . . . Chen, Z. (2013). Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy*, 9(9), 1321-1333. doi:10.4161/auto.25132
47. Zhang, Y., Guo, X., Wang, G., Liu, J., Liang, P., Wang, H., . . . Wu, Q. (2020). Effects of rhodioloside on the neurological functions of rats with total cerebral ischemia/reperfusion and cone neuron injury in the hippocampal CA1 region. *PeerJ*, 8, e10056. doi:10.7717/peerj.10056
48. Zhang, Z. L., Qin, P., Liu, Y., Zhang, L. X., Guo, H., Deng, Y. L., . . . Hou, W. G. (2017). Alleviation of ischaemia-reperfusion injury by endogenous estrogen involves maintaining Bcl-2 expression via the

Figures

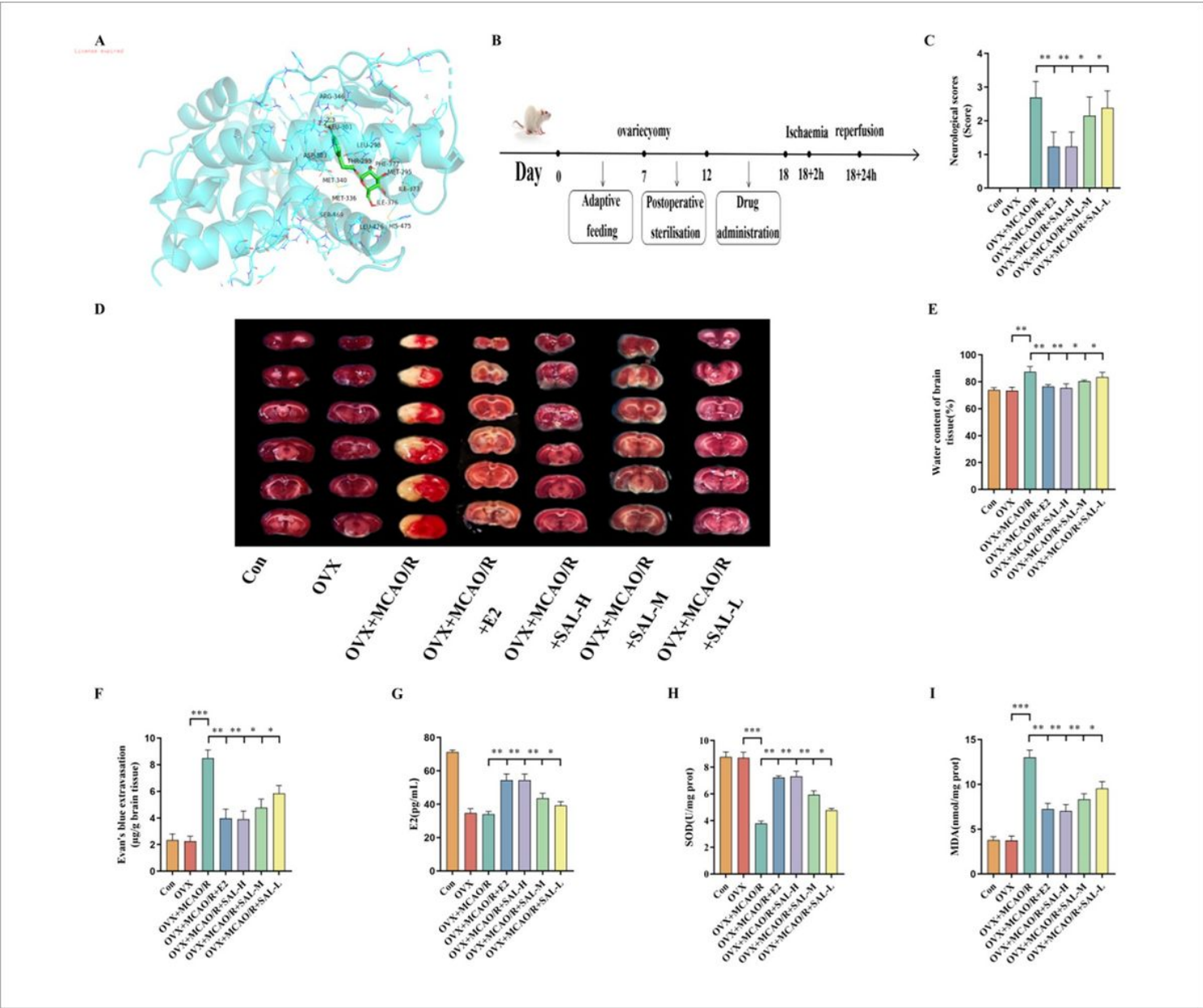


Figure 1

Note: SAL protects neuronal cells in CIRI rats. (A):The result of molecular docking.(B):Groups of rats and drug administration. (C):Neurological loss was assessed using the Longa score (n=13/group). (D):Cerebral infarct volume was determined by TTC staining (n=3/group). (E):Brain water content was determined by wet-dry method (n=3/group). (F):EB content was quantified using a spectrophotometer (n=3/group). Tissues on the ischemic side were subjected to E2(G), SOD(H), and MDA(I) content determination using the appropriate kits (n=6/group). * $P<0.05$; ** $P<0.01$. Comparisons between each group are marked in the Figure.

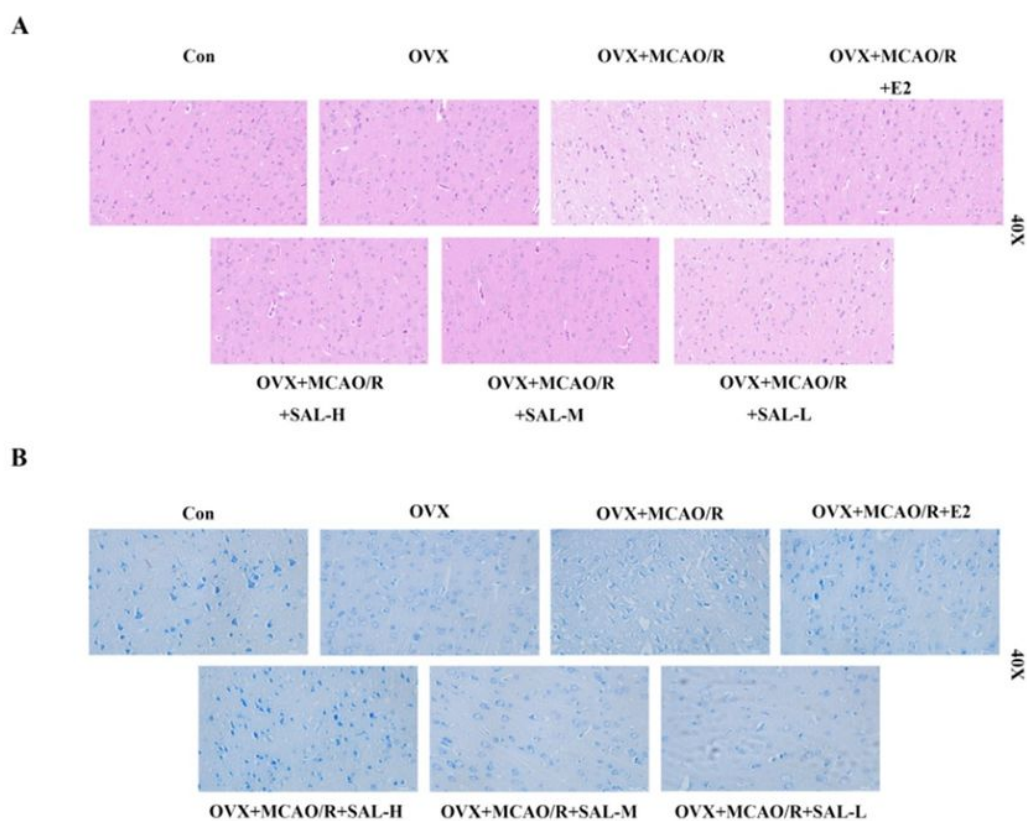


Figure 2

Note: SAL reduces neuronal damage in rats after CIRI. (A):HE staining to detect neuronal structural damage. (B):Nissl staining to detect neuronal morphology and structure.

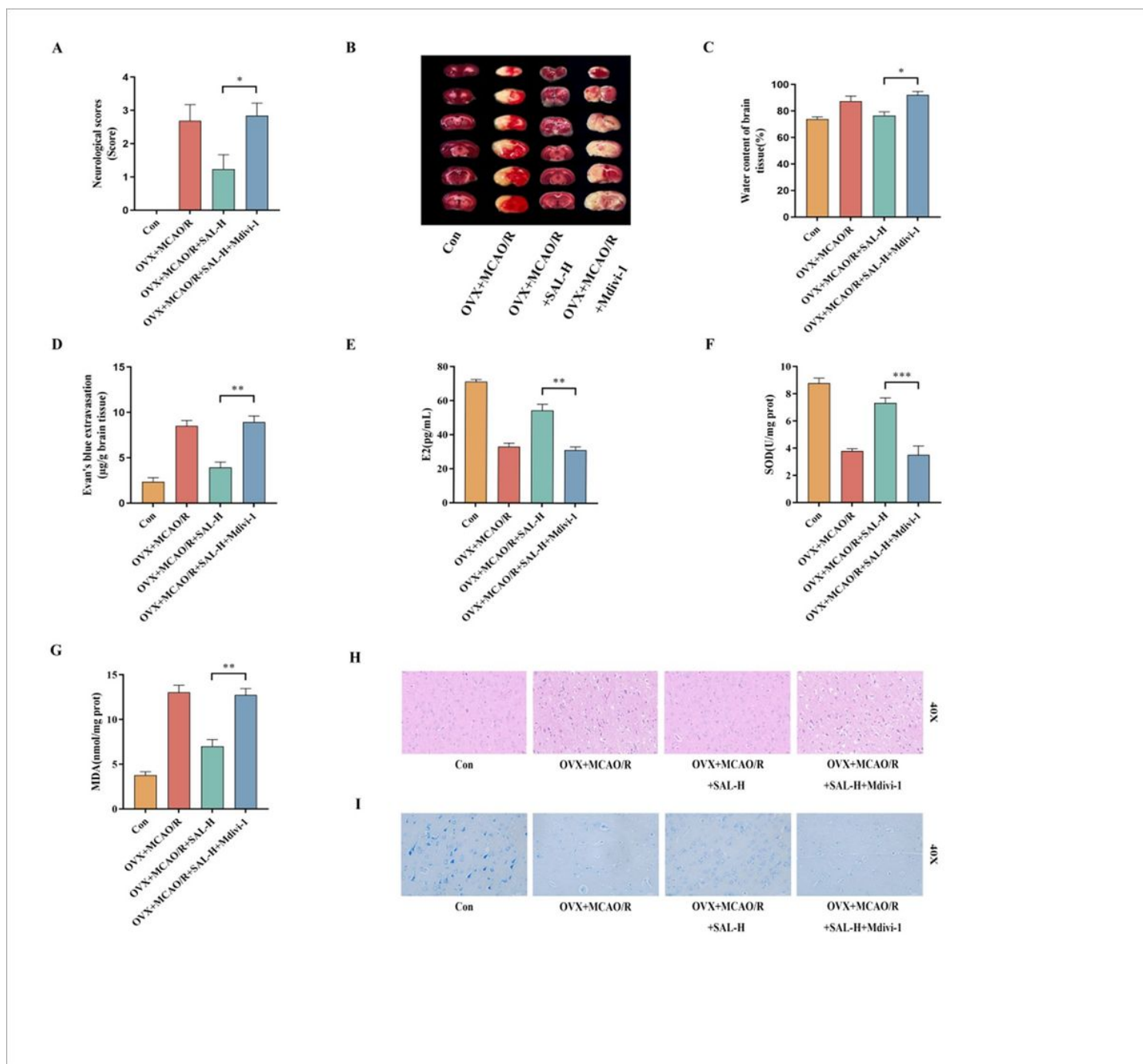


Figure 3

Note: SAL attenuated CIRI in rats by inhibiting mitochondrial autophagy. (A) Neurological function loss was assessed using Longa score (n=13/group). (B): Cerebral infarct volume was determined by TTC staining (n=3/group). (C): Brain water content was determined by wet-dry method (n=3/group). (D): EB content was quantified using a spectrophotometer (n=3/group). Tissues on the ischemic side were subjected to E2 (E), SOD (F), and MDA (G) content determination using the appropriate kits (n=6/group). (H): HE staining to detect neuronal structural damage. (I): Nissl staining to detect neuronal morphology and structure.

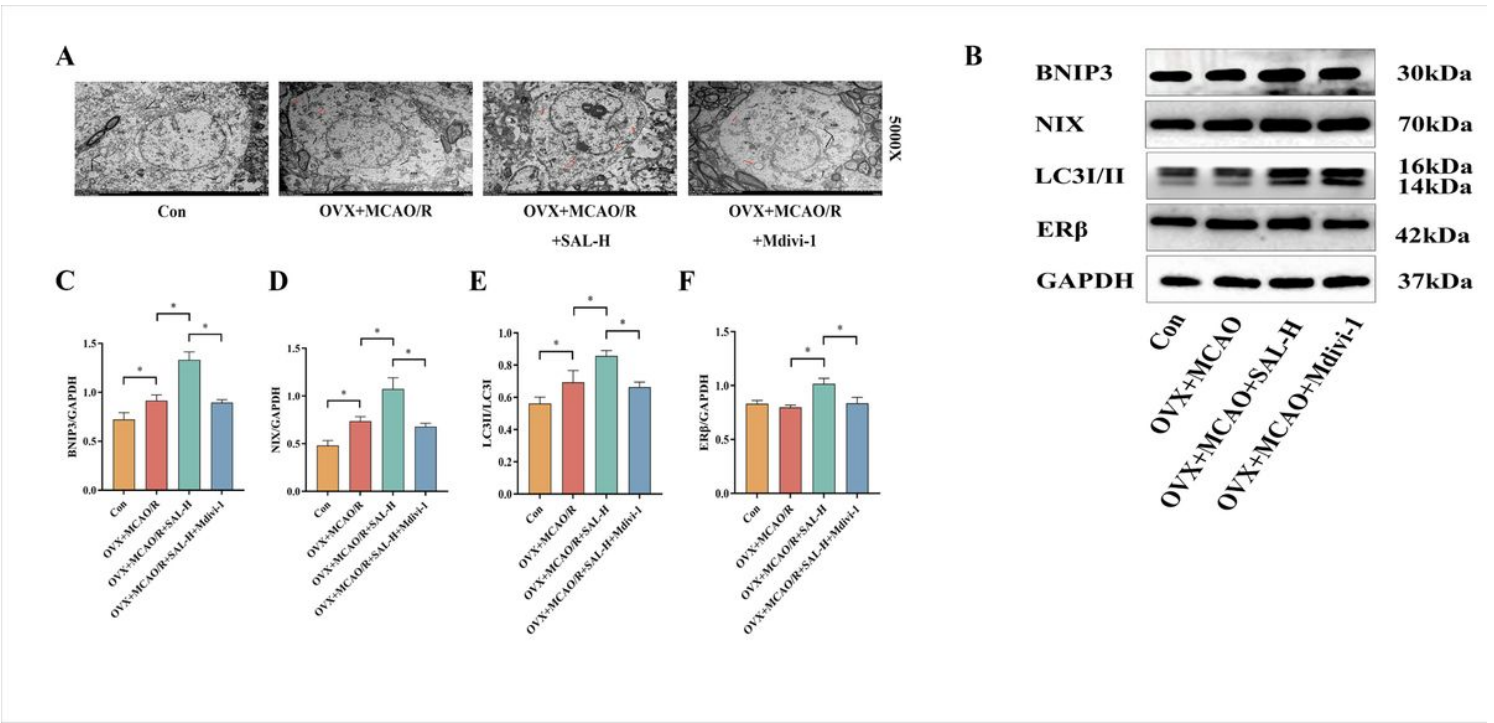


Figure 4

Note: Molecular mechanisms of mitochondrial autophagy in MCAO/R injury. (A):Mitochondrial autophagy was examined using TEM. (B-G):Detection of autophagy proteins by Western bolt method (n=6/group). * $P<0.05$,** $P<0.01$. Comparison between each group is marked in the figure.

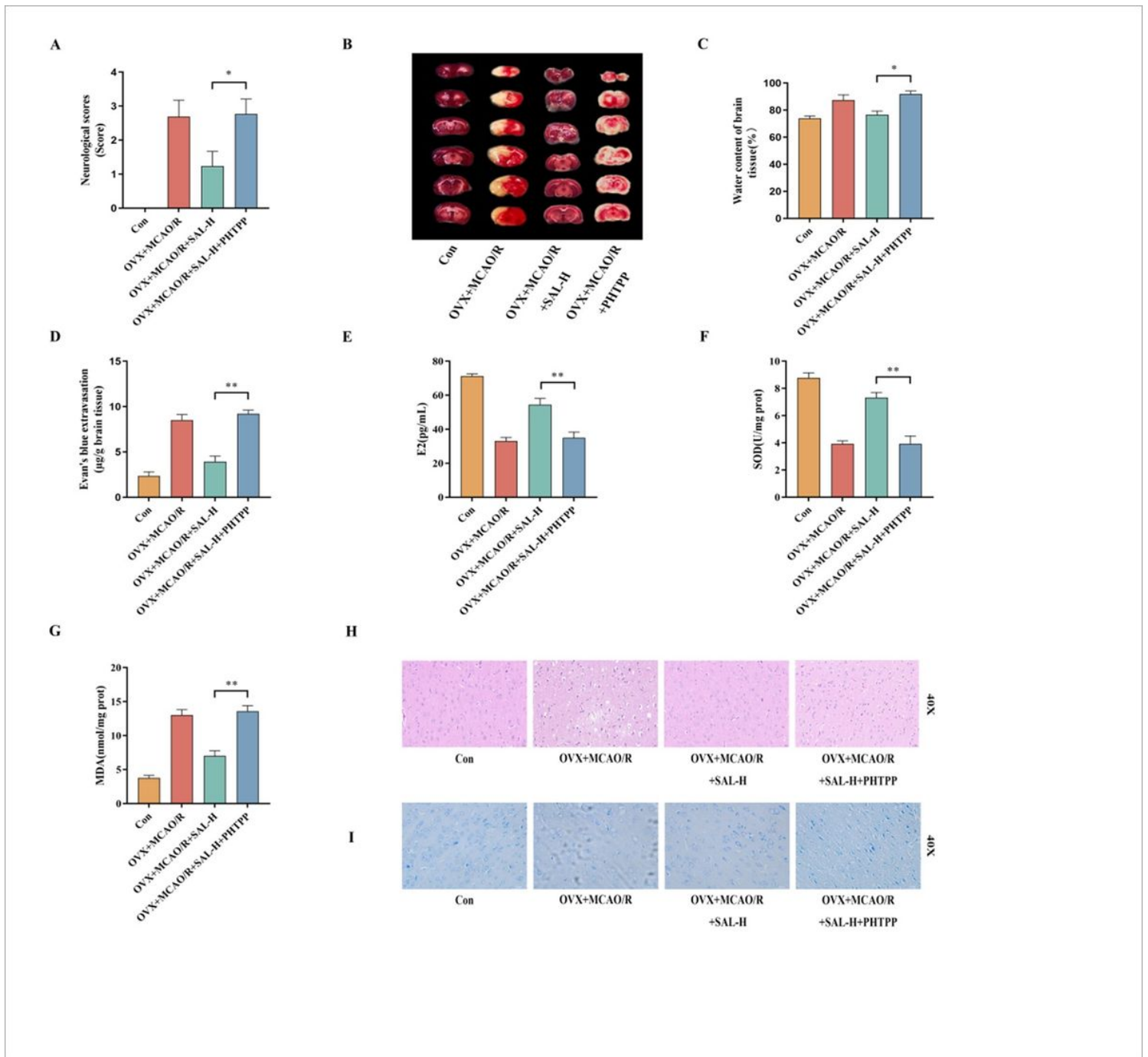


Figure 5

Note: ER β alleviated CIRI-induced neuronal damage. SAL attenuated CIRI in rats by inhibiting mitochondrial autophagy. (A): Neurological function loss was assessed using the Longa score (n=13/group). (B): Cerebral infarct volume was determined by TTC staining (n=3/group). (C): Brain water content was determined by wet-dry method (n=3/group). (D): EB content was quantified using a spectrophotometer (n=3/group). Tissues on the ischemic side were subjected to E2 (E), SOD (F), and MDA (G) content determination using the appropriate kits (n=6/group). (H): HE staining to detect neuronal structural damage. (I): Nissl staining to detect neuronal morphology and structure.

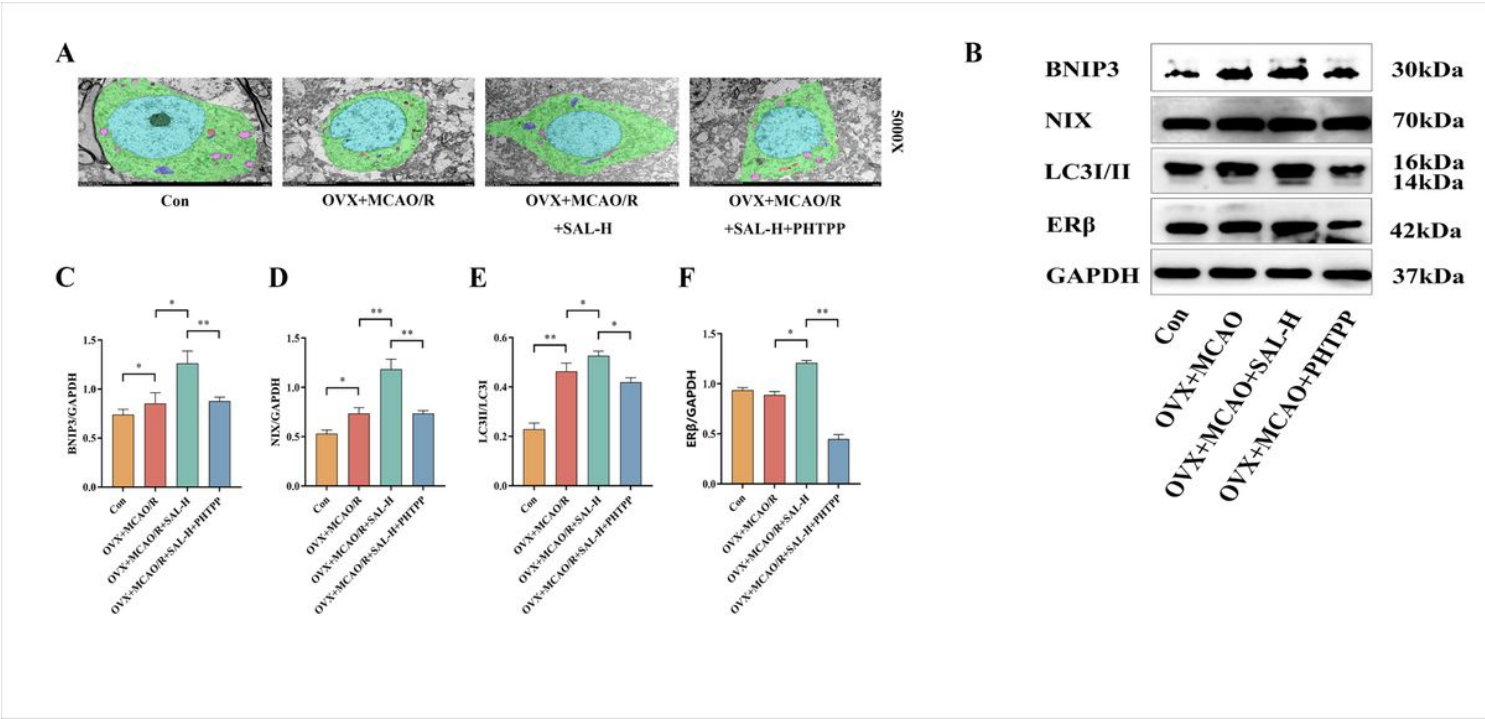


Figure 6

Note: Effects of ERβ on mitochondrial autophagic structures and proteins. (A):Examination of mitochondrial autophagy using TEM. (B-G):Detection of autophagy proteins by Western bolt assay (n=6/group). * $P<0.05$,** $P<0.01$. Comparison between each group is marked in the figure.

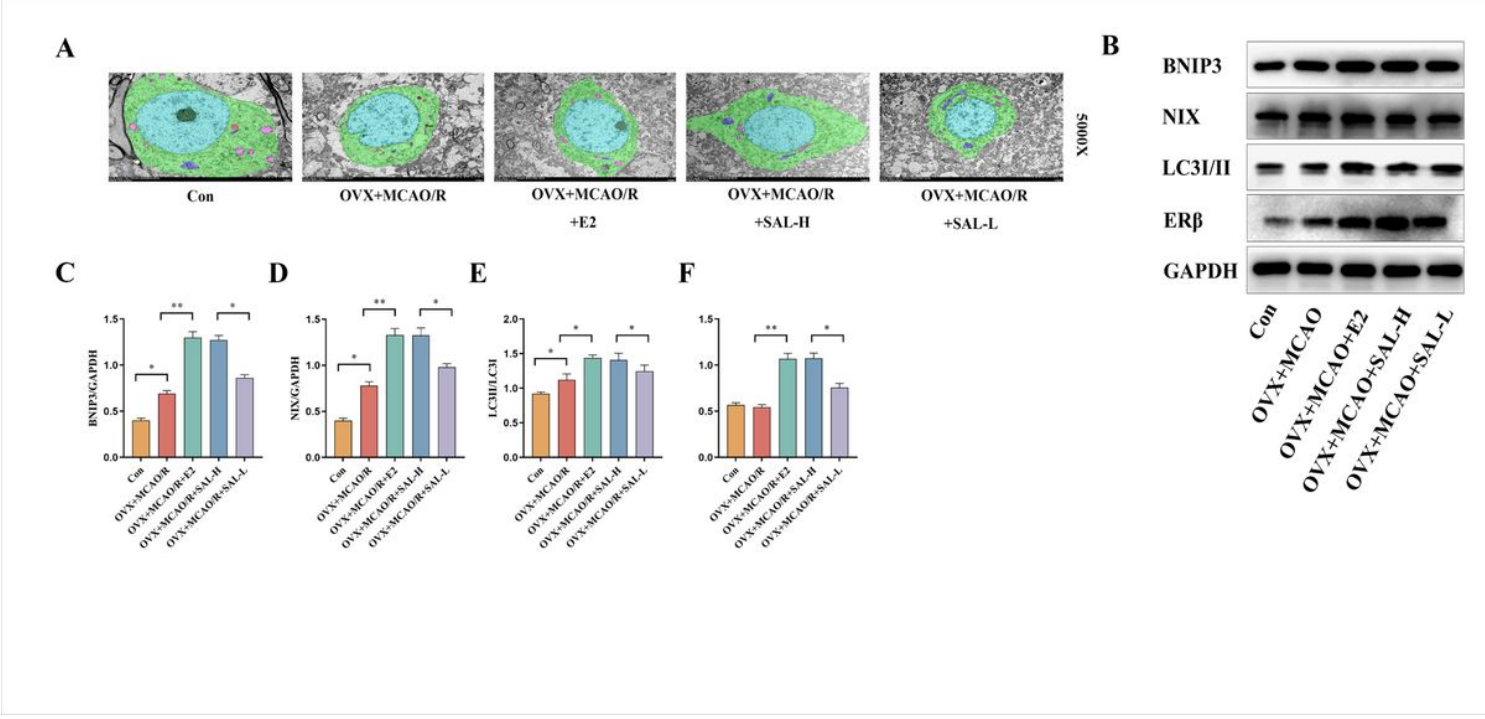


Figure 7

Note: SAL promotes mitochondrial autophagy and its associated protein expression by increasing ER β . (A):Mitochondrial autophagy was examined using TEM. (B-G):Detection of autophagy proteins by Western bolt assay (n=6/group). * $P<0.05$; ** $P<0.01$. Comparisons between each group are marked in the figure.