



Salidroside inhibited cerebral ischemia/reperfusion-induced oxidative stress and apoptosis via Nrf2/Trx1 signaling pathway

Fuyuan Li¹ · Qianqian Mao¹ · Jinyu Wang² · Xiaoying Zhang¹ · Xinyan Lv¹ · Bo Wu² · Tingxu Yan² · Ying Jia²

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Abstract

Cerebral ischemia reperfusion injury (CIRI) is still a serious problem threatening human health. Salidroside (SAL) is a natural phenylpropanoid glycoside compound with antioxidant, anti-inflammatory, and anti-ischemic properties. This study investigated the protective mechanism of SAL on middle cerebral artery occlusion (MCAO)- and oxygen-glucose deprivation/reoxygenation (OGD/R) model-induced CIRI via regulating the nuclear factor erythroid 2-related factor 2 (Nrf2)/thioredoxin 1 (Trx1) axis. The results indicated that SAL (50 mg/kg or 100 mg/kg, intraperitoneal injection) not only effectively alleviated infarction rate, improved histopathological changes, relieved apoptosis by strengthening the suppression of cleaved caspase-3 and Bax/Bcl-2 proteins and decreased malondialdehyde (MDA) formation, but also increased superoxide dismutase (SOD) and catalase (CAT) activities and upregulated the expressions of Nrf2 and Trx1 on MCAO-induced CIRI rats. SAL also efficiently inhibited apoptosis and decreased oxidative stress in OGD/R-stimulated PC12 cells. Furthermore, blocking the Nrf2/Trx1 pathway using tretinoin, an Nrf2 inhibitor, significantly reversed the protective effect of SAL on OGD/R-induced oxidative stress. Moreover, SAL reduced the expression of apoptosis signal-regulating kinase-1 (ASK1) and mitogen-activated protein kinase (MAPK) family proteins. These results demonstrated that SAL inhibited oxidative stress through Nrf2/Trx1 signaling pathway, and subsequently reduced CIRI-induced apoptosis by inhibiting ASK1/MAPK.

Keywords Cerebral ischemia/reperfusion · Salidroside · Nrf2/Trx1 · Oxidative stress · Apoptosis

Introduction

Cerebral ischemia or ischemic stroke is an acute cerebrovascular disease with high rates of mortality and disability, which has caused a huge burden to humans (Roth et al. 2020). It is caused by a lack of blood supply, which results in the immediate depletion of oxygen and glucose in brain tissues. At present, the effective clinical treatment for this disease is thrombolysis (Group. 1995), but the rapid recovery

of blood flow and nutrients during thrombolysis may aggravate brain injury and lead to cerebral ischemia/reperfusion injury (CIRI). The development of CIRI involves complex pathophysiological events, including free radical injury, oxidative stress, inflammatory response, intracellular calcium overload, and apoptosis (Meng et al. 2019; Wang et al. 2016; Ye et al. 2012; Wu et al. 2020). Therefore, finding a more effective treatment for CIRI has been a hot and challenging topic in this field.

Apoptosis, also known as programmed cell death, caused by oxidative stress and excitotoxicity, plays a crucial role in CIRI (Fangma et al. 2021). Moreover, excessive reactive oxygen species in brain tissues after reperfusion will cause oxidative stress. Nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidative responses, can play a protective role by coordinating the basal and stress-inducible activation of multiple cytoprotective genes (Vomund et al. 2017). It is reported that caffeic acid phenethyl ester 4-O-glucoside could inhibit oxidative stress to ameliorate neurodegenerative diseases by activating the

✉ Tingxu Yan
yantingxu@126.com

✉ Ying Jia
jiayingyaphu@126.com

¹ School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, China

² School of Functional Food and Wine, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, China

Nrf2 pathway (Wan et al. 2019). Furthermore, Nrf2 could also alleviate CIRI by promoting antioxidant activity. A previous study has shown that Nrf2 played a protective role by regulating the expression of thioredoxin 1 (Trx1) during CIRI (Hou et al. 2018). Trx1 is a cellular redox enzyme that plays an indispensable role in cell redox homeostasis. After CIRI, Trx1 silencing increased the production of reactive oxygen species (ROS), where its expression level was probably in part regulated by Nrf2 (Li et al. 2015). Trx1 can interact with apoptosis signal-regulating kinase-1 (ASK1), and the knockdown of Trx1 could promote phosphorylation of ASK1 to induce apoptosis (Wu et al. 2015). ASK1 is a crucial effector in ROS-induced cell death, and blocking the ASK1/MAPK signaling pathway is considered to be able to inhibit apoptosis (Yoon et al. 2020). The Nrf2/Trx1 axis is a specific target of therapeutic intervention. Consequently, targeting the Nrf2/Trx1 axis is an attractive therapeutic strategy for stroke prevention and reversal.

Salidroside (SAL, Fig. 1A shows the chemical structure of SAL) is a phenylpropanoid glycoside compound isolated from *Rhodiola rosea* L. growing in high-altitude areas. Additionally, SAL has extensive pharmacological activities, such as anti-inflammatory, antioxidant, anti-apoptosis, and cardioprotective (Chen et al. 2019; Huang et al. 2019; Rong et al. 2020; Wu et al. 2019). Many studies have shown that SAL has protective effects on cell damage and apoptosis through different cell signal transduction pathways. For example, SAL could protect human umbilical vein endothelial cells (HUVECs) from oxidized low-density lipoprotein (ox-LDL) damage by inhibiting oxidative stress (Dongming et al. 2019). A recent study has indicated that SAL could alleviate hepatic oxidative stress induced by hypoxia through the Nrf2 signaling pathway (Xiong et al. 2021). Here, SAL could cross the blood-brain barrier when given intraperitoneally (Zuo et al. 2018). Moreover, SAL could be used to treat neurodegenerative diseases, and its neuroprotective effect is involved in the Nrf2 pathway (Zhong et al. 2018). This study aims to explore whether SAL plays an antioxidant stress role through the Nrf2/Trx1 pathway in CIRI, thus playing an anti-apoptosis role.

Materials and methods

Animals

All the animal experiments were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University. Experiments were performed with SPF male, 50 Sprague-Dawley (SD) rats weighing 250–300 g from the Central Animal House of Shenyang Pharmaceutical University (Shenyang, China). Rats were kept in plastic cages in

standard laboratory conditions (temperature $23 \pm 2^\circ\text{C}$, 12 h light/dark cycle) accessing to adequate food and water, and they were meanwhile allowed to adjust the environment for 7 days before the experiment.

Middle cerebral artery occlusion (MCAO) injury operation

All groups of rats except for rats in the sham group were treated with modified Longa's method (Longa. 1989). The rats were anesthetized and placed on a surgical plate. Subsequently, an incision was made along the median line of the neck and the right common, external (ECA) and internal (ICA) carotid arteries were carefully exposed and separated. After the ligation of the distal end of the ECA and its branches, a silicone-coated nylon monofilament (Beijing Cinontech Co., Ltd., China) was inserted through the ECA stump into the ICA and gently advanced to block the middle cerebral artery. After 2 h of MCAO, the filament was removed to restore cerebral perfusion. The body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating lamp throughout the surgery and occlusion period. In the sham group, all surgical procedures were the same as above except for the insertion of the nylon monofilament.

Groupings

The experimental procedures of the in vivo experiments are shown in Fig. 1B. All rats were randomly divided into 5 groups ($n = 10$) including: sham, MCAO, MCAO + 100 mg/kg SAL (purity > 98% by HPLC, Cat. #MB5843, Meilun Biotech, China) (Zhong et al. 2019), MCAO + 50 mg/kg SAL, MCAO + 5 mg/kg edaravone (ED, positive control group) (purity > 98% by HPLC, Cat. #MB1441, Meilun Biotech, China), no rats were dead. Seven days before MCAO, all groups of rats except for the sham group were injected intraperitoneally once a day. SAL and ED were dissolved in normal saline solution, respectively before use. The sham group was given normal saline.

2,3,5-Triphenyltetrazolium (TTC) staining

Rat brains were quickly removed and coronally sectioned to a thickness of 2 mm. Subsequently, the sections were quickly immersed in a solution containing 2% TTC (Meilun Biotech, China) at 37°C in the dark for 20 min and fixed in 4% paraformaldehyde overnight. TTC staining of each brain section of rats in each group (the unstained area was identified as infarct area) was used to quantitatively evaluate the percentage of infarct volume with Image-Pro Plus software.

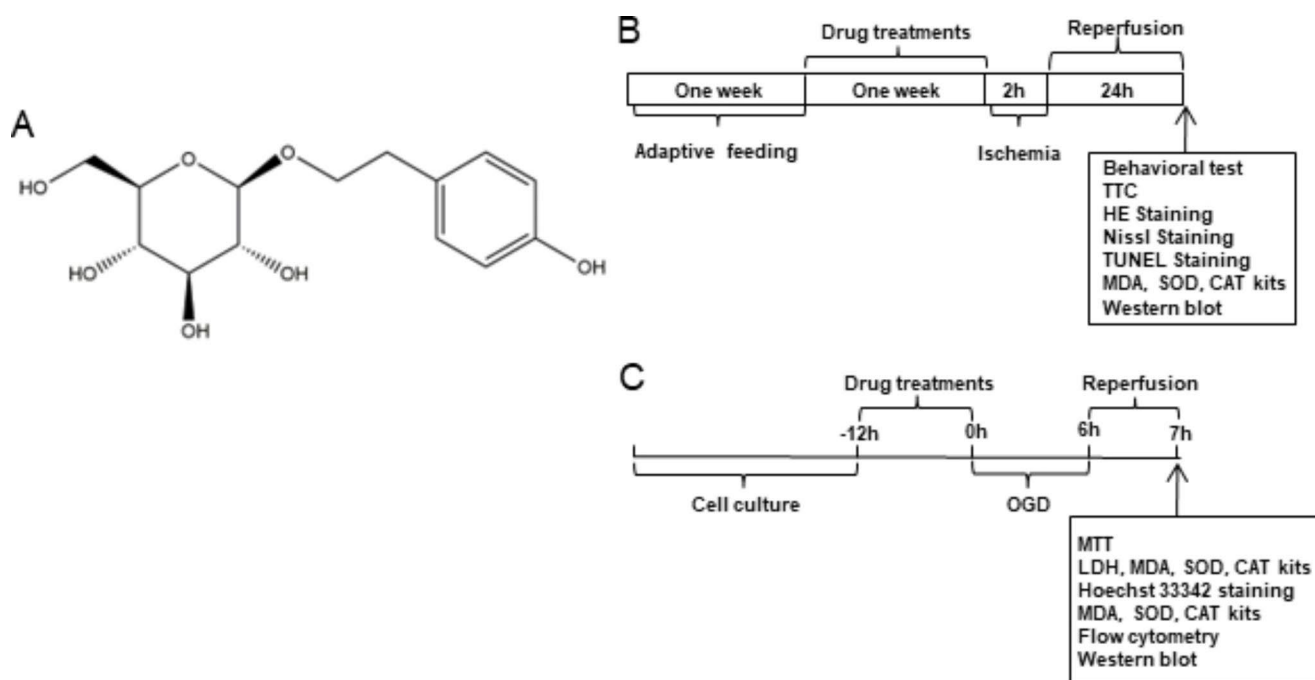


Fig. 1 (A) Chemical structure of SAL. (B) Flow chart of the study design of rats. (C) Flow chart of the study design of PC12 cells

Neurological deficits assessment

Neurological deficits were determined using Longa's methods (Longa, 1989) with minor revision, and the score ranged from 0 to 4 (0, no obvious nerve injury; 1, failed to stretch forepaw fully; 2, circling to the contralateral side; 3, falling to the left or no spontaneous motor activity; 4, do not walk spontaneously). The neurological test was conducted at 24 h after MCAO by three blinded observers.

Tissue preparation and hematoxylin and eosin (HE), Nissl, and TdT-mediated dUTP nick end labeling (TUNEL) staining

Deeply anesthetized rats were perfused first with cold normal saline (4 °C) until forelimbs became pale and the brains were collected gently and fixed in cold 4% paraformaldehyde for 24 h at 4 °C. After dehydration in gradient ethanol, the samples were made transparent with xylene, and the brain cortex was embedded in paraffin. Subsequently, the coronal slices (3–4 μm) were stained with HE staining, Nissl, and TUNEL staining. For histological staining, sections were stained with hematoxylin and eosin (Servicebio, China) for HE staining and Nissl staining solution (Servicebio, China) for Nissl staining, respectively. Apoptosis was assessed histologically and with a TUNEL reagent kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. A microscope (Nikon, Tokyo, Japan) was used to collect images.

Cell culture

The PC12 cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in RPMI-1640 (Meilun Biotech, China) complete medium containing 10% FBS (Meilun Biotech, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Meilun Biotech, China). Cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere. SAL, ED and tretinoin were dissolved in DMSO to make 200 mM stock solution. The stock solution was then diluted with RPMI-1640 complete medium so that the concentration of DMSO was kept below 0.1% to avoid toxic effects on cells in culture. Cells were pretreated with freshly prepared SAL, ED (10 μM) and tretinoin (12.5 μM) (Meilun Biotech, China) for 12 h, before oxygen-glucose deprivation (OGD) and during OGD/reoxygenation (OGD/R). The experimental procedures of the in vitro experiments are shown in Fig. 1C.

OGD/R treatment

Before OGD, the PC12 cells were washed three times with PBS and were then replaced with glucose-free RPMI-1640 (Meilun Biotech, China) and then incubated with 95% N₂/5% CO₂ for 6 h. Cells were then reoxygenated by placing them in fresh normal RPMI-1640 and an incubator with 95% air/5% CO₂ (normoxia) for 1 h, respectively. Control cells were incubated with a normal medium for 6 h and

replaced with a normal medium with normoxic conditions in a manner identical to that for OGD/R (Wang et al. 2020).

Cell viability assay

MTT assay was conducted to determine the cell viability of PC12 cells. In brief, PC12 cells were seeded into 96-well cell culture microplates at an approximate density of 8×10^3 cells per well and incubated overnight. After the treatment with SAL and ED for 12 h, OGD/R was carried out as described above. The cells were incubated with an additional 20 μ L of MTT (Meilun Biotech, China) solution (5 mg/mL) at 37 °C for 3 h. After that, 100 μ L of a solution containing 10% SDS, 5% isopropanol and 0.12 M HCl was added to each well to dissolve the formazan crystals. Finally, the absorbance of each well was read on a microplate reader (Spectra maxm2, Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Detection of LDH, SOD, MDA, CAT

The activities of lactate dehydrogenase (LDH) assay kit (Cat. # A020, Nanjing Jiancheng Bioengineering Institute, China), superoxide Dismutase (SOD) assay kit (Cat. # S0103, Beyotime, China), catalase (CAT) assay kit (Cat. # S0051, Beyotime, China), and malondialdehyde (MDA) assay kit (Cat. # S0131, Beyotime, China) in the brain cortex and PC12 cells were measured according to the protocol recommended.

Reactive oxygen species production assay

2',7'-dichlorofluorescein diacetate (DCFH-DA) (Meilun Biotech, China) staining method was applied to measure the generation of reactive oxygen species. PC12 cells were incubated with 10 μ M DCF-DA for 20 min at 37 °C in the dark. The cell suspension was loaded into the flow-specific tube and analyzed by flow cytometry at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Hoechst 33,342 staining

PC12 cells were seeded into 12-well plates at a density of 10,000 cells per well and cultured for 24 h. Then, the cells were treated with SAL and/or tretinoin for 12 h before OGD/R. Subsequently, cells were washed with cold PBS once. Hoechst 33,342 (Meilun Biotech, China) was added to cells for 20 min incubation at 37 °C in dark. The apoptotic cells with the fragmented nuclei were observed under a fluorescence microscope (MCTIO, Xiamen, China).

Western blot

Total proteins extracted from the ischemic side cerebral cortex and PC12 cells were collected and fractionated on SDS-PAGE gels. Then, the protein was incubated with primary antibodies against Bax (CAT. # 50,599, Proteintech, China), Bcl-2 (CAT. # 26,593, Proteintech, China), cleaved caspase-3 (CAT. # 19,677, Proteintech, China), p-ASK1 (CAT. # 320,030, Zen-Bioscience, China), ASK1 (CAT. # 28,201, Proteintech, China), p-JNK (CAT. # 80,024, Proteintech, China), JNK (CAT. # 66,210, Proteintech, China), Nrf2 (CAT. # 16,396, Proteintech, China), Trx1 (CAT. # 14,999, Proteintech, China), p-ERK1/2 (CAT. # 50,599, Proteintech, China), ERK1/2 (CAT. # 11,257, Proteintech, China), p38 (CAT. # 66,234, Proteintech, China), p-p38 (CAT. # 310,069, Zen-Bioscience, China), and β -actin (CAT. # 20,536, Proteintech, China). After that, the membranes were incubated with the corresponding secondary antibodies (CAT. # SA00001, Proteintech, China) and scanned using the Tamon imaging system (Shanghai, China). The relative band intensity was normalized to that of β -actin. Band intensities were quantified using Image-Pro 6.0 software (Media Cybernetics, Baltimore, MD, USA).

Statistical analysis

Data are expressed as means \pm SEM. Differences between the groups were analyzed by one-way ANOVA with Tukey's multiple comparisons test by GraphPad Prism (SanDiego, CA, USA). The differences among the groups were considered statistically significant at $P < 0.05$.

Results

Neuroprotective effects of SAL against CIRI

To study the potential neuroprotective effect of SAL on CIRI, the histopathological changes in the brain tissues were evaluated using TTC staining following treatment of MCAO rats with SAL. The largest visible infarct area was found in the MCAO group (Fig. 2 A-B), which was significantly reduced with the treatment of high-dose SAL ($P < 0.001$). As shown in Fig. 2 C, the sham group showed no symptoms of neurological impairment, and the neurological function score was 0. On the contrary, the neurological deficit score of the MCAO group was significantly higher than that of the sham group ($P < 0.001$), suggesting that the neurological function of the rats was severely damaged and the modeling was successful. The symptoms of the SAL group were less than those of the MCAO group ($P < 0.001$), suggesting that SAL could ameliorate neurological impairment.

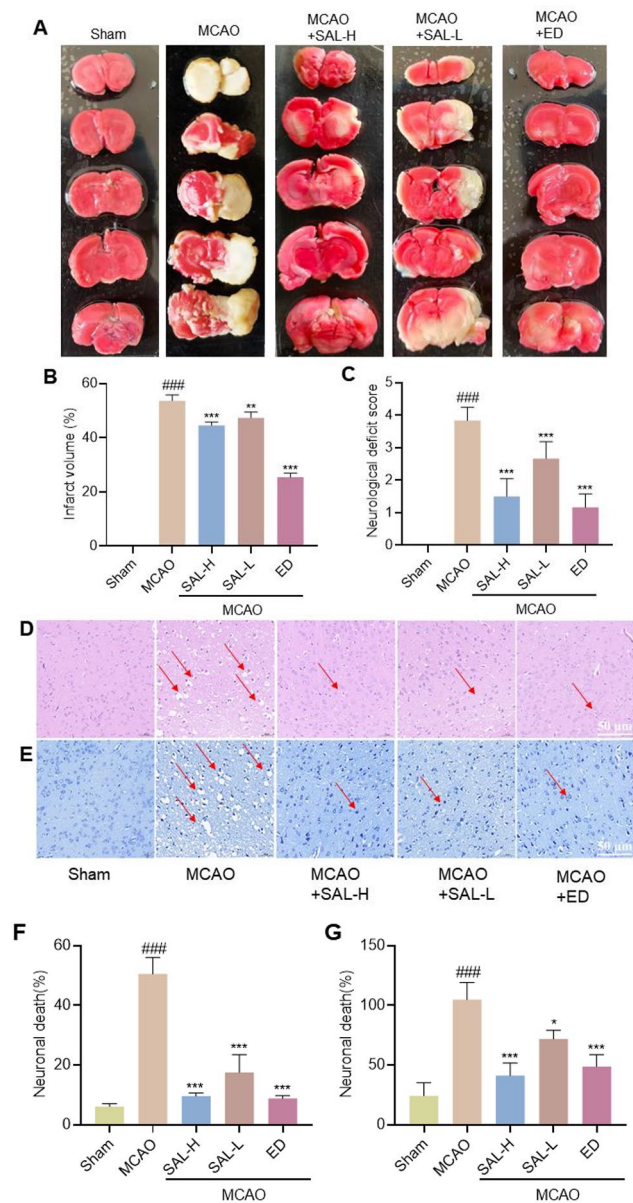


Fig. 2 Neuroprotective effects of SAL against CIRI. (A and B) Cerebral infarction volume in each group was evaluated by using TTC staining. Normal brain tissue (red) and infarction area (white), scale bar = 2 mm. (C) Statistical graph of neurological deficit score after reperfusion. Histopathological changes in the brain tissues were evaluated using HE staining (D) and Nissl staining (E) images. (F) was quantitative analysis of HE staining. (G) was quantitative analysis of Nissl staining Magnification, x200. Values are presented as mean \pm SEM (n=6). ### P <0.001 versus sham group; * P <0.05, ** P <0.01 and *** P <0.001 versus MCAO group

The effects of SAL on neuronal morphology of cortical neurons in rats after MCAO were determined by HE staining and Nissl staining (Fig. 2D-E). The structure of brain tissue was normal, the cells were arranged orderly with clear cell outline, necrotic cells were not observed, Nissl bodies, nuclei and nucleoli were clearly distinguishable in the sham

group. Instead, in the MCAO group, the brain tissues were arranged irregularly, stained unevenly, the intercellular space increased, the number of cells decreased obviously, Nissl bodies shrank and were distributed disorderly, and the nuclei were small and even disappeared. However, SAL could reduce damage by reducing the number of damaged cells (P <0.001). Taken together, these results suggested that SAL had neuroprotective effects on CIRI.

Effects of SAL on cellular apoptosis in the brain of rats in CIRI

Apoptosis was detected by the TUNEL assay. Compared with the sham group, the number of apoptotic cells was increased after MCAO in brain tissue (P <0.001) (Fig. 3 A-B). The cell apoptosis induced by MCAO was better reduced by high-dose SAL (P <0.001). The ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 increased in the MCAO group (P <0.001) (Fig. 3 C-E), while SAL obviously reversed their expression compared with the MCAO group (Bax/Bcl-2, P <0.001; cleaved caspase-3, P <0.05). These results indicated that SAL could inhibit CIRI-induced apoptosis.

Effects of SAL on oxidative stress in the brain of rats in CIRI

As shown in Fig. 4 A, compared with the sham group, the activities of SOD and CAT were decreased, and the activity of MDA was increased after MCAO, while high-dose SAL treatment better reversed MCAO-induced changes in brain tissues (P <0.001). Meanwhile, it was found that the expression of Nrf2 and its downstream factor Trx1 were significantly reduced in the MCAO group in brain tissues of rats compared with the sham group. SAL obviously increased their expression compared with the MCAO group, and the effect of high-dose SAL was better (P <0.001) (Fig. 4B-D). Our results showed that SAL attenuated oxidative stress in the brain of rats after MCAO.

Effects of SAL on OGD/R induced apoptosis of PC12 cells

The effects of SAL on the survival of PC12 cells were examined using the MTT assay. The results showed that PC12 cells treated with 0–50 μ M SAL did not affect cells viability (Fig. 5 A). LDH assay was then performed for the detection of cytotoxicity, and the result showed that SAL led to a marked decrease in the cytotoxicity of OGD/R induced by PC12 cells (P <0.001) (Fig. 5B). Besides, the expression of apoptosis-related proteins was detected by western blot. The results showed that PC12 cells treated with OGD/R

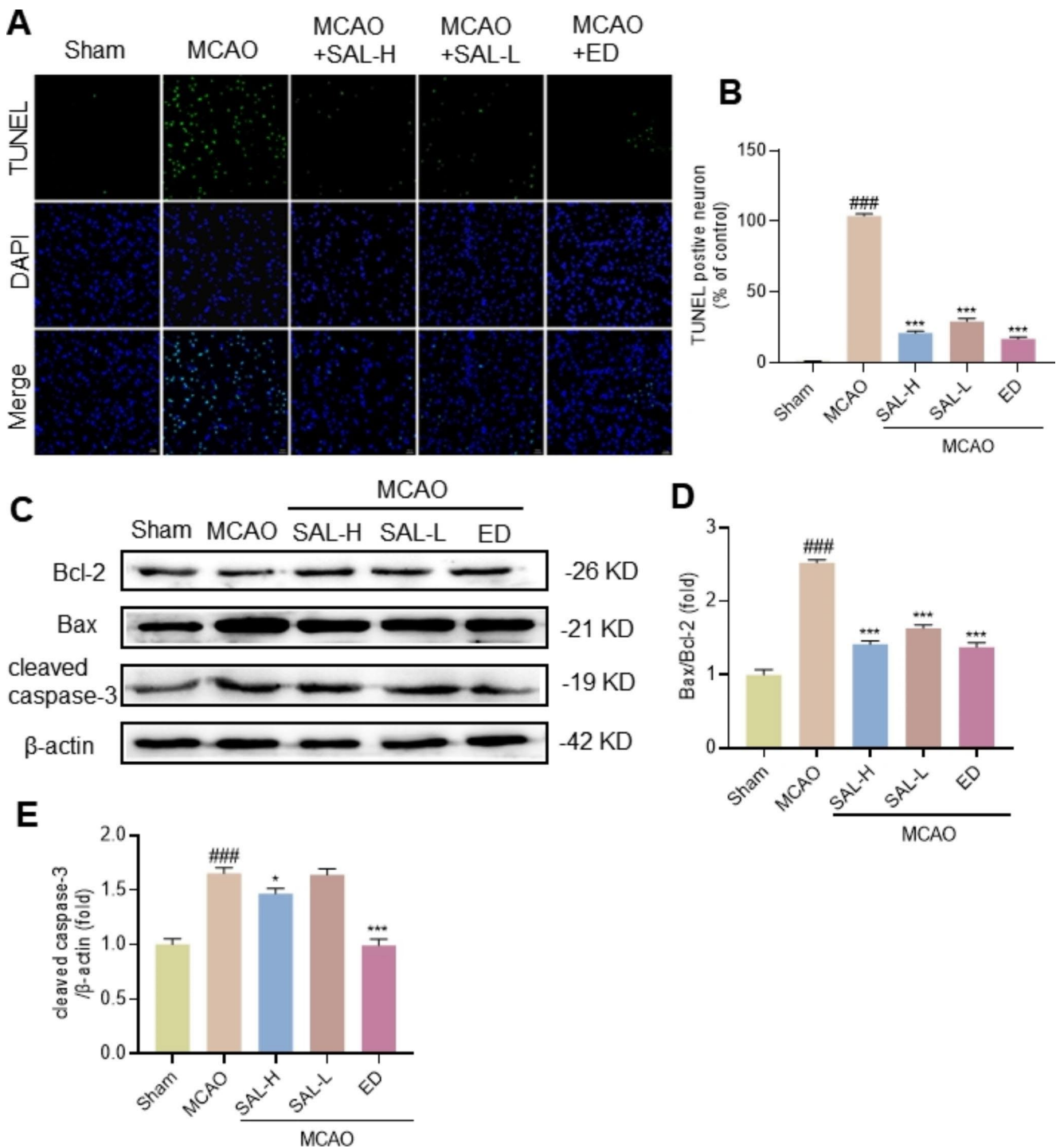


Fig. 3 Effects of SAL on cellular apoptosis in the brain of rats after MCAO. (A–B) The apoptotic neurons were detected by TUNEL (green)/DAPI (blue) staining. The proportion of apoptotic cells was analyzed quantitatively. Magnification, $\times 200$. (C–E) The expression of Bax, Bcl-2, and cleaved caspase-3 proteins. Values are presented as mean \pm SEM. ^{###} $P < 0.001$ versus sham group; ^{*} $P < 0.05$ and ^{***} $P < 0.001$ versus MCAO group

significantly increased the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 ($P < 0.001$) (Fig. 5 C–E). Their expression induced by OGD/R were reversed by SAL, with the most significant effect at the dose of 50 μ M ($P < 0.001$).

These results showed that SAL played an anti-apoptosis role *in vitro*.

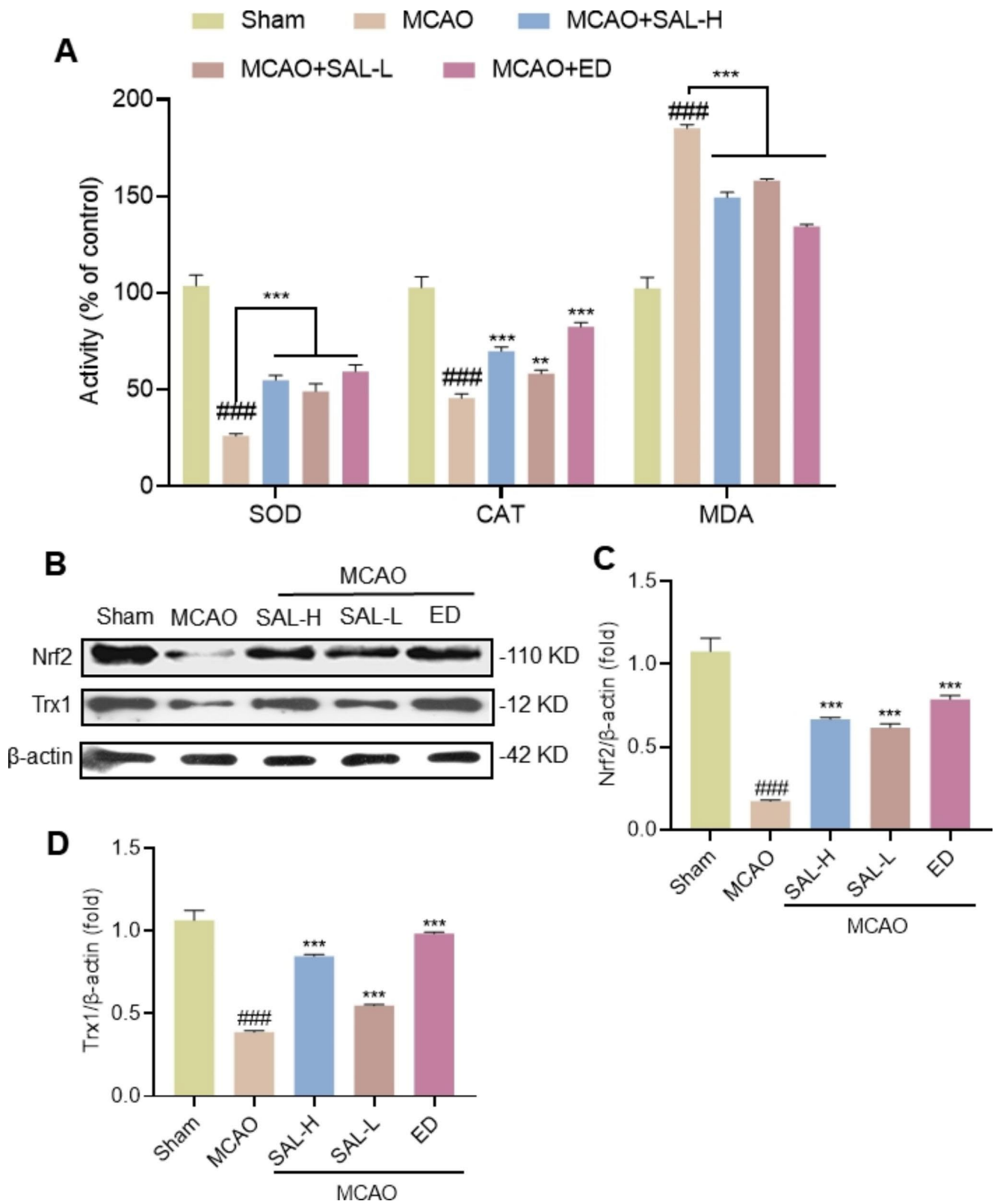


Fig. 4 Effects of SAL on oxidative stress in the brain of rats after MCAO. (A) SOD, CAT, and MDA commercial kits were used to detect the activities of SOD, CAT, and MDA in the brain of rats. (B–D) Expression of Nrf2 and Trx1 proteins. Values are presented as mean \pm SEM. ### $P < 0.001$ versus sham group; ** $P < 0.01$ and *** $P < 0.001$ versus MCAO group

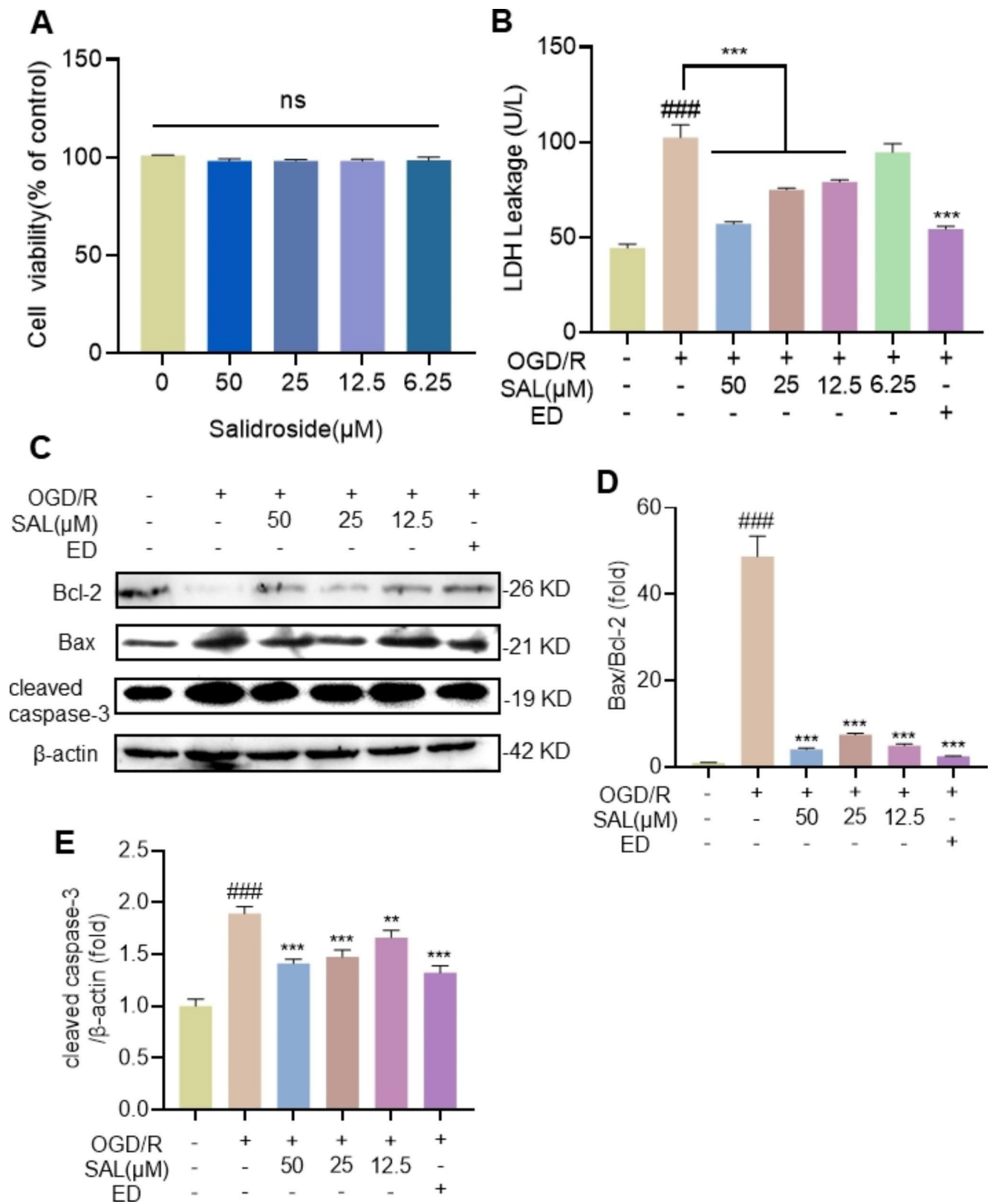


Fig. 5 Effects of SAL on OGD/R induced apoptosis of PC12 cells. **(A)** Cell viability of PC12 cells treated with SAL (0–50 μM). **(B)** The LDH release of PC12 cells treated with SAL (0–50 μM) followed by OGD/R stimulation. **(C–E)** The expression of Bax, Bcl-2, and cleaved caspase-3 proteins. Values are presented as mean ± SEM. ####*P* < 0.001 versus control group; ***P* < 0.01 and ****P* < 0.001 versus OGD/R group

Effects of SAL on oxidative stress in OGD/R treated PC12 cells

To further explore the potential effects of SAL on OGD/R-induced PC12 cells, indicators of oxidative stress were examined *in vitro*. As shown in Fig. 6 A, compared with the control group, the SOD and CAT activities of the OGD/R group significantly decreased, and the MDA activity increased, while this change was significantly reversed after SAL treatment, and the reversal effect at the dose of 50 μM was even more pronounced ($P < 0.001$). Subsequently, the level of ROS in PC12 cells was detected by a DCFH-DA probe. OGD/R was shown to increase the level of ROS in PC12 cells, while treatment with SAL decreased ROS production ($P < 0.001$) (Fig. 6B-C). Meanwhile, it was found that OGD/R significantly reduced the expression of Nrf2 and its downstream factor Trx1 in PC12 cells compared with the control, but after treatment with SAL, their expression increased significantly (Nrf2, $P < 0.01$; Trx1, $P < 0.001$) (Fig. 6D-E). The results demonstrated that SAL inhibited oxidative stress in OGD/R-treated PC12 cells.

Effects of SAL on oxidative stress in OGD/R treated PC12 cells by Nrf2/Trx1 pathway

To determine whether the Nrf2 pathway is involved in the inhibitory effect of SAL on OGD/R, we exposed PC12 cells to tretinoin, an Nrf2 inhibitor, to detect the downstream related indicators. Indicators of oxidative stress in PC12 cells were detected (Fig. 7 A-C). OGD/R was shown to reduce the activities of SOD and CAT and increase the activity of MDA, thus producing oxidative stress, while SAL treatment showed an opposite effect. Moreover, tretinoin eliminated the inhibitory effect of SAL on oxidative stress ($P < 0.001$). And it was found that SAL reversed the downregulation of Nrf2 and Trx1 expression induced by OGD/R, while tretinoin inhibited the SAL-activated Nrf2/Trx1 pathway ($P < 0.001$) (Fig. 7D-E). The results further indicated that SAL inhibited oxidative stress in PC12 cells by the Nrf2/Trx1 pathway.

Effects of SAL mediated Nrf2/Trx1 signaling on oxidative stress-driven apoptosis

Finally, we determined whether the mechanism behind how SAL reduced apoptosis driven by oxidative stress was directly involved in Nrf2 up-regulation. As shown in Fig. 8 A, compared with the SAL group, tretinoin resulted in extensive apoptotic nuclear fragmentation of PC12 cells. Excessive ROS accumulation stimulates the activation of ASK1, which can induce apoptosis through the MAPK signaling pathway. Since MAPK signaling is a well-recognized

canonical apoptosis signaling pathway, thus we further examined the activation of MAPK family proteins, including JNK, ERK, and p38 signaling pathways. As shown in Fig. 8B-H, the results showed that OGD/R significantly increased the phosphorylation expression of JNK, ERK, and p38 ($P < 0.001$). After pre-treatment with SAL, it was found that the expression levels of p-JNK ($P < 0.05$), p-ERK ($P < 0.001$), and p-p38 ($P < 0.001$) decreased significantly, while tretinoin could reverse this situation ($P < 0.001$). In addition, tretinoin decreased the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 ($P < 0.001$). The data indicated that SAL-mediated Nrf2/Trx1 signaling suppressed oxidative stress-driven apoptosis.

Discussion

This study investigated the role of SAL in CIRI-induced oxidative stress and apoptosis *in vivo* and *in vitro*. The MCAO and OGD/R models were used to mimic the pathophysiology of ischemia/reperfusion to investigate the neuroprotective effects of SAL both *in vivo* and *in vitro*. We found that SAL and ED could equally reduce infarct size, decrease neurological severity score, improve pathological changes, and reduce LDH release, and that high-dose SAL was more effective, suggesting that SAL has neuroprotective effects on CIRI induced by MCAO and OGD/R. The present study showed that SAL treatment inhibited oxidative stress by reducing ROS production, thereby reducing apoptosis caused by CIRI, which might be mediated by the regulation of the Nrf2/Trx1 axis.

During CIRI, a great number of neuron cells is apoptotic, which mainly occurs in the penumbra around the necrotic core of stroke focus. The Bcl-2 protein family and its members play significant roles in regulating and controlling apoptosis. Also, when cell apoptosis occurs, caspase-3 will turn into its active form cleaved caspase-3 (Porter et al. 1999). This study mainly focused on Bax, Bcl-2, and cleaved caspase-3, and the results demonstrated that, under both *in vivo* and *in vitro* conditions, high-dose SAL better regulated their expression to exert anti-apoptotic effects. Oxidative stress is an essential factor and plays a vital role in CIRI. The mass-produced ROS, which cannot be scavenged, can induce neuronal apoptosis (Davies. 1999). Therefore, reducing the damage caused by oxidative stress can effectively alleviate the occurrence of CIRI. Here, biochemical parameters related to oxidative stress in the brain and PC12 cells were tested. SAL and ED equally reversed the MCAO- and OGD/R-induced decrease in the activities of SOD and CAT and the increase in the activity of MDA and the level of ROS, and the therapeutic effect of the high-dose SAL was more significant than that of ED. These results indicated that SAL

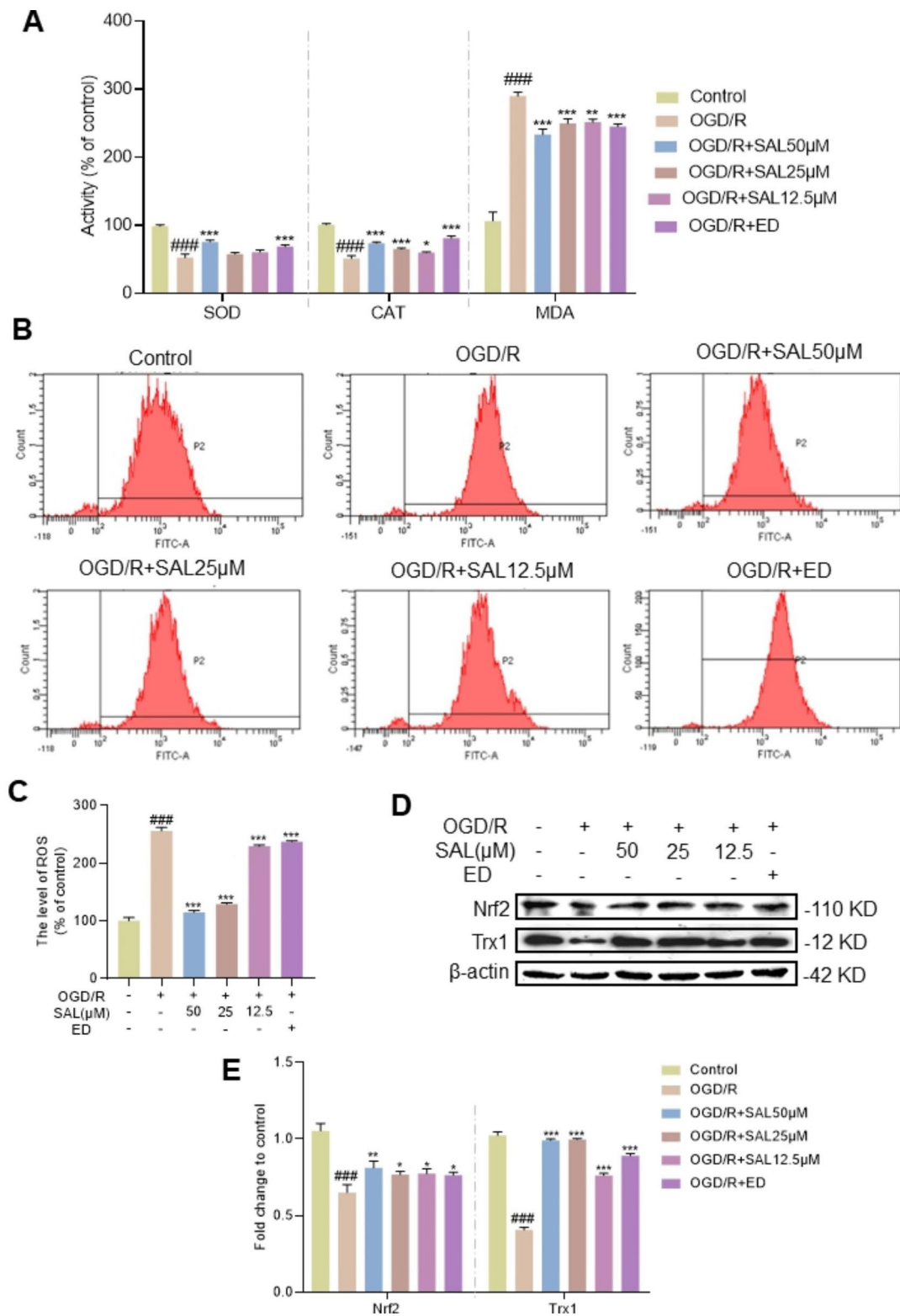


Fig. 6 Effects of SAL on oxidative stress in OGD/R treated PC12 cells. **(A)** SOD, CAT, and MDA commercial kits were used to detect the activities of SOD, CAT, and MDA in PC12 cells. **(B–C)** The intracellular ROS levels in PC12 cells were measured by flow cytometry. **(D–E)** Expression of Nrf2 and Trx1 proteins. Values are presented as mean \pm SEM. ### P <0.001 versus control group; * P <0.05, ** P <0.01 and *** P <0.001 versus OGD/R group

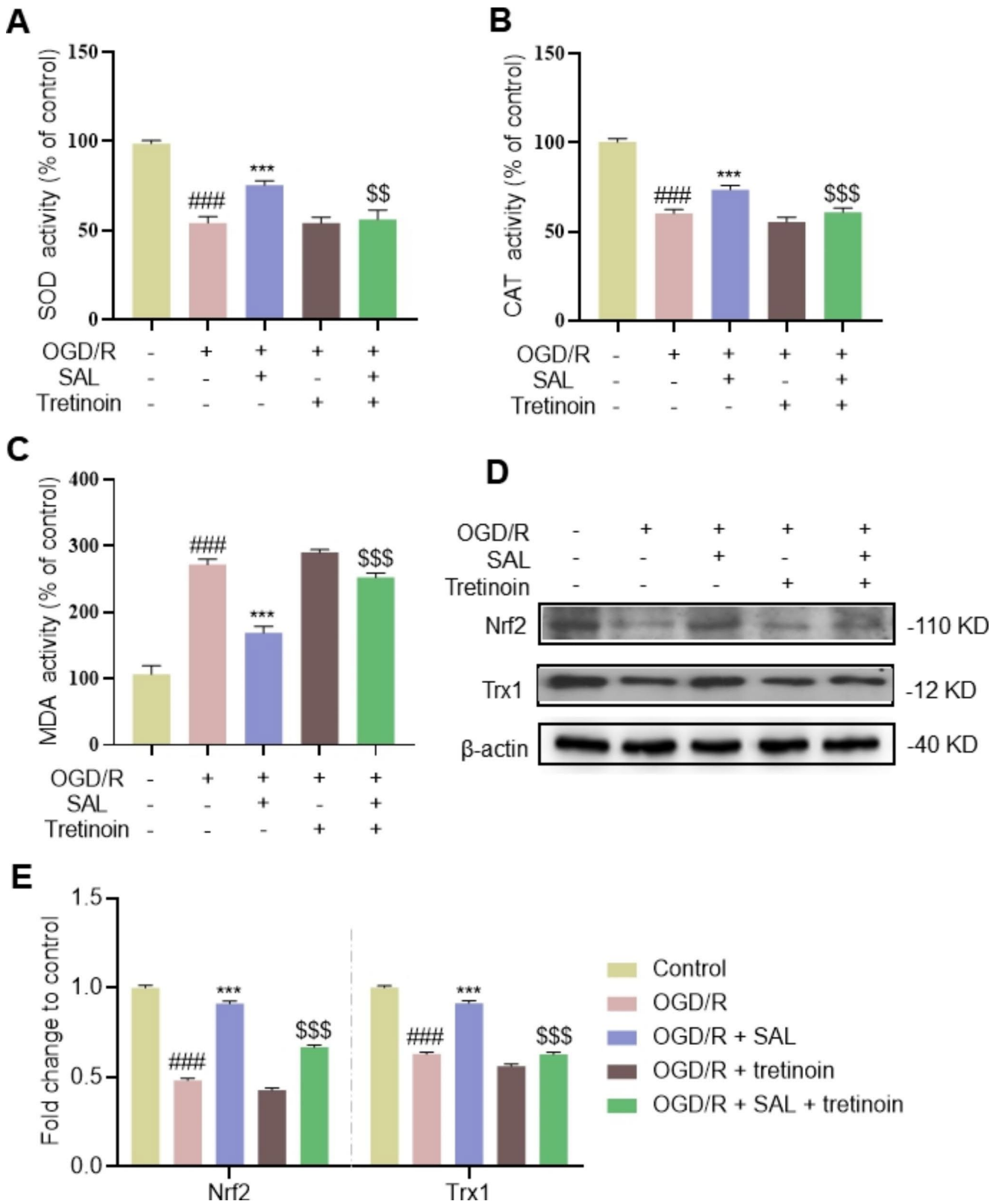


Fig. 7 Effects of SAL on oxidative stress in OGD/R treated PC12 cells by Nrf2/Trx1 pathway. (A-C) SOD, CAT, and MDA commercial kits were used to detect the activities of SOD, CAT, and MDA in PC12 cells. (D-E) Expression of Nrf2 and Trx1 proteins. Values are presented as mean ± SEM. ###*P* < 0.001 versus control group; ****P* < 0.001 versus OGD/R group; \$\$\$*P* < 0.01, \$\$\$*P* < 0.001 versus OGD/R + SAL group

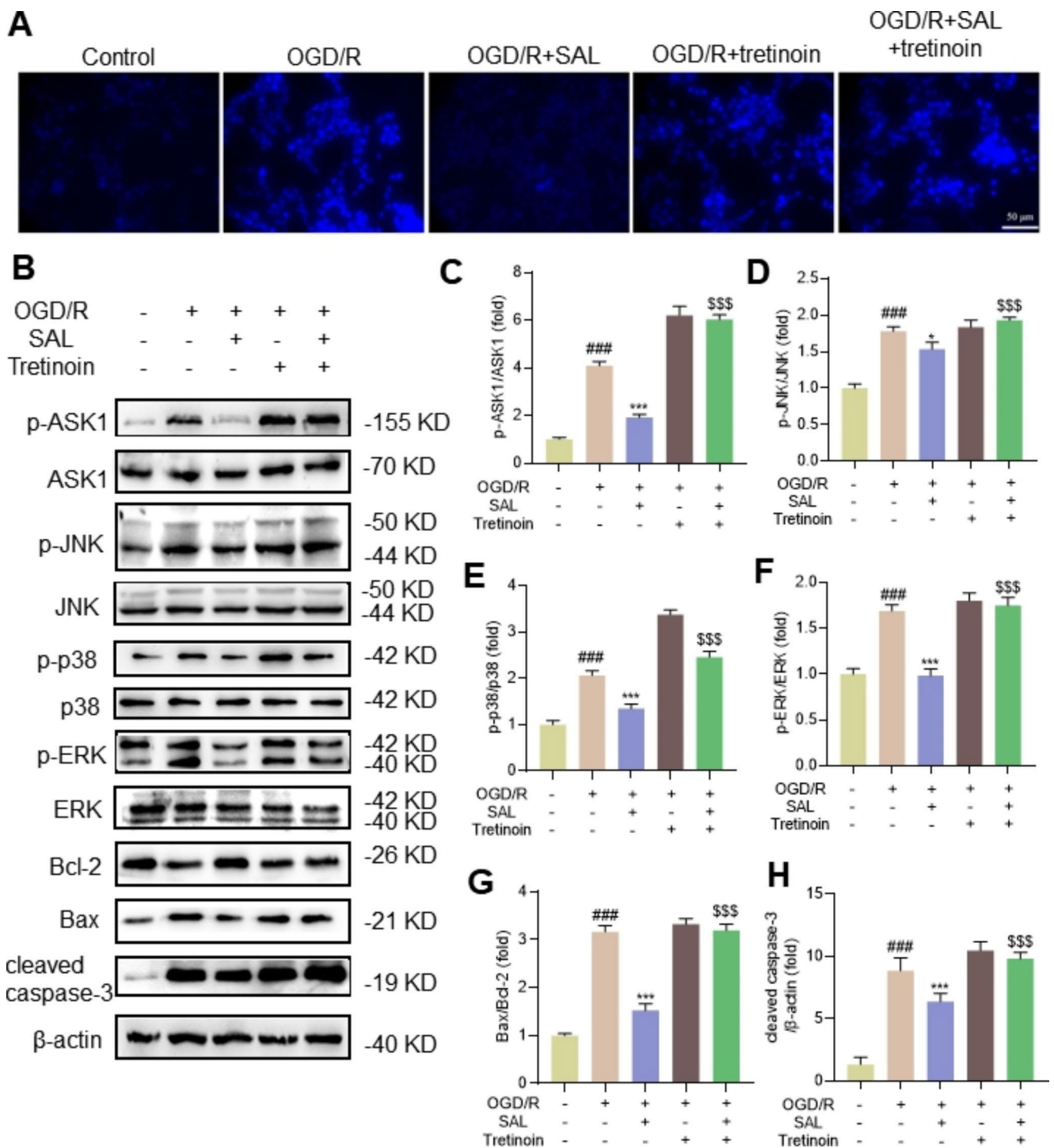


Fig. 8 Effects of SAL mediated Nrf2/Trx1 signaling on oxidative stress-driven apoptosis. **(A)** Apoptosis was detected by Hoechst 33,342 staining (200 × magnification, Scale bar = 50 μm). **(B–H)** Expression of p-ASK1, ASK1, p-JNK, JNK, p-p38, p38, p-ERK, ERK, Bcl-2, Bax, and cleaved caspase-3 proteins. Values are presented as mean ± SEM. ###*P* < 0.001 versus control group; **P* < 0.05, ****P* < 0.001 versus OGD/R group; \$\$\$*P* < 0.001 versus OGD/R + SAL group

could first enhance antioxidant capacity and then inhibit oxidative stress.

Nrf2 is a key molecule responsible for properly regulating the antioxidant system, which can induce the expression

of antioxidant enzymes and play a pivotal role in the protection of antioxidant stress (Vomund et al. 2017). Previous studies have demonstrated that SAL could improve hypoxia-induced oxidative stress through the Nrf2 signaling

pathway (Xiong et al. 2021). Trx1, a cell redox protein, is the key protein in maintaining the steady-state of cell redox level, thus protecting cells from oxidative stress (Li et al. 2021). In addition, many studies show that the expression of Trx1 can be up-regulated by enhancing the Nrf2 signal (Im et al. 2012). Trx1 protects oxidative stress-induced neuronal cell death through the ASK1/MAPK signaling pathway (Yeo et al. 2021). The results of the present study showed that SAL could regulate the expression of Nrf2 and Trx1 proteins *in vivo* and *in vitro*. To further evaluate how SAL inhibited oxidative stress and apoptosis activated by CIRI, tretinoin, an Nrf2 inhibitor (Zhang et al. 2020), was used to inhibit the expression of Nrf2 in PC12 cells to explain the potential relationship and determine whether apoptosis was directly related to the activation of the Nrf2 signaling pathway. Tretinoin could reverse the antioxidant effects of SAL, suggesting that SAL inhibited oxidative stress by the Nrf2/Trx1 pathway.

Trx1 binds directly to the N-terminal region of ASK1. Under the condition of oxidative stress, excessive ROS accumulation stimulates the activation of ASK1 and activates many downstream signaling pathways, including the MAPK pathway. MAPK family proteins include ERK, JNK, and p38 (You et al. 2019), which are involved in cell growth, proliferation and apoptosis. The MAPK signaling pathway is activated by ASK1, and it is the key signaling pathway leading to neuronal cell death (Yeo et al. 2021; Chen et al. 2021; Lin et al. 2013). In this study, the specific mechanism of SAL on oxidative stress-induced apoptosis was explored. SAL reduced the increase in ASK1/MAPK induced by OGD/R, while tretinoin could reverse this situation, indicating that SAL plays an antioxidant role by up-regulating the Nrf2/Trx1 pathway, and subsequently inhibits apoptosis by inhibiting the ASK1/MAPK signaling pathway.

Conclusion

Our results showed that SAL had a neuroprotective effect by inhibiting CIRI-induced oxidative stress and apoptosis. This study showed that SAL reduced cell apoptosis caused by CIRI by inhibiting ASK1/MAPK, which might be through inhibiting oxidative stress regulated by Nrf2/Trx1 signaling pathway. This present study expanded the current understanding of the pathophysiology of brain injury, thereby providing a new therapeutic option for CIRI.

Author contributions Fuyuan Li: Methodology, Project administration, Writing - original draft. Qianqian Mao: Data curation and investigation. Jinyu Wang: Methodology. Xiaoying Zhang: Methodology. Xinyan Lv: Methodology. Bo Wu: Project administration. Tingxu Yan: Methodology, Project administration, Writing - review & editing, Funding acquisition. Ying Jia: Methodology, Project administration, Writing - review & editing, Funding acquisition.

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Data Availability The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The study was carried out in compliance with the National Institutes of Health and Institutional Guidelines for the Humane Care of Animals and was approved by the Animal Care Committee of Shenyang Pharmaceutical University (protocol no.: SYPU-IACUC-C2019-11-2-301).

Consent for publication All authors approved the submission of the manuscript for publication.

Conflict of interest The authors declare no conflict of interest.

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