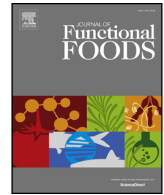
Journal of Functional Foods 71 (2020) 104019

Contents lists available at ScienceDirect

Journal of Functional Foods

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



Pinosylvin provides neuroprotection against cerebral ischemia and reperfusion injury through enhancing PINK1/Parkin mediated mitophagy and Nrf2 pathway

Hui Xu[a], Ruixia Deng[b], Edmund T.S. Li[a], Jiangang Shen[b], Mingfu Wang[a,*]

[a] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[b] School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Sassoon Road, Hong Kong, China

ARTICLE INFO

Keywords:
Cerebral ischemia
Neuroprotection
Pinosylvin
Mitophagy
Nrf2

ABSTRACT

Neuroprotection is one common strategy to reduce injuries from cerebral ischemia. This study aims to evaluate neuroprotective activity of several representative stilbenes with different substituted groups to preliminarily know about their structure/activity relationship and action mechanisms. Among the stilbenes tested, only pinosylvin showed strong neuroprotective effects, evidenced by the decreased cell death in OGD/R-damaged PC12 cells and improved brain function in MCAO/R rats after pinosylvin treatment. Pinosylvin reduced the rate of depolarized cells (low mitochondrial membrane potential) in OGD/R-damaged PC12 cells, implying its role in improving mitochondrial function. Further studies indicated that pinosylvin induced PINK1/Parkin mediated protective mitophagy and activated Nrf2 pathway, suggested by the elevated protein levels of LC3 II, Beclin1, PINK1 and Parkin, and Nrf2 translocation to nucleus. In conclusion, pinosylvin exhibited neuroprotective effects by inducing PINK1/Parkin mediated mitophagy to remove damaged mitochondria and activating Nrf2 pathway to ameliorate oxidative stress-induced mitochondrial dysfunction.

1. Introduction

Cerebral ischemia is a very common clinical condition, involved in many diseases, such as stroke and cardiac arrest (Lee et al., 2018). Cerebral ischemia leads to the deprivation of glucose and oxygen in the blocked brain tissue, and causes a series of damaging events to neurons, including energy depletion, excitotoxicity, oxidative stress, inflammation and apoptosis (Kunz, Dirnagl, & Mergenthaler, 2010). The major approach to fight against this condition is to restore the blood flow as soon as possible, via a process known as reperfusion (Rodrigo et al., 2013). However, reperfusion might further promote oxidative stress and inflammation induced damages, causing secondary injury to neurons. Therefore, drugs are needed to protect neurons from ischemia and reperfusion (I/R) damages by decreasing the deleterious events and controlling programmed cell death, a strategy called neuroprotection (Xing, Arai, Lo, & Hommel, 2012).

Natural stilbene compounds are potential neuroprotective agents. In the past, some stilbenes have been evaluated for their neuroprotective effect. Resveratrol, one of the most studied stilbenes, has been shown to possess neuroprotective effects both *in vitro* and *in vivo*. As an example, resveratrol showed protective effects after administration to some elderly rats undergoing two mild transient middle cerebral artery occlusion (MCAO) (Zenkov et al., 2016). One *in vitro* study with Oxygen and glucose deprivation/reperfusion (OGD/R) damaged PC12 cells indicated that resveratrol decreased OGD/R induced cell damage by reducing oxidative stress and mitochondrial dysfunction (Liu et al., 2016). Another popular stilbene, pterostilbene from blueberries was also reported to exhibit neuroprotective effects after 90 min MCAO and then reperfusion in mice (Zenkov et al., 2016). However, these previous studies only focused on a very limited number of stilbenes, and their structure/activity relationship and action mechanism for neuroprotection remained unclear.

Mitochondrial respiratory function is impaired during I/R, resulting in excessive reactive oxygen species (ROS) production and the release

Abbreviations: BafA1, bafilomycin A1; CCK-8, cell counting kit-8; Cyt. c, cytochrome *c*; DCFH-DA, dichloro-dihydro-fluorescein diacetate; GCLM, glutamate-cysteine ligase modifier subunit; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NQO1, quinone oxidoreductase; Nrf2, nuclear factor erythroid 2 related factor 2; OGD/R, oxygen and glucose deprivation/reperfusion; PINK1, PTEN-induced putative kinase 1; PSY, pinosylvin; ROS, reactive oxygen species; SOD, superoxide dismutase

* Corresponding author.
E-mail address: mfwang@hku.hk (M. Wang).

https://doi.org/10.1016/j.jff.2020.104019
Received 23 February 2020; Received in revised form 7 May 2020; Accepted 11 May 2020
Available online 26 May 2020
1756-4646/ © 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license
(http://creativecommons.org/licenses/BY-NC-ND/4.0/).

of mitochondrial apoptotic factors, which are critical to the programmed cell death (Niizuma, Endo, & Chan, 2009; Sims & Muyderman, 2010). Therefore, improving the mitochondrial function or decreasing its harmful effects may be important neuroprotective strategies. Mitochondrial health can be moderated by mitochondrial autophagy (mitophagy) through the timely removal of damaged mitochondria (Fang et al., 2014). Mitophagy has been reported to be activated in different I/R models, including the MCAO rat model as well as the OGD neuronal model with PINK1 (PTEN-induced putative kinase 1) and Parkin as two key mediators (Feng, Chen, & Shen, 2017). Some stilbenes are reported to regulate autophagy/mitophagy under different conditions. For instance, resveratrol was reported to activate autophagy in diabetic cardiomyopathy and mitophagy in Alzheimer's disease offering protective effects (Kou & Chen, 2017; Xu et al., 2018). Recently, resveratrol was also shown to activate AMPK mediated mitophagy in ischemic stroke models (Pineda-Ramirez et al., 2020). In addition, pinosylvin was found to induce AMPK-mediated autophagy to prevent necrosis in bovine aortic endothelial cells (Park, Pyee, & Park, 2014). Hence, the induction of mitophagy may be a potential mechanism for the neuroprotection of some stilbenes against cerebral ischemia and reperfusion injury.

In the present study, we compared the neuroprotective effects of four resveratrol analogues including pinosylvin, pterostilbene, pinostilbene and 4-methoxy-*trans*-stilbene using the oxygen and glucose depletion/reperfusion model in PC12 cells and clarified the functional mechanisms of the one with the strongest neuroprotective effect in PC12 cells and MCAO/R rats.

2. Materials and methods

2.1. Reagents and chemicals

Pinosylvin, pterostilbene, pinostilbene and 4-methoxy-*trans*-stilbene were purchased from Greet Forest Biomedical Ltd. (Hangzhou, China) and their purities were over 98% as analyzed by HPLC method. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco (Gaithersburg, MD, USA). The cell counting kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). Phosphate buffered saline (PBS), bovine serum albumin (BSA), dichloro-dihydro-fluorescein diacetate (DCFH-DA), ML385, and 2,3,4-triphenyltetrazolium chloride (TTC) were supplied by Sigma (St. Louis, MO, USA). Hoechst 33,342 was purchased from BD Biosciences (San Jose, CA, USA). Caspases3, Bax, NQO1, Beclin1, LC3A/B, PINK1, Parkin, Histone H3, COX IV, and β-actin primary antibodies were obtained from Cell signaling technology (Boston, MA, USA). Nrf2, cytochrome *c*, BCL-2, GCLM, and HO-1 primary antibodies, as well as horseradish peroxidase-conjugated anti-rabbit secondary antibody, were Abcam (Cambridge, UK) products. Horseradish peroxidase-conjugated anti-mouse secondary antibody and bafilomycin A1 (BafA1) were supplied by Santa Cruz Biotechnology (Dallas, Texas, USA).

2.2. Cell line, OGD/R model and drug treatment

PC12 cell line was purchased from American Type Culture Collection (ATCC, VA, USA). They were cultured in a high glucose DMEM medium with 10% FBS and 1% penicillin-streptomycin at 37 °C in a relatively humidified atmosphere with 5% CO_2.

OGD/R model was established according to Feng et al. (2017). Briefly, PC12 cells were washed with PBS for three times. Then, normal cultural medium (high glucose DMEM with 10% FBS) was changed to low glucose (2.5 mM) DMED and cells were immediately transferred to a hypoxia chamber (Billups-Rothenberg, Delmar, CA, USA) to maintain the oxygen concentration at 1% by perfusing with the mixed gas of 5% CO_2 and 95% N_2. After incubated at 37 °C for 10 h, cells were then cultured with the normal cultural medium at an atmosphere with 5% CO_2 and 95% O_2 for another 14 h for reoxygenation. The control group cells were always cultured with the normal cultural medium under 5% CO2 and 95% O2.

Cells were pretreated with pinosylvin, pterostilbene, pinostilbene, or 4-methoxy-*trans*-stilbene for 24 h and then subjected to OGD/R damages without adding those stilbenes. To block the autophagic flux, PC12 cells were treated with 20 nM BafA1 at the beginning of reoxygenation.

2.3. Detection of cell death

After OGD/R damages, cell viability was then measured with the cell counting kit-8 (CCK-8) method, and lactate dehydrogenase (LDH) level in the cultural supernatant was tested using a pierce LDH cytotoxicity assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Apoptosis was detected with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay kit (Beyotime Institute of Biochemistry, Shanghai, China). Briefly, PC12 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, treated with 0.3% Triton X-100 for 5 min and then stained with a mixture of terminal deoxynucleotidyl transferase and FITC-dUTP for 1 h at 37 °C. Images were captured with a Nikon 80i phase-contrast fluorescence microscope.

2.4. Detection of mitochondrial function

Mitochondrial membrane potential (MMP) was detected by a JC-1 probe kit (Beyotime Institute of Biochemistry, Shanghai, China). Briefly, PC12 cells were washed with PBS (for qualitative analysis) or harvested with trypsinization (for quantitative analysis), stained with 10 μg/mL JC-1 in the dark for 20 min at 37 °C and then washed with PBS for three times. Fluorescence was then observed with a Nikon 80i phase-contrast fluorescence microscope or analyzed by flow cytometer with the excitation/emission wave of 490/530 nm for JC-1 monomers and 525/590 nm for JC-1 aggregates.

Cytochrome *c* (cyt. c) release was detected with the Cyt. c releasing apoptosis assay kit (Abcam, Cambridge, UK) according to the manufacturers' instructions. Briefly, cells were collected, homogenized and centrifuged to get the cytosolic fraction. The centrifugal pellet was lysed with the mitochondrial extraction buffer to obtain the mitochondrial fraction. After that, cyt. c content in the cytosolic and mitochondrial fraction was analyzed by the standard Western blotting procedure with the monoclonal mouse anti-cyt. c antibody.

2.5. Intracellular oxidative stress

Intracellular redox status was measured with the DCFH-DA fluorescent probe. After reoxygenation, cells were incubated with 10 μM DCFH-DA in the dark for 30 min at 37 °C and washed three times with PBS. Mitochondrial superoxide accumulation was measured with the MitoSOX™ red indicator (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, PC12 cells were stained with 5 μM MitoSOX™ red reagent for 20 min at 37 °C in the dark and then washed with warm Hank's balanced salt solution for three times. Fluorescence was observed and imaged with a Nikon 80i phase-contrast fluorescence microscope or analyzed by flow cytometer.

2.6. Detection of superoxide dismutase (SOD) and malondialdehyde (MDA) level

PC12 cells were homogenized with the Dounce tissue grinder and then intracellular SOD and MDA level were measured with the SOD and MDA detecting kits (Beyotime Institute of Biochemistry, Shanghai, China), respectively. SOD was measured with the WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-

benzene disulfonate sodium salt) method, while MDA was detected with the thiobarbituric (TBA) method. Protein contents of different groups were also measured using the BCA (bicinchonininc acid) method to normalize the SOD and MDA levels.

2.7. Nuclear Nrf2 measurement

Cytoplasmic and nuclear fraction were extracted with the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA). Nrf2 content in the cytoplasmic and nuclear extracts was further tested by the standard Western blot procedure with rat anti-Nrf2 antibody. Histone H3 (HH3) and β-actin were used as the internal control for nuclear extract and cytoplasmic extract, respectively. Data were analyzed with the image J software.

2.8. Animal model and drug treatment

The intraluminal suture middle cerebral artery occlusion (MCAO) model is the most commonly used in ischemic stroke research. To induce cerebral ischemia, a monofilament is inserted into the internal carotid artery (CCA) and advanced to the origin of MCA to block the blood flow.

Adult male Sprague Dawley rats (250–280 g) were obtained from the Laboratory Animal Unit, The University of Hong Kong. The use of animals in this study was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong. The rats were maintained under 12–12 h light-dark conditions at a temperature of 22 ± 2 °C. Rats were anesthetized with 4% isoflurane (Abbott, USA) at the induction of anesthesia and 2% isoflurane during the whole operation process. The MCAO/R model was performed according to Longa, Weinstein, Carlson, and Cummins (1989). Briefly, the rats were placed on a heating pad to maintain their body temperature and the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed under a microscope. Nerves were carefully dissected from the arteries and the blood flow of the CCA and ICA was temporarily blocked with the clamps. The ECA was tied with suture, cut and then a monofilament coated with silicon on the tip (0.36 mm, Jialing Co. Ltd., China) was inserted from the ECA to ICA, and advanced to block the blood flow to the middle cerebral artery (MCA). After ischemia for 2 h, the monofilament was removed to achieve reperfusion. The Sham rats were operated on like the MCAO rats except that no monofilament was inserted to block the MCA. The rats were sacrificed after 24 h of reperfusion.

Rats were randomly divided into three groups (n = 8 per group): the control group, the MCAO/R group and the PSY-treated group (50 mg/kg). PSY dissolved in the mixture of ethanol (45%), PEG400 (25%), and saline (30%) was intraperitoneally injected upon reperfusion. The control- and MCAO/R-group rats were intraperitoneally injected with the mixture solution (45% ethanol, 25% PEG400 and 30% saline).

2.9. Evaluation of infarct volume and neurological deficits

The neurological deficits were scored with the modified neurological severity score (mNSS) scale at 24 h after reperfusion (Mao et al., 2013). The mNSS scale includes motor, sensory, and reflex tests ranging from 1 to 18 points. A higher score indicates more severe neurological damages.

The infarct volume was evaluated with TTC staining. Brain tissue was collected after cardiac perfusion with PBS. Brain tissue was sliced into 2 mm coronal section and stained with 2% TTC solution at 37 °C for 20 min. The stained slices were photographed and analyzed with Image J. Infarct volume was calculated as follows: infarct volume (%) = (the size of right hemisphere − red size of left hemisphere)/(right hemisphere size × 2) × 100%.

2.10. Western blotting assay

PC12 cells or brain tissues were lysed, and centrifuged; proteins were also collected. The proteins (10 μg) were then separated by SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane (Bio-rad, Hercules, CA, USA). The membrane was blocked with 5% nonfat milk or BSA and incubated with primary antibodies overnight at 4 °C. After being washed with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse (1:2000) or anti-rabbit (1:5000) secondary antibodies. Protein bands were visualised using an enhanced chemiluminescence (ECL) substrate kit (Bio-rad, Hercules, CA, USA) and quantified with the Image J software. The primary antibody used in this research were as follows. Antibodies from Abcam: Cyt. c (ab110325, 1:250), Nrf2 (ab31163, 1:1000), HO-1 (ab13243, 1:2000), BCL-2 (ab59348, 1:500) and GCLM (ab153967, 1:2000). Antibodies from Cell signaling technology: Caspases3 (9662, 1:500), Bax (2772, 1:1000), Beclin1 (3495, 1:1000), LC3A/B (12741, 1:1000), PINK1 (6946, 1:500), Parkin (2132, 1:500), NQO1 (62262, 1:2000), Histone H3 (4499, 1:2000), COX IV (4850, 1:1000), or β-actin (3700, 1:5000).

2.11. Statistical analysis

All data were presented as mean ± standard deviation (SD) of at least three independent experiments using the SPSS statistics 22 (IBM). Differences between the means of the individual groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey's test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of the stilbenes tested against OGD/R damages in PC12 cells

OGD/R model is the most common model to mimic the I/R conditions *in vitro* (Durukan & Tatlisumak, 2007). In this study, the OGD/R model was applied to research the neuroprotective effects of four stilbenes, namely, pinosylvin, pterostilbene, pinostilbene and 4-methoxy-*trans*-stilbene. The protective effects of those stilbenes were firstly measured with H_2O_2 damage (100 μM for 4 h) before they were tested in OGD/R model. It was found that the protective effects of those stilbenes against H_2O_2 damage were strongest at 10 μM (data not shown), so this concentration was used in OGD/R model. Results showed that pinosylvin and pterostilbene significantly protected PC12 cells from OGD/R damages, while pinostilbene and 4-methoxy-*trans*-stilbene could not attenuate OGD/R-induced cell death (Fig. 1B). Among these four stilbenes, the pretreatment with pinosylvin (10 μM) showed the strongest neuroprotective effect, decreasing the OGD/R induced cell death by 69.07% compared to only OGD/R treated cells (Fig. 1B). Therefore, the following functional mechanism studies only focused on pinosylvin.

3.2. Pinosylvin exhibited neuroprotection both in vitro and in vivo

Apoptosis is one of the major reasons for neuronal death after I/R injury (Broughton, Reutens, & Sobey, 2009). As shown in Fig. 2A, OGD/R treatment increased the number of TUNEL positive cells. After pinosylvin (10 μM) pretreatment, the number of TUNEL positive cells was reduced compared to the OGD/R group. To further confirm the anti-apoptotic effect of pinosylvin, the level of cleaved caspase 3 was measured, as cleaved caspase 3 was the active form of caspase 3 and regarded as an important marker of the initiation of the apoptotic signaling pathway. Data indicated that protein level of cleaved caspase 3 was significantly increased by OGD/R treatment compared to the control group, which was then decreased after pinosylvin (10 μM) pretreatment by 45.46% (Fig. 2B). The Bcl-2 family of proteins, consisting of proteins with pro-apoptotic effect (Bax) and anti-apoptotic

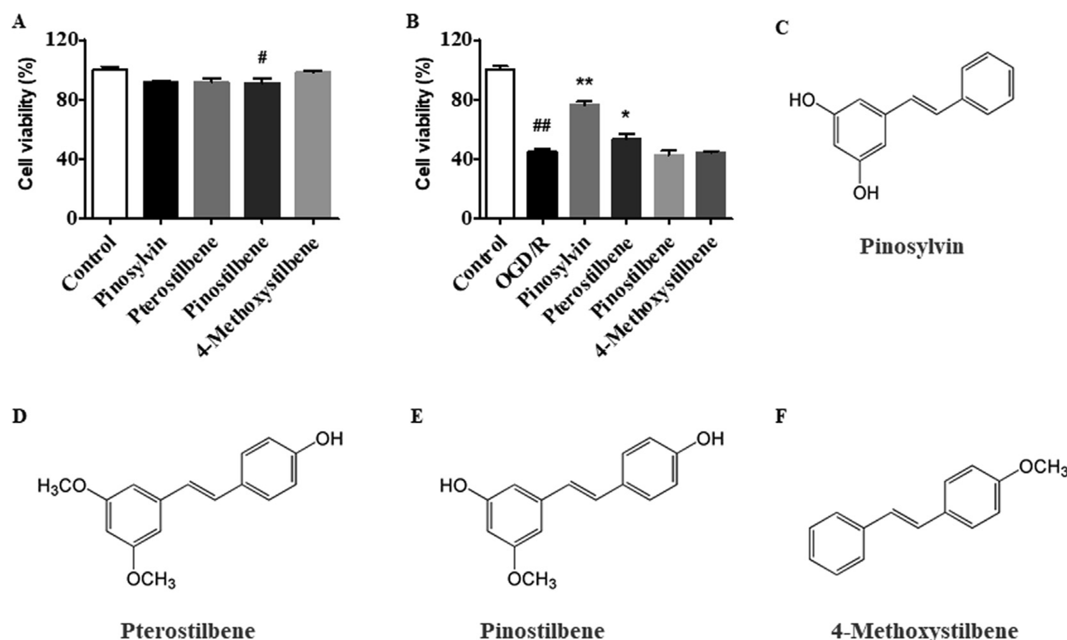


Fig. 1. Effects of the stilbenes tested against OGD/R damages in PC12 cells. (A) Cytotoxicity of the stilbenes tested (10 μM) was measured with CCK-8 method. (B) Neuroprotective effects of the stilbenes tested. PC12 cells were treated with 10 μM of the stilbenes tested for 24 h and then subjected to OGD/R damages. Cell viability was then measured with CCK-8 method. (C–F) Chemical structures of the stilbenes tested. All results were expressed as means ± SD of at least three independent experiments. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *versus* control group; $^{*}P < 0.05$; $^{**}P < 0.01$ *versus* OGD/R group.

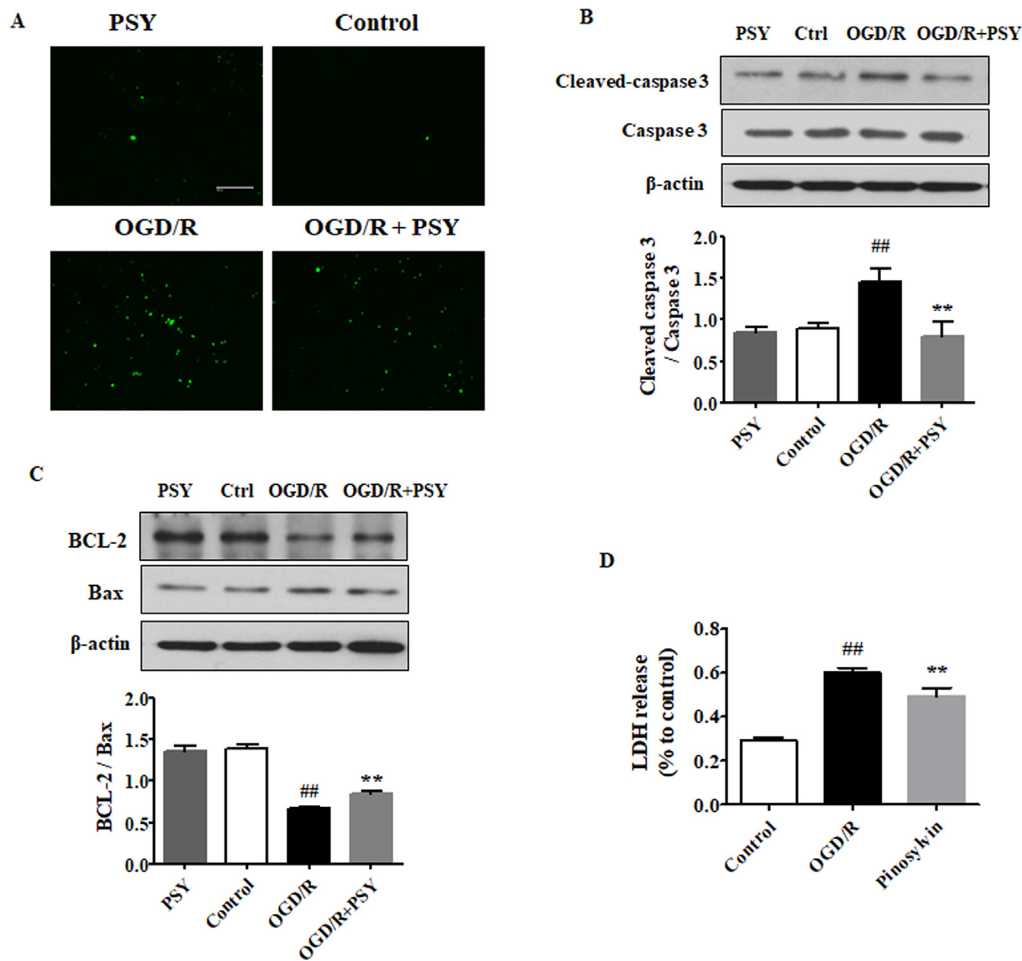


Fig. 2. Pinosylvin decreased OGD/R-induced cell death. PC12 cells were treated with pinosylvin for 24 h and then subjected to OGD/R damages. (A) TUNEL assay was performed, and cells were imaged under a Nikon 80i phase-contrast fluorescence microscope. Scale bar: 100 μm. (B) LDH level in the supernatant was measured with a LDH kit. (C–D) Proteins were collected and protein levels of caspase 3, cleaved caspase 3, BCL-2 and Bax were analyzed with western blotting. β-actin was used as the internal control. The bands were semi-quantitatively analyzed using Image J software. All results were expressed as means ± SD of at least three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* OGD/R group. PSY: Pinosylvin.

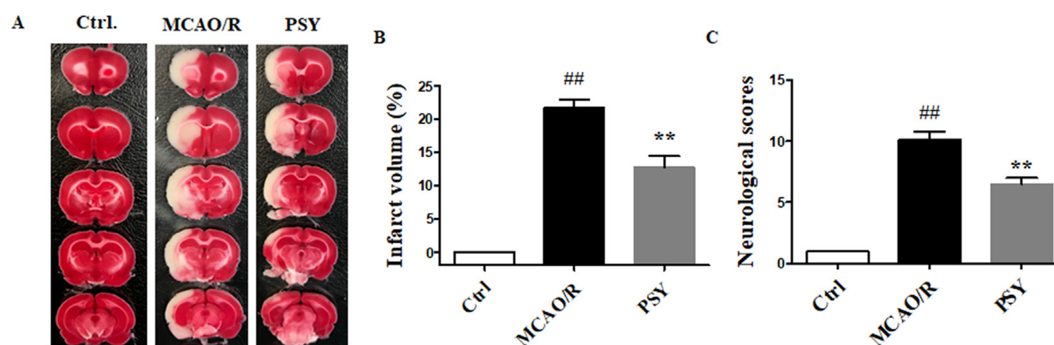


Fig. 3. Pinosylvin showed neuroprotection in MCAO/R rats. Rats (n = 8) were intraperitoneally injected with 50 mg/kg pinosylvin at the onset of reperfusion after 2 h of cerebral ischemia. (A–B) Brain tissues were collected after 24 h of reperfusion and infract volume was measured with TTC staining and analyzed with Image J software. (C) Neurological deficits were scored with the mNSS scale after 24 h of reperfusion. All results were expressed as means ± SD of at least eight independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* OGD/R group. PSY: Pinosylvin.

effect (BCL-2), are important regulators of mitochondria-mediated apoptosis (Siddiqui, Ahad, & Ahsan, 2015). Pinosylvin was observed to increase the protein expression ratio of BCL-2 and Bax that was decreased by OGD/R damages (Fig. 2C). Necrosis is another form of cell death induced by I/R injury. To detect the necrosis of PC12 cells, LDH levels in the cultural supernatant were measured. Pinosylvin was found to reduce the LDH level when compared to OGD/R damaged cells, proving that pinosylvin decreased OGD/R induced necrotic cell death (Fig. 2D). The neuroprotective effect of pinosylvin was then further verified in the MCAO/R rats. Pinosylvin was found to significantly reduce the cerebral infarct volume (Fig. 3A, B) and improve the neurological deficit (Fig. 3C) of MCAO/R rats at a dosage of 50 mg/kg. This dosage was selected according to Wei *et al.* who reported that 50 mg/kg of resveratrol showed significant neuroprotective activity in MCAO/R rats when administrated immediately after reperfusion (Wei et al., 2015). Those data indicated that pinosylvin reduced OGD/R induced apoptotic and necrotic cell death in PC12 cells, and cerebral damages of MCAO/R rats, proving its neuroprotective activity both *in vitro* and *in vivo*.

3.3. Pinosylvin improved OGD/R induced mitochondrial dysfunction

Mitochondrial function was evaluated by mitochondrial membrane potential (MMP) and measured with a JC-1 fluorescence probe in PC 12 cells. JC-1 could enter the mitochondrial matrix forming aggregates to emit bright red fluorescence at normal membrane potential, while exist as monomers outside mitochondria with a fluorescence of green at low potential (Li et al., 2011). Fig. 4A showed that OGD/R damaged cells exhibited strong green fluorescence and a weak red signal. After pinosylvin (10 μM) pretreatment, mitochondrial function was partially recovered, as evidenced by the stronger red fluorescence and the weaker green signal than the OGD/R group cells. Quantitative analysis was also performed with flow cytometry. It was found that OGD/R significantly decreased the percentage of cells that emitted lower red signal compared to the OGD/R group (Fig. 4B, C). These data indicated the role of pinosylvin in ameliorating OGD/R-induced mitochondrial dysfunction.

The release of cyt. c is one of the major events happening in the damaged mitochondria, so it can serve as an indicator for mitochondria function (Kluck, Bossy-Wetzel, Green, & Newmeyer, 1997). Fig. 4D revealed that after treated with pinosylvin (10 μM), cells showed lower cyt. c level in cytosolic fraction and higher cyt. c level in mitochondrial fraction than OGD/R damaged cells, suggesting that pinosylvin decreased the cyt. c release from mitochondria. The released cyt. c is also the key regulator to initiate the mitochondria-mediated apoptosis (Kluck et al., 1997). It was speculated from these data that pinosylvin could improve the mitochondria function, and thus decrease the cyt. c release from damaged mitochondria, and further inhibiting the OGD/R-induced apoptosis.

3.4. Pinosylvin decreased OGD/R induced oxidative stress

In addition to cyt. c release, the other major event that happened in the damaged mitochondria is the dramatically elevated level of superoxide after reperfusion (Allen & Bayraktutan, 2009). As shown in Fig. 5, both mitochondrial superoxide and intracellular ROS were elevated in OGD/R treated cells, as evidenced by the stronger MitoSOX™ red and DCFH-DA fluorescent intensity. Pinosylvin (10 μM) treatment was found to decrease the OGD/R-induced ROS production, as both the MitoSOX™ red and the DCFH-DA fluorescent intensity were significantly lowered in pinosylvin-treated cells.

MDA is a by-product of membrane lipid peroxidation and thus could be used as a marker to reflect oxidative damage (Mateos, Lecumberri, Ramos, Goya, & Bravo, 2005). Fig. 6A indicated that the MDA content in OGD/R treated cells was much higher than that in control cells (increased by 64.17%), while pinosylvin (10 μM) lowered the cellular MDA content by 23.79% compared with OGD/R group. The activity or expression of the key antioxidant enzymes was then measured in OGD/R damaged PC12 cells. Pinosylvin (10 μM) could significantly enhance the activity of SOD and up-regulate the expression of GCLM (Glutamate cysteine ligase regulatory subunit), NQO1 (NADPH quinone dehydrogenase 1), and HO-1 (Heme oxygenase 1) compared to OGD/R damaged cells (Fig. 6B-C). It can be concluded from these data that pinosylvin might reduce the ROS production by improving mitochondrial function and activating the intracellular antioxidant enzymes to reduce the OGD/R-induced oxidative stress.

3.5. Pinosylvin induced PINK1/Parkin-mediated mitophagy

Mitophagy plays a central role in removing damaged mitochondria after OGD/R damage. Zhang *et al.* reported that inhibition of mitophagy by 3-methyladenine (3MA), an autophagosome formation inhibitor in reperfusion phase enhanced the cerebral I/R injuries both *in vitro* and *in vivo* (Zhang et al., 2008). Another study showed that I/R induced injury would worsen after administration of a mitophagy inhibitor, mdivi-1, in reperfusion phase (Rami, 2009). Beclin 1 and LC3 II are two important markers for autophagosome formation (Wang et al., 2012). It was found that OGD/R enhanced the protein levels of both Beclin 1 and LC3 II in PC 12 cells, which were further increased by pinosylvin (10 μM) in our study (Fig. 7A). LC3 II was also up-regulated by pinosylvin in the MCAO/R rats (Fig. 7B), indicating the effect of pinosylvin to activate autophagy. PINK1 and Parkin are two regulators that mediated the selective removal of damaged mitochondria by mitophagy. Once stimulated, PINK1 is stabilized on the mitochondrial membrane, recruiting Parkin to the mitochondria to initiate mitophagy. It is demonstrated that Parkin is translocated to mitochondria during reperfusion (Yuan, Zhang, Zheng, & Chen, 2015). Our results showed that pinosylvin elevated the mitochondrial protein level of PINK1 and

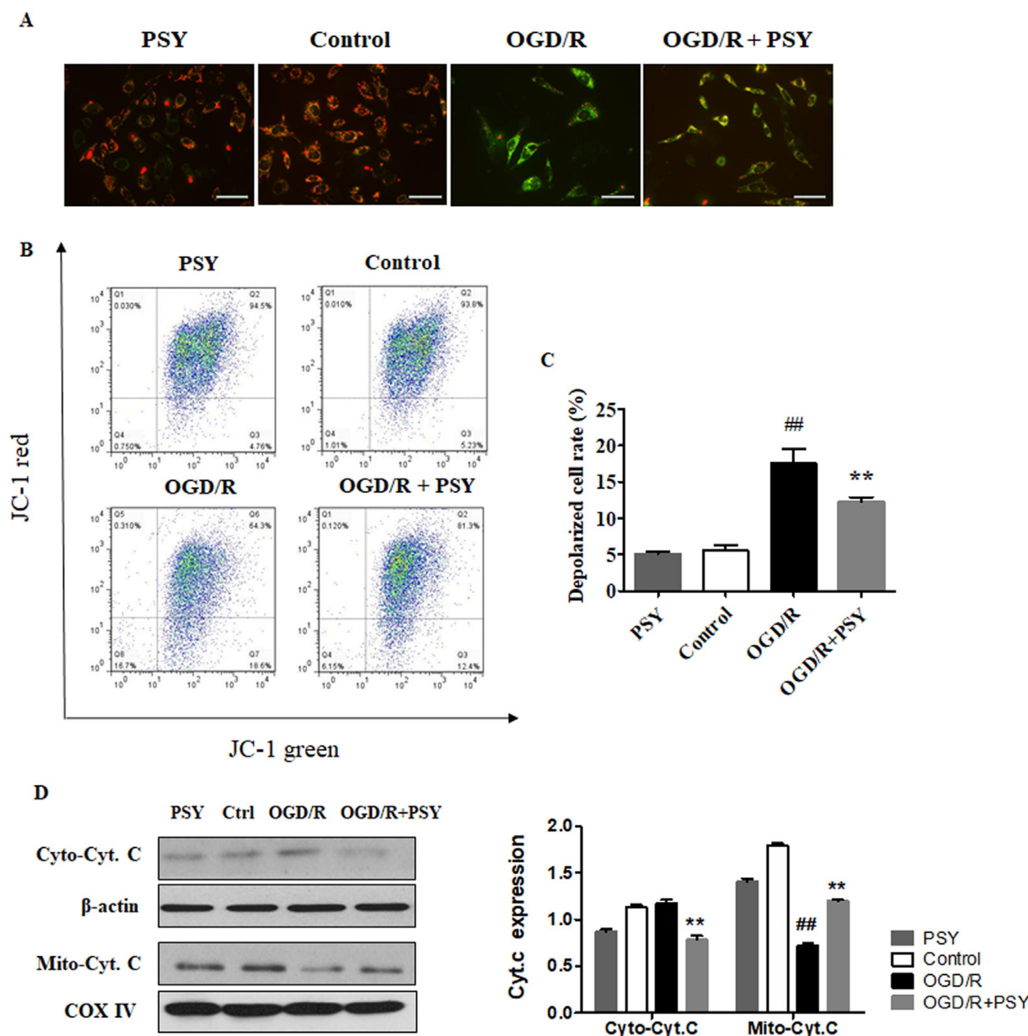


Fig. 4. Pinosylvin improved OGD/R induced mitochondrial dysfunction. PC12 cells were treated with pinosylvin for 24 h, and then subjected to OGD/R damages. (A-C) JC-1 staining was performed. Pictures of JC-1 stained cells imaged under a Nikon 80i phase-contrast fluorescence microscope (A); Red fluorescence represents cells with normal MMP; Green fluorescence represents cells with normal MMP. Scale bar: 25 μm. Quantitative analysis of JC-1 stained cells with flow cytometry (B, C). (D) Mitochondrial and cytosolic fraction were separated and then protein level of cyt. c was measured by western blotting. β-actin was used as the internal control for cytosolic fraction and COX IV for mitochondrial fraction. All results were expressed as means ± SD of at least three independent experiments. ##P < 0.01 *versus* control group; **P < 0.01 *versus* OGD/R group. PSY: Pinosylvin.

Parkin after OGD/R injuries, suggesting that pinosylvin enhanced PINK1/Parkin-mediated mitophagy (Fig. 7C). This inference was further verified in the animal system, as the expression of PINK1 and Parkin was also enhanced by pinosylvin in MCAO/R rats (Fig. 7D).

To investigate the role of mitophagy in pinosylvin-mediated neuroprotection, BafA1 was used to block the autophagic flux in PC 12 cells. BafA1 is a lysosomal protease inhibitor, which blocks the autolysosome formation, leading to the accumulation of autophagosome in the cytoplasm (Zhao et al., 2016). It is observed that endogenous LC3-II accumulation is augmented after cells were treated with BafA1 at the beginning of reperfusion. Cells exposed to pinosylvin and BafA1 together showed more LC3-II accumulation than BafA1 treated cells (Fig. 7E), suggesting that pinosylvin could promote autophagosome formation. Moreover, BafA1 promoted damage of OGD/R, and weakened the neuroprotective effect of pinosylvin (Fig. 7F), implying that mitophagy might contribute to pinosylvin's neuroprotective effect. These data provided evidence that the neuroprotective effect of pinosylvin against I/R damage might be exhibited *via* enhancing the induction of PINK1/Parkin-mediated mitophagy to remove damaged mitochondria.

3.6. Pinosylvin activated Nrf2 pathway in PC12 cells

Nrf2 (Nuclear factor erythroid 2 related factor 2) is an important transcriptional factor to regulate the gene expression of diverse cytoprotective proteins, such as antioxidant enzymes, including GCLM, NQO1, and HO-1. Recent studies show that it also plays a prominent role in the structural and functional integrity of mitochondria (Dinkova-Kostova & Abramov, 2015). Nrf2 is kept in the cytoplasm at normal condition, and then translocated into the nucleus, binding to the ARE promoter regions and activating its target genes once stimulated (Liu, Locascio, & Dore, 2019). Data showed that the content of Nrf2 in the nucleus was significantly elevated by pinosylvin (Fig. 8), indicating that pinosylvin promoted the activation of the Nrf2 pathway. Nrf2 was reported to affect the mitochondrial function though multiple ways, including regulating the cellular redox homeostasis, affecting mitochondrial respiration and MMP, enhancing mitochondrial metabolism, and enhancing mitochondrial biogenesis and integrity (Dinkova-Kostova & Abramov, 2015). Therefore, apart from mitophagy, pinosylvin might also improve the mitochondrial function by activating the Nrf2 pathway.

To further explore the role of Nrf2 pathway in pinosylvin-mediated neuroprotection, PC12 cells were treated with the Nrf2 inhibitor ML385. ML385 specifically interacts with Nrf2 protein, blocking its transcriptional activity and expression (Singh et al., 2016). It was found that ML385 significantly lowered the Nrf2 expression at the concentration of 20 μM (Fig. 8B), and the neuroprotective ability of pinosylvin was decreased after ML385 treatment (Fig. 8C). It can be concluded from the data that the Nrf2 pathway participated in the pinosylvin-mediated neuroprotection against OGD/R damages.

4. Discussion

Pinosylvin (3,5-dihydroxy-*trans*-stilbene), a natural stilbene, is

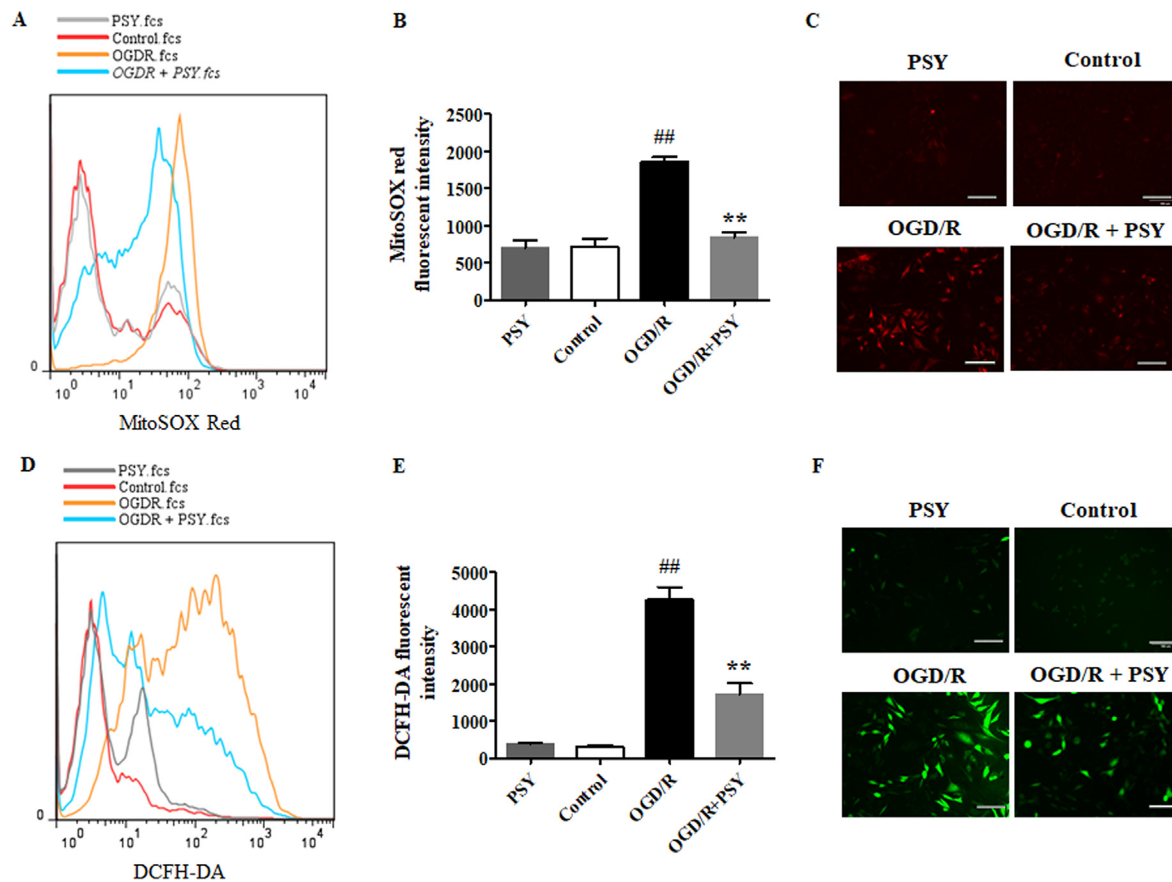


Fig. 5. Pinosylvin reduced OGD/R induced ROS production. PC12 cells were treated with pinosylvin for 24 h and then subjected to OGD/R damages. (A-C) Mitochondria superoxide was detected by MitoSOX™ red indicator with flow cytometry or a Nikon 80i phase-contrast fluorescence microscope. (D-F) Intracellular oxidative stress was detected with DCFH-DA by flow cytometry or a Nikon 80i phase-contrast fluorescence microscope. Scale bar: 100 µm. $^{##}P < 0.01$ *versus* control group; $^{**}P < 0.01$ *versus* OGD/R group. PSY: Pinosylvin.

found in the heartwood and leaves of pine. It possesses a similar structure as resveratrol, only lacking a hydroxyl group located at position C-4′. Although the two have different number of hydroxyl groups, pinosylvin and resveratrol were reported to possess equal intracellular antioxidative, as pinosylvin was more lipophilic and exhibited higher bioavailability (Perecko, Jancinova, Drábiková, Nosal', & Harmatha, 2008). Previously, pinosylvin was reported for its antioxidant and antifungal activities. More recently, some studies demonstrated its bioactivities in regulating endothelial cells adhesion and cancers (Lee et al., 2005; Park et al., 2004). In the current study, we compared the neuroprotective effects of four stilbenes against OGD/R damage. Results indicated that pinosylvin showed the strongest neuroprotective

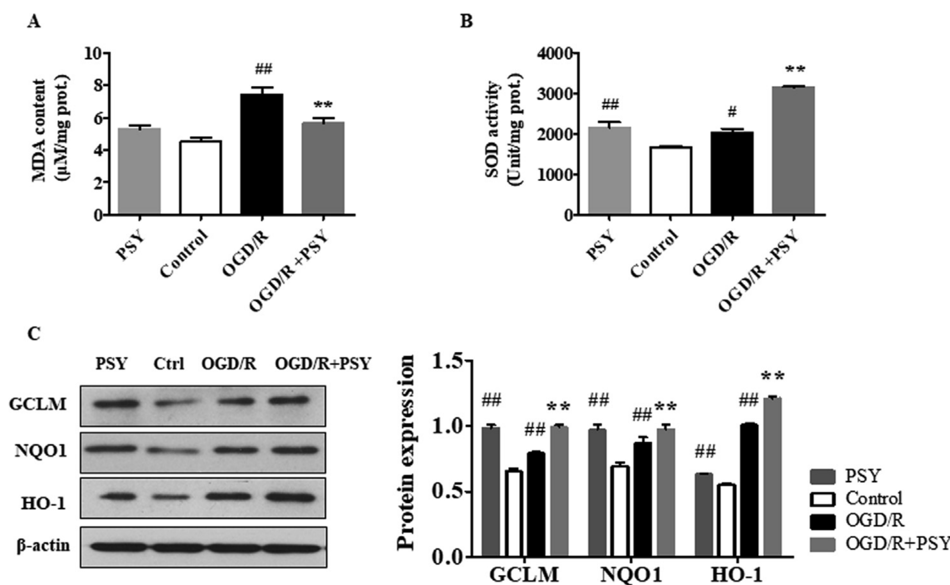


Fig. 6. Pinosylvin activated the anti-oxidant enzymes. (A-B) PC12 cells were treated with pinosylvin for 24 h and then subjected to OGD/R damages. Intracellular MDA and SOD content was measured by the corresponding commercial kit and normalized by protein content in each group. (C) Proteins of PC12 cells were collected and protein levels of the antioxidant enzymes GCLM, NQO1 and HO-1 were detected with western blotting. β-actin was used as an internal control. $^{##}P < 0.01$ *versus* control group; $^{**}P < 0.01$ *versus* OGD/R group. PSY: Pinosylvin.

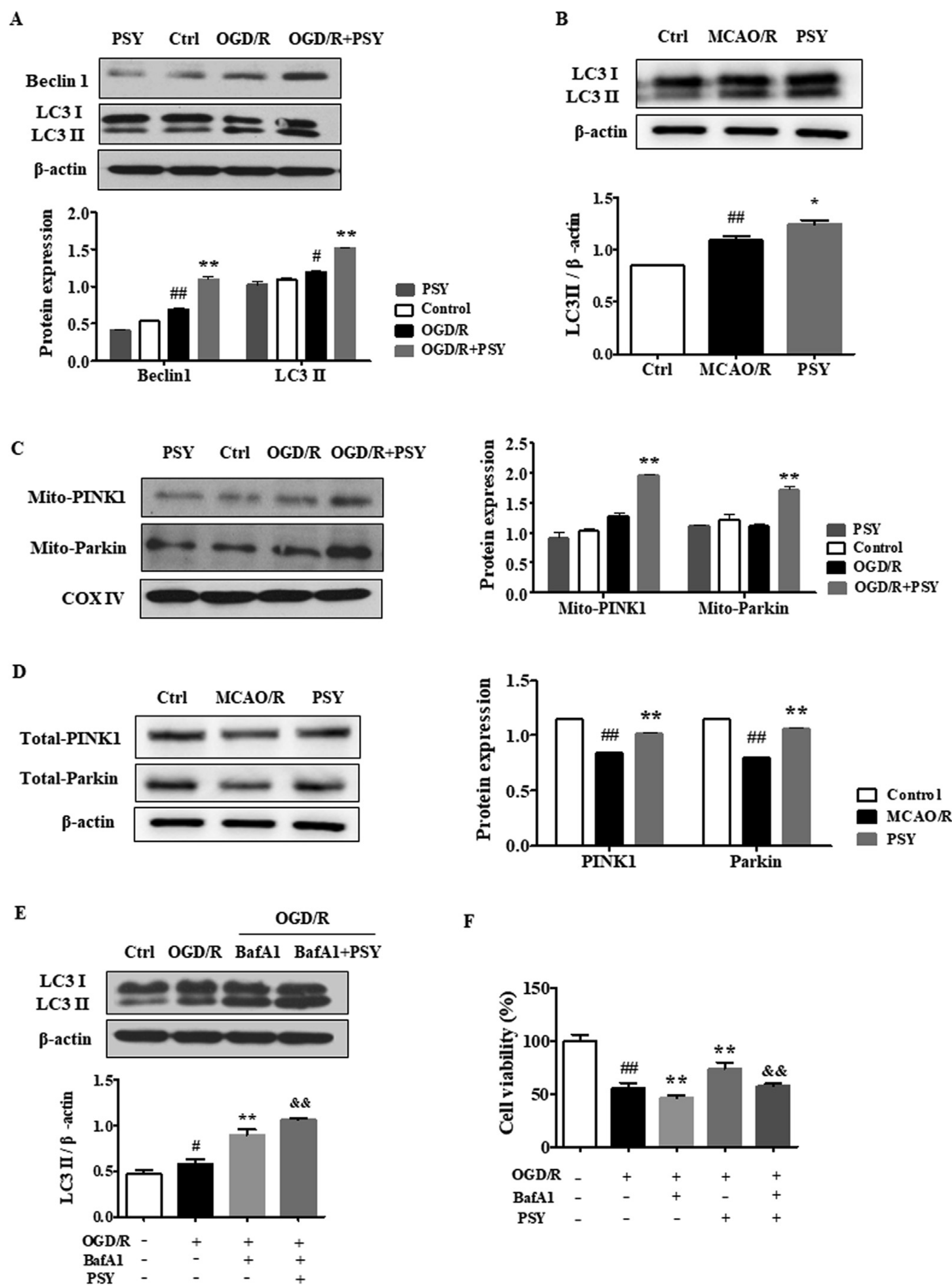


Fig. 7. Pinosylvin induced PINK1/Parkin-mediated mitophagy. (A) PC12 cells were treated with pinosylvin for 24 h and then subjected to OGD/R damages. Proteins were collected and protein levels of Beclin1 and LC3 I/II were detected with western blotting. (B, E) Proteins of rats were collected and protein levels of LC3 I/II, PINK1 and Parkin were detected with western blotting. β-actin was used as an internal control. (D) Cytosolic and mitochondrial fractions of PC12 cells were separated with a commercial kit and protein levels of the mitochondrial PINK1 and Parkin were then measured by western blotting. COX IV was used as an internal control. PC12 cells were treated with 20 nM bafilomycin A1 (BafA1) at the beginning of reperfusion. (E) Proteins were collected and protein levels of LC3 I/II were detected with western blotting. β-actin was used as an internal control. All the western blotting bands were semi-quantitatively analyzed using Image J software. (F) Cell viability was measured with CCK-8 method. All results were expressed as means ± SD of at least three independent experiments. All the western blotting bands were semi-quantitatively analyzed using Image J software. All results were expressed as means ± SD of at least three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* control group; $^{**}P < 0.01$, $^{*}P < 0.05$ *versus* OGD/R or MCAO/R group; $^{\&\&}P < 0.01$ *versus* OGD/R + PSY group. PSY: Pinosylvin.

effect, attenuating OGD/R induced cell death, MCAO/R induced cerebral infarction, and neurological scores. When compared to some previous studies, the neuroprotective effect of pinosylvin was much stronger than that of resveratrol, a well-studied neuroprotective agent. Liu *et al.* and Agrawal *et al.* found that the pretreatment of PC12 cells with 10 μM resveratrol increased the cell viability by 37.6% after 6 h OGD and 24 h reoxygenation (Liu et al., 2016), which was much lower than that of 10 μM pinosylvin (63.8%). It was thus suggested that pinosylvin might be an efficient neuroprotective agent against cerebral I/R damages, so its functional mechanisms were further investigated.

I/R damages could lead to brain cells death through necrosis or apoptosis. Necrosis predominantly occurs in the acute and permanent ischemia, while the death of neurons in the ischemic penumbra is preferentially induced by apoptotic-like mechanisms as cells in this region underwent milder injury and preserved energy (Lo, Dalkara, & Moskowitz, 2003). In the present study, pinosylvin could reduce OGD/R induced cell damage by inhibition of both necrosis and apoptosis, evidenced by the reduced LDH level, decreased TUNEL positive cells, and down-regulated cleaved caspase 3 levels in PC 12 cells with pinosylvin treatment. Releasing cyt. c from the damaged mitochondria is a central event in regulating apoptosis induced by ischemia and reperfusion damages. After released, cyt. c leads to the cleavage of downstream caspase 3 and activated the apoptotic pathway (Chan, 2005). Our data showed that pinosylvin significantly decreased the cyt. c release from mitochondria. The release of cyt. c depends on the integrity of the outer mitochondrial membrane. Bcl-2 family of proteins, consisting of proteins with pro-apoptotic effect (Bax, Bak, Bad, Bim and Bid) and anti-apoptotic effect (BCL-2, BCL-XL, BCL-w), are a group of

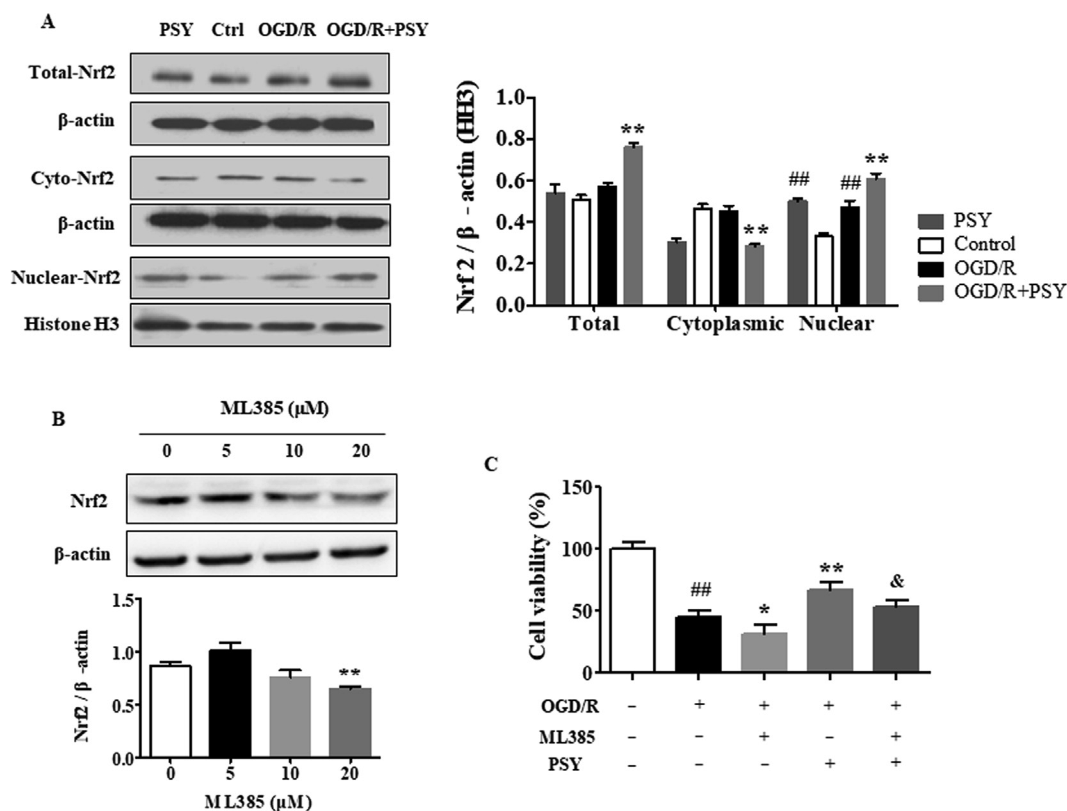


Fig. 8. Pinosylvin activated Nrf2 pathway. (A) Cytosolic and nuclear fractions of PC12 cells were separated with a commercial kit and protein levels of the total Nrf2, cyto-Nrf2 (cytoplasmic Nrf2) and nuclear Nrf2 were measured by western blotting. β-actin was used as an internal control for total and cyto Nrf2 and Histone H3 (HH3) for nuclear Nrf2. (B-C) PC12 cells were treated with the Nrf2 inhibitor ML385 and then the Nrf2 expression and cell viability were measured. All the western blotting bands were semi-quantitatively analyzed using Image J software. All results were expressed as means ± SD of at least three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* control group; $^{**}P < 0.01$ *versus* OGD/R group; $^{\&}P < 0.05$ *versus* OGD/R + PSY group. PSY: Pinosylvin.

important regulators of mitochondrial permeability (Bagci, Vodovotz, Billiar, Ermentrout, & Bahar, 2006). Bax and/or Bak promotes the pore formation in the mitochondrial outer membrane via oligomerization, while BCL-2 and Bcl-XL could combine with Bax, thus inhibiting the pore formation (Bernardi & Di Lisa, 2015). Pinosylvin was found to enhance BCL-2 expression and decrease Bax expression, indicating that the neuroprotective effect of pinosylvin might act through reducing the mitochondrial damages induced by OGD/R.

As discussed above, mitochondria play an important role in the development of I/R damages. Several studies have demonstrated the altered mitochondrial respiratory function in both global and focal cerebral ischemia (Moro, Almeida, Bolaños, & Lizasoain, 2005). It was found that ADP-stimulated or uncoupled respiration was reduced by 15–40% in the penumbra region, while 45–60% in the core tissue after 2 h of MCA occlusion in rats. Mitochondrial respiratory function was almost completely recovered in the first hour of reperfusion, but then declined rapidly (Chan, 2004). It was noteworthy that the delayed alteration in mitochondrial respiratory function after reperfusion was found to occur earlier than the development of irreversible cell dysfunction, which implied its contribution to the neuron death (Chan, 2004). A study taking electron micrographs of the brain tissue that experienced 3 h of focal ischemia and then 2 h reperfusion observed obvious mitochondrial structural abnormalities in neurons (Moro et al., 2005). Mitochondrial membrane potential ($\Delta\psi_m$) is a commonly used biomarker for mitochondrial function. Mitochondria isolated from the ischemic and reperfusion damaged brain tissue showed a lower $\Delta\psi_m$ when incubated at either basal or ADP-stimulated condition (Chan, 2005). In agreement with the previous studies, our study showed that OGD/R damages markedly reduced the $\Delta\psi_m$ of PC12 cells, as proved by the decreased red fluorescence signal and elevated green fluorescence of JC-1 probe, while pinosylvin significantly ameliorated the OGD/R-induced MMP decrease, suggesting its role in improving the mitochondrial function. These findings indicated the role of mitochondria in pinosylvin mediated neuroprotective effect.

As mitochondrial dysfunction results in excessive ROS generation and cell apoptosis leading to neuron death, the quality control of mitochondria is essential for maintaining cellular homeostasis during I/R conditions (Wei, Liu, & Chen, 2015). Mitophagy, the main way for cells to eliminate the damaged mitochondria, has been reported to be activated in different I/R models including MCAO/R rat model and OGD/R neuronal model (Zhang et al., 2013; Zuo et al., 2014). In addition, PINK1/Parkin pathway induced mitophagy has been showed to be involved in ischemic stroke (Jin & Youle, 2012). As an example, a study reported that the increase of reperfusion time after 1 h MCAO reduced the protein level of Parkin, and meanwhile increased the cell injury, which might attribute to impaired mitophagy (Cui et al., 2012). Our present results suggested that in PC12 cells, pinosylvin could enhance the protein level of LC3 II and Beclin1, implying its role in activating autophagy (LC3 II was also up-regulated by pinosylvin in the MCAO/R rats). PINK1/Parkin mediated mitophagy was also induced by pinosylvin as mitochondrial PINK1 and Parkin protein levels were further increased. Moreover, an autophagy inhibitor, BafA1 exacerbated the OGD/R induced cell death and weakened the neuroprotective effect of pinosylvin. These data implied that pinosylvin could induce cell mitophagy to remove the damaged mitochondria to eliminate their harmful effects.

Mitochondria are the major site where superoxide is produced under normal and pathological conditions. During ischemia, the elevated Ca^{2+} accumulation in the mitochondria activates many Ca^{2+}-dependent phosphatase, leading to the dephosphorylated OxPhos

complexes (Mattson & Kroemer, 2003). It is indicated that the phosphorylation status of the OxPhos complexes was important to regulate balanced cell respiration, while dephosphorylation of them would hyperactivate the ETC (Mattson & Kroemer, 2003). After reperfusion, the substrate for OxPhos is restored and the hyperactivated ETC would result in an increased $\Delta\psi_m$, which dramatically enhances ROS generation (Sanderson, Reynolds, Kumar, Przyklenk, & Hüttemann, 2013). The free radical produced then further damages the ETC, causing the vicious cycle in mitochondrial function (Sims & Muyderman, 2010). Our results showed that pinosylvin could reduce the superoxide accumulation in the mitochondria and MDA content as well as enhanced the suppressed SOD activity, implying its antioxidative activity. The Nrf2 pathway is regarded as the primary host defense, especially under oxidative stress conditions, and has been associated with the cytoprotective functions in a series of human diseases including ischemic stroke (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Under basal conditions, Nrf2 is rapidly degraded by the ubiquitin ligase systems, such as the Kelch-like ECH-associated protein 1 (Keap1)-Cullin 3/ Rbx1 system. When exposed to oxidative or electrophilic stress, Keap1 is conformationally changed, resulting in the accumulation of Nrf2 in cytosol (Zhu, Zhang, Liu, Shi, & Gu, 2016). The accumulated Nrf2 then translocates to the nucleus and binds to the antioxidant response elements (ARE), activating hundreds of target genes, including GCLC, GCLM, NQO1 and HO-1 (Suzuki & Yamamoto, 2015). It was found that levels of NADPH and GSH in Nrf2-knockout mice or cells were lower than their wide-type counterparts, while the level of ROS was higher, proving the important role of Nrf2 in fighting against oxidative stress (Loboda et al., 2016). According to our data, pinosylvin enhanced the nuclear translocation of Nrf2 and the expression of its target genes including HO-1, NQO1, and GCLM, implying that pinosylvin could activate the Nrf2 pathway to reduce the intracellular oxidative stress and further ameliorate mitochondrial dysfunction.

5. Conclusion

In conclusion, our findings indicated that cerebral I/R injuries led to mitochondrial dysfunction, which then promoted the ROS generation and apoptosis. Pinosylvin showed significant neuroprotective effect. The effect might be exhibited through inducing PINK1/Parkin mediated mitophagy to remove the I/R damaged mitochondria, as well as activating the Nrf2 pathway to ameliorate oxidative stress-induced mitochondrial dysfunction.

Ethics statements

Animal experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. The use of animals in this study was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (CULATR 4765-18).

CRediT authorstship contribution statement

Hui Xu: Conceptualization, Data curation, Formal analysis, Writing-original draft. **Ruixia Deng:** Conceptualization, Writing-review & editing. **Edmund T.S. Li:** Supervision. **Jiangang Shen:** Supervision. **Mingfu Wang:** Conceptualization, Writing-review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was partially supported by HKU Seed Fund for Translational and Applied Research (201811160003).

References

Allen, C. L., & Bayraktutan, U. (2009). Oxidative stress and its role in the pathogenesis of ischaemic stroke. *International Journal of Stroke, 4*(6), 461–470.
Bagci, E., Vodovotz, Y., Billiar, T., Ermentrout, G., & Bahar, I. (2006). Bistability in apoptosis: Roles of bax, bcl-2, and mitochondrial permeability transition pores. *Biophysical Journal, 90*(5), 1546–1559.
Bernardi, P., & Di Lisa, F. (2015). The mitochondrial permeability transition pore: Molecular nature and role as a target in cardioprotection. *Journal of Molecular Cellular Cardiology, 78*, 100–106.
Broughton, B. R., Reutens, D. C., & Sobey, C. G. (2009). Apoptotic mechanisms after cerebral ischemia. *Stroke, 40*(5), e331–e339.
Chan, P. H. (2004). Mitochondria and neuronal death/survival signaling pathways in cerebral ischemia. *Neurochemical Research, 29*(11), 1943–1949.
Chan, P. H. (2005). Mitochondrial dysfunction and oxidative stress as determinants of cell death/survival in stroke. *Annals of the New York Academy of Sciences, 1042*(1), 203–209.
Cui, D., Wang, L., Qi, A., Zhou, Q., Zhang, X., & Jiang, W. (2012). Propofol prevents autophagic cell death following oxygen and glucose deprivation in PC12 cells and cerebral ischemia-reperfusion injury in rats. *PloS One, 7*(4).
Dinkova-Kostova, A. T., & Abramov, A. Y. (2015). The emerging role of Nrf2 in mitochondrial function. *Free Radical Biology Medicine, 88*, 179–188.
Durukan, A., & Tatlisumak, T. (2007). Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacology Biochemistry Behavior, 87*(1), 179–197.
Fang, E. F., Scheibye-Knudsen, M., Brace, L. E., Kassahun, H., SenGupta, T., Nilsen, H., & Bohr, V. A. (2014). Defective mitophagy in XPA via PARP-1 hyperactivation and NAD +/SIRT1 reduction. *Cell, 157*(4), 882–896.
Feng, J., Chen, X., & Shen, J. (2017). Reactive nitrogen species as therapeutic targets for autophagy: Implication for ischemic stroke. *Expert Opinion on Therapeutic Targets, 21*(3), 305–317.
Jin, S. M., & Youle, R. J. (2012). PINK1-and Parkin-mediated mitophagy at a glance. *Journal of Cell Science, 125*(4), 795–799.
Kluck, R. M., Bossy-Wetzel, E., Green, D. R., & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science, 275*(5303), 1132–1136.
Kou, X., & Chen, N. (2017). Resveratrol as a natural autophagy regulator for prevention and treatment of Alzheimer's disease. *Nutrients, 9*(9), 927.
Kunz, A., Dirnagl, U., & Mergenthaler, P. (2010). Acute pathophysiological processes after ischaemic and traumatic brain injury. *Best Practice & Research: Clinical Anaesthesiology, 24*(4), 495–509.
Lee, R. H., Lee, M. H., Wu, C. Y., e Silva, A. C., Possoit, H. E., Hsieh, T. H., & Lin, H. W. (2018). Cerebral ischemia and neuroregeneration. *Journal of Neural Regeneration Research, 13*(3), 373.
Lee, S., Lee, H., Min, H., Park, E., Lee, K., Ahn, Y., & Pyee, J. (2005). Antibacterial and antifungal activity of pinosylvin, a constituent of pine. *Fitoterapia, 76*(2), 258–260.
Li, B., Yuan, Y., Hu, J., Zhao, Q., Zhang, D., & Chen, N. (2011). Protective effect of Bu-7, a flavonoid extracted from Clausena lansium, against rotenone injury in PC12 cells. *Acta Pharmacologica Sinica, 32*(11), 1321–1326.
Liu, L., Locascio, L. M., & Dore, S. (2019). Critical role of Nrf2 in experimental ischemic stroke. *Frontiers in Pharmacology, 10*, 153.
Liu, X., Zhu, X., Chen, M., Ge, Q., Shen, Y., & Pan, S. (2016). Resveratrol protects PC12 cells against OGD/R-induced apoptosis via the mitochondrial-mediated signaling pathway. *Acta Biochimica et Biophysica Sinica, 48*(4), 342–353.
Lo, E. H., Dalkara, T., & Moskowitz, M. A. (2003). Mechanisms, challenges and opportunities in stroke. *Nature Reviews Neuroscience, 4*(5), 399–414.
Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., & Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: An evolutionarily conserved mechanism. *Cellular Molecular Life Sciences, 73*(17), 3221–3247.
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.
Mao, L., Jia, J., Zhou, X., Xiao, Y., Wang, Y., Mao, X., & Cheng, J. (2013). Delayed administration of a PTEN inhibitor BPV improves functional recovery after experimental stroke. *Neuroscience, 231*, 272–281.
Mateos, R., Lecumberri, E., Ramos, S., Goya, L., & Bravo, L. (2005). Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress: Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. *Journal of Chromatography B, 827*(1), 76–82.
Mattson, M. P., & Kroemer, G. (2003). Mitochondria in cell death: Novel targets for neuroprotection and cardioprotection. *Trends in Molecular Medicine, 9*(5), 196–205.
Moro, M. A., Almeida, A., Bolaños, J. P., & Lizasoain, I. (2005). Mitochondrial respiratory chain and free radical generation in stroke. *Free Radical Biology Medicine, 39*(10), 1291–1304.
Niizuma, K., Endo, H., & Chan, P. H. (2009). Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *Journal of Neurochemistry, 109*, 133–138.
Park, E. J., Min, H. Y., Ahn, Y. H., Bae, C. M., Pyee, J. H., & Lee, S. K. (2004). Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E2 production in

lipopolysaccharide-induced mouse macrophage cells. *Bioorganic Medicinal Chemistry Letters, 14*(23), 5895–5898.

Park, J., Pyee, J., & Park, H. (2014). Pinosylvin at a high concentration induces AMPK-mediated autophagy for preventing necrosis in bovine aortic endothelial cells. *Canadian Journal of Physiology Pharmacology, 92*(12), 993–999.

Perecko, T., Jancinova, V., Drábiková, K., Nosal', R., & Harmatha, J. (2008). Structure-efficiency relationship in derivatives of stilbene. Comparison of resveratrol, pinosylvin and pterostilbene. *Neuroendocrinology Letters, 29*(5), 802.

Pineda-Ramirez, N., Alquisiras-Burgos, I., Ortiz-Plata, A., Ruiz-Tachiquin, M. E., Espinoza-Rojo, M., & Aguilera, P. (2020). Resveratrol activates neuronal autophagy through AMPK in the ischemic brain. *Molecular Neurobiology, 57*(2), 1055–1069.

Rami, A. (2009). Autophagy in neurodegeneration: Firefighter and/or incendiarist? *Neuropathology Applied Neurobiology, 35*(5), 449–461.

Rodrigo, R., Fernández-Gajardo, R., Gutiérrez, R., Manuel Matamala, J., Carrasco, R., Miranda-Merchak, A., & Feuerhake, W. (2013). Oxidative stress and pathophysiology of ischemic stroke: Novel therapeutic opportunities. *CNS Neurological Disorders-Drug Targets, 12*(5), 698–714.

Sanderson, T. H., Reynolds, C. A., Kumar, R., Przyklenk, K., & Hüttemann, M. (2013). Molecular mechanisms of ischemia–reperfusion injury in brain: Pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Molecular Neurobiology, 47*(1), 9–23.

Siddiqui, W. A., Ahad, A., & Ahsan, H. (2015). The mystery of BCL2 family: Bcl-2 proteins and apoptosis: An update. *Archives of Toxicology, 89*(3), 289–317.

Sims, N. R., & Muyderman, H. (2010). Mitochondria, oxidative metabolism and cell death in stroke. *Biochimica et Biophysica Acta -Molecular Basis of Disease, 1802*(1), 80–91.

Singh, A., Venkannagari, S., Oh, K. H., Zhang, Y.-Q., Rohde, J. M., Liu, L., & Gajghate, S. (2016). Small molecule inhibitor of NRF2 selectively intervenes therapeutic resistance in KEAP1-deficient NSCLC tumors. *ACS Chemical Biology, 11*(11), 3214–3225.

Suzuki, T., & Yamamoto, M. (2015). Molecular basis of the Keap1–Nrf2 system. *Free Radical Biology Medicine, 88*, 93–100.

Wang, P., Guan, Y., Du, H., Zhai, Q., Su, D., & Miao, C. (2012). Induction of autophagy contributes to the neuroprotection of nicotinamide phosphoribosyltransferase in cerebral ischemia. *Autophagy, 8*(1), 77–87.

Wei, H., Liu, L., & Chen, Q. (2015). Selective removal of mitochondria via mitophagy: Distinct pathways for different mitochondrial stresses. *Biochimica et Biophysica Acta -Molecular Cell Research, 1853*(10), 2784–2790.

Wei, H., Wang, S., Zhen, L., Yang, Q., Wu, Z., Lei, X., & Xue, R. (2015). Resveratrol attenuates the blood-brain barrier dysfunction by regulation of the MMP-9/TIMP-1 balance after cerebral ischemia reperfusion in rats. *Journal of Molecular Neuroscience, 55*(4), 872–879.

Xing, C., Arai, K., Lo, E. H., & Hommel, M. (2012). Pathophysiologic cascades in ischemic stroke. *International Journal of Stroke, 7*(5), 378–385.

Xu, K., Liu, X., Ke, Z., Yao, Q., Guo, S., & Liu, C. (2018). Resveratrol modulates apoptosis and autophagy induced by high glucose and palmitate in cardiac cells. *Cellular Physiology Biochemistry, 46*(5), 2031–2040.

Yuan, Y., Zhang, X., Zheng, Y., & Chen, Z. (2015). Regulation of mitophagy in ischemic brain injury. *Neuroscience Bulletin, 31*(4), 395–406.

Zenkov, N., Chechushkov, A., Kozhin, P., Kandalintseva, N., Martinovich, G., & Menshchikova, E. (2016). Plant phenols and autophagy. *Biochemistry, 81*(4), 297–314.

Zhang, H., Bosch-Marce, M., Shimoda, L. A., Tan, Y. S., Baek, J. H., Wesley, J. B., & Semenza, G. L. (2008). Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *Journal of Biological Chemistry, 283*(16), 10892–10903.

Zhang, X., Yan, H., Yuan, Y., Gao, J., Shen, Z., Cheng, Y., & Hu, W. (2013). Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy, 9*(9), 1321–1333.

Zhao, Y., Fan, D., Ru, B., Cheng, K., Hu, S., Zhang, J., & Wang, M. (2016). 6-C-(E-phenylethenyl) naringenin induces cell growth inhibition and cytoprotective autophagy in colon cancer cells. *European Journal of Cancer, 68*, 38–50.

Zhu, Y., Zhang, Y., Liu, W., Shi, A., & Gu, N. (2016). Salidroside suppresses HUVECs cell injury induced by oxidative stress through activating the Nrf2 signaling pathway. *Molecules, 21*(8), 1033.

Zuo, W., Zhang, S., Xia, C., Guo, X., He, W., & Chen, N. (2014). Mitochondria autophagy is induced after hypoxic/ischemic stress in a Drp1 dependent manner: The role of inhibition of Drp1 in ischemic brain damage. *Neuropharmacology, 86*, 103–115.