

## PHYLLODULCIN PROTECTS PC12 CELLS AGAINST THE INJURY INDUCED BY OXYGEN AND GLUCOSE DEPRIVATION-RESTORATION

CHANG-LONG HU<sup>1</sup>, LU GE<sup>2</sup>, YONG TANG<sup>1</sup>, JIE LI<sup>1</sup>, CHUN-HUI WU<sup>1</sup>,  
JIANG-HONG HU<sup>2</sup>, JIN-TAO YUAN<sup>1</sup> and YONG-ZHONG FAN<sup>1\*</sup>

<sup>1</sup>Department of Neurosurgery, <sup>2</sup>Department of Gastroenterology, The People's Hospital of Danyang,  
Danyang, Jiangsu 212300, China

**Abstract:** Phyllo dulcin is a natural coumarin derivative found in *Hydrangea macrophylla*. To discover effective therapeutics for cerebral ischemia-reperfusion injury, we investigated the protective effects of phyllo dulcin on PC12 cells injury induced by oxygenation and glucose deprivation/restoration (OGD/R). As a result, phyllo dulcin can improve the cell viability and attenuate the extracellular lactate dehydrogenase release. Meanwhile, phyllo dulcin can ameliorate the dysfunction of mitochondria by reducing reactive oxygen species production, decreasing the intracellular calcium level and increasing the mitochondrial membrane potential. ROS-associated OGD/R can induce cell apoptosis through the mitochondrial pathway. Phyllo dulcin can inhibit apoptosis of PC12 cells through down-regulation of Caspase-3 and Bax as well as up-regulation of Bcl-2. These results indicate phyllo dulcin can protect PC12 cells against the damage induced by OGD/R. This investigation gives promising evidences for the therapy of cerebral ischemia-reperfusion injury.

**Keywords:** phyllo dulcin, PC12 cells, oxygen and glucose deprivation/restoration, apoptosis, oxidative stress

Cerebral ischemia-reperfusion injury is a leading cause of long term disability and death all over the world (1). The embolism of blood flow in the brain will lead to the deprivation of oxygen and glucose as well as the following brain tissue injury, then the restoration of oxygen and glucose will exacerbate the neuronal injury (2). Pathogenesis of cerebral ischemia-reperfusion includes oxidative stress, apoptosis and so on (3, 4). Oxidative stress plays an important role in the process of cerebral ischemia-reperfusion (5). Cerebral ischemia-reperfusion can directly affect the function of mitochondria and break the metabolic homeostasis to induce oxidative stress for brain is susceptible to ischemic hypoxia (6). Reactive oxidative species (ROS) are produced in mitochondria and increased when the dysfunction of mitochondria and oxidative stress appear. The dysfunction of mitochondria will not only result in the overproduction of ROS but also an overload of intracellular calcium followed by neuronal apoptosis through the mitochondrial pathway (7-9). Meanwhile, LDH will be released into cells culture medium from cytoplasm when the cells die and the next cytoplasm membranes break (10). At present, few therapeutic approaches are available to cerebral

ischemia-reperfusion injury for the complicated mechanisms. In the discovery of efficient therapeutics for cerebral ischemia-reperfusion injury, bioactive phytochemicals showed promising evidences such as aloin (10), acetylpuerarin (11), genistein (12) and so on.

Phyllo dulcin (Fig. 1) is a natural coumarin derivative found in *Hydrangea macrophylla* as a natural sweetener (13). Previous pharmacological investigations have revealed phyllo dulcin showed potential for regulating obesity (14), sensitizing cancer chemotherapy (15), relieving diabetes (16), blocking degranulation of mast cells (17), inhibiting phosphodiesterase (18) and suppressing fungus (19).

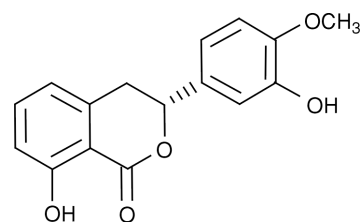


Figure 1. Chemical structure of phyllo dulcin.

\* Corresponding author: e-mail: fyzdy123@126.com

In our program to search bioactive phytochemicals for the treatment of cerebral ischemia-reperfusion injury, we have evaluated the neuroprotective effects of phyllodulcin on PC12 cells induced by oxygen and glucose deprivation-restoration (OGD/R). Herein we report these effects and related mechanisms.

## MATERIALS AND METHODS

### Chemicals and reagents

Phyllodulcin was purchased from Yuanye Biotechnology Ltd. (Shanghai, China). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Gibco Co. (Grand Island, NY, USA). ROS, lactate dehydrogenase (LDH) and BCA assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rhodamine-123, Fluo-3 AM and Caspase-3 assay kit as well as cleaved Caspase-3, Bcl-2, Bax and  $\beta$ -actin antibodies were purchased from Beyotime Biotechnology Institute (Nantong, China).

### Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) were supplied by Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM with 10% fetal bovine serum, 1% penicillin and streptomycin under a humid 5% CO<sub>2</sub> and 95% air condition at 37°C. To mimic the cerebral ischemia-reperfusion model *in vitro*, OGD/R in PC12 cells was carried out as previously described (20). In brief, the culture medium was replaced with glucose-free DMEM after washed. Then the cells were incubated in glucose-free DMEM at a 95% N<sub>2</sub> and 5% CO<sub>2</sub> atmosphere with or without certain concentrations of phyllodulcin (0.1, 1 and 10  $\mu$ M) for 4 h. And then the cells were cultured with DMEM in the absence or presence of phyllodulcin under a normal condition (5% CO<sub>2</sub> and 95% air) for 24 h. The cells as control group were incubated in DMEM at normal atmosphere.

### MTT assay

To evaluate the effect of phyllodulcin on PC12 cell viability, the MTT assay was employed. PC12 cells were added into 96-well microplates and adjusted to  $1 \times 10^5$  per well. After treated as above, MTT was added into each well to be 0.5 mg/mL and

the incubation was maintained for 4 h at 37°C. Then the medium was removed and DMSO was used to dissolve the formazan. After 10 min, the absorbance was measured at 570 nm on a microplate reader. Cell viability was expressed as a relative percentage of optical density (OD) values compared with the control group.

### LDH release assay

The leakage of LDH in the extracellular matrix indicates the cells' injury (10). To determine the activity of LDH in PC12 cells culture medium, the LDH assay kit was employed. After treatment and incubation, 20  $\mu$ L supernatant in PC12 cells culture was sucked each time and treated according to the manufacturer's instructions. Then the absorbance was recorded on a microplate reader at 450 nm. The activity of LDH can be calculated from the absorbance and expressed as U/L.

### Measurement of ROS

The production of ROS was determined by the fluorescence method according to the manufacturer's instructions. After treatment, the medium was removed and the cells were washed with PBS. Then the 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) in DMEM was added and incubation was implemented at 37°C for 30 min. The cells were washed with PBS again and the fluorescence intensity was recorded on a fluorescence microplate reader at 485 nm (excitation wavelength) and 520 nm (emission wavelength).

### Measurement of intracellular calcium

The level of intracellular calcium was determined by Fluo-3 AM molecular fluorescence probe according to the manufacturer's instruction. Briefly, pre-treated PC12 cells were incubated with Fluo-3 AM at 37°C for 1 h in dark, and then were washed with PBS for three times to remove the extracellular dye. The fluorescence was excited at 488 nm and emission wavelength was set at 525 nm on the fluorescence microplate reader.

### Determination of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was also measured through a fluorescence probe, rhodamine-123. Rhodamine-123 can accumulate in mitochondria of normal cells, but it will be released into the cytosol and emit fluorescence while the MMP of apoptotic cells reduces (21). Following the treatment, 2 mM rhodamine-123 was added into PC12 cells and incubated for 15 min. Then the fluo-

rescence intensity was read at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. MMP was expressed as the percentage of fluorescence intensity compared to the control group.

**Western blot analysis**

The PC12 cells were treated as the description in “cell culture and treatment” section and then subjected to Western blot analysis for the expression of

Caspase-3, Bcl-2, and Bax. In short, the cells were lysed with lysis buffer including 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. Then the lysed cells were centrifuged at 12 000 × g and 4°C for 15 min, and the supernatant was collected as the total protein for the analysis of cleaved Caspase-3, Bcl-2, and Bax. After quantified by a BCA assay kit, the samples were separated on 15%

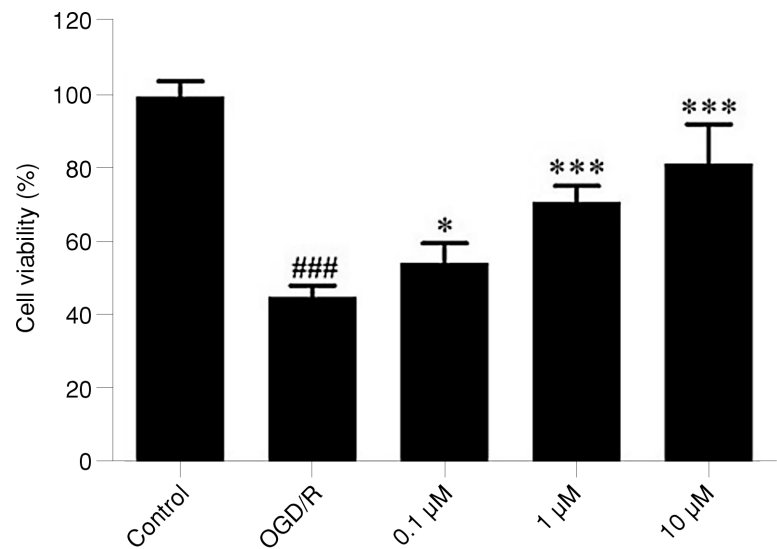


Figure 2. Effect of phyllodulcin on the PC12 cell viability. PC12 cells were treated by OGD with or without phyllodulcin (0.1, 1 and 10 μM) for 4 h, and then exposed to R for 24 h in the presence or absence of phyllodulcin. The cell viability was performed by MTT assay. n = 6, ###p < 0.001 vs control group, \*p < 0.05, \*\*\*p < 0.001 vs OGD/R group.

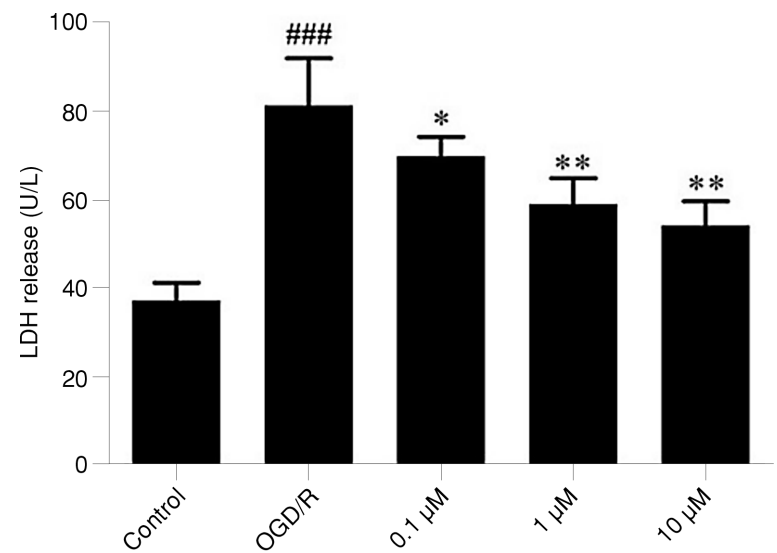


Figure 3. Effect of phyllodulcin on extracellular LDH. PC12 cells were treated by OGD with or without phyllodulcin (0.1, 1 and 10 μM) for 4 h, and then exposed to R for 24 h in the presence or absence of phyllodulcin. The activity of LDH in the supernatant of PC12 cells culture was determined by the LDH assay kit. n = 6, ###p < 0.001 vs control group, \*p < 0.05, \*\*p < 0.01 vs OGD/R group.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocked with 5% defatted milk, the membranes were incubated overnight with primary antibody of cleaved Caspase-3 (1 : 1000), Bcl-2 (1 : 1000), Bax (1 : 1000) and  $\beta$ -actin (1 : 1000) at 4°C. The membranes were tested with respective secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h and detected by enzyme-link chemiluminescence substrate.  $\beta$ -actin was used as internal control. Densitometric analysis was imple-

mented by Image J software to obtain the relative intensities of bands.

Caspase-3 activity

The activity of Caspase-3 was quantified by a colorimetric assay kit according to the manufacturer's instruction. The PC12 cells were pretreated as above and washed with PBS. Then they were lysed and centrifuged at  $16\,000 \times g$  and 4°C for 10 min. The supernatant was incubated with substrate (Ac-DEVD-pNA) at 37°C for 2 h. The OD values were measured on a microplate reader at 405 nm.

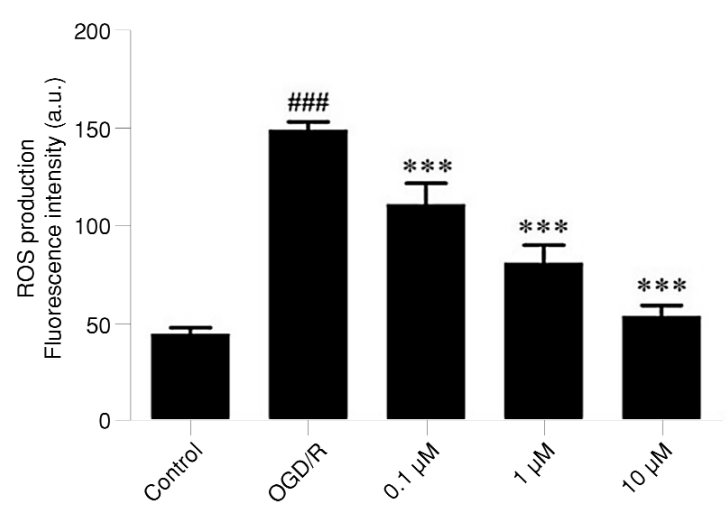


Figure 4. Effect of phyllodulcin on ROS production. PC12 cells were treated by OGD with or without phyllodulcin (0.1, 1 and 10  $\mu$ M) for 4 h, and then exposed to R for 24 h in the presence or absence of phyllodulcin. The ROS production was presented as fluorescence intensity of hydrolyzed DCF. n = 6, ###p < 0.001 vs control group, \*\*\*p < 0.01 vs OGD/R group.

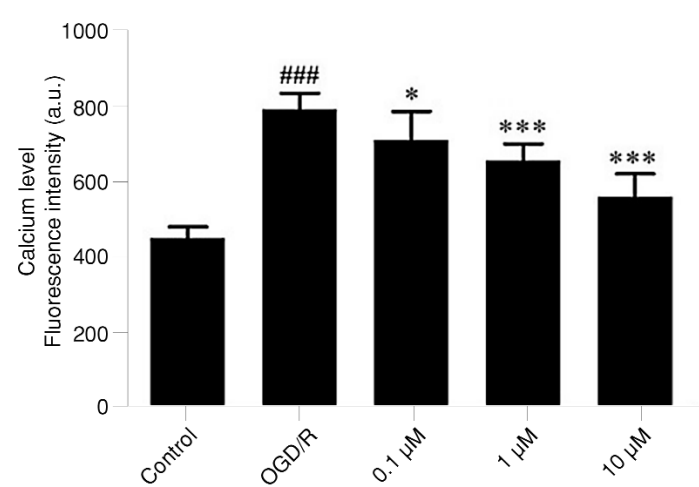


Figure 5. Effect of phyllodulcin on intracellular calcium. PC12 cells were treated by OGD with or without phyllodulcin (0.1, 1 and 10  $\mu$ M) for 4 h, and then exposed to R for 24 h in the presence or absence of phyllodulcin. The intracellular calcium level was expressed as fluorescence intensity of Fluo-3 AM. n = 6, ###p < 0.001 vs control group, \*p < 0.05, \*\*\*p < 0.001 vs OGD/R group.

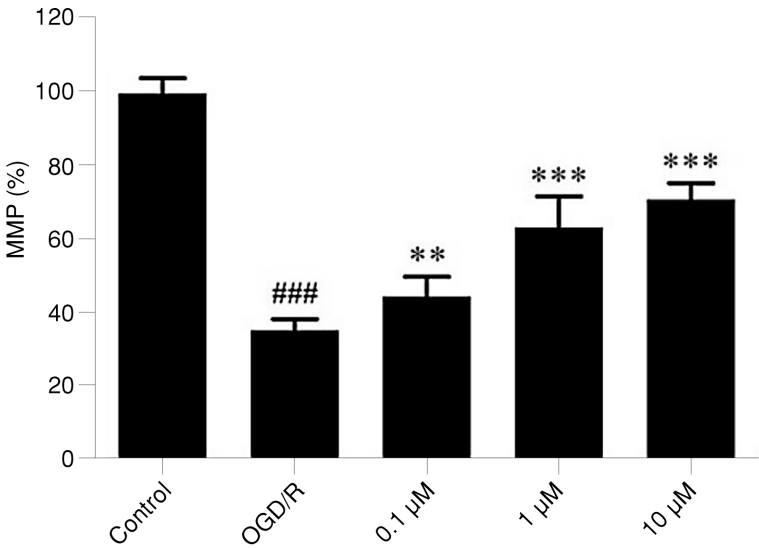


Figure 6. Effect of phyllodulcin on MMP. PC12 cells were treated by OGD with or without phyllodulcin (0.1, 1 and 10 μM) for 4 h, and then exposed to R for 24 h in the presence or absence of phyllodulcin. The MMP was expressed as the percentage compared with the control group. n = 6, ###p < 0.001 vs control group, \*\*p < 0.01, \*\*\*p < 0.001 vs OGD/R group.

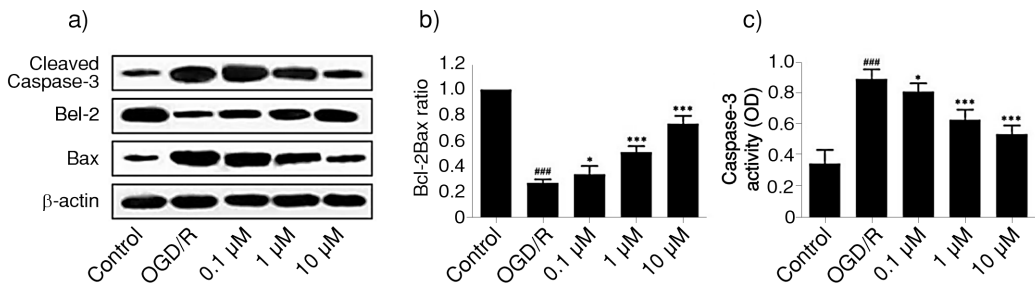


Figure 7. Effect of phyllodulcin on the apoptosis induced by OGD/R. (a) Western blot analysis of the expression of Caspase-3, Bcl-2 and Bax, (b) Densitometric analysis of the ratio between Bcl-2 and Bax derived from the Western blot, (c) The activity of Caspase-3 in PC12 cells, n = 6, ###p < 0.001 vs control group, \*p < 0.05, \*\*\*p < 0.001 vs OGD/R group.

Statistical analysis

All results were expressed as the means ± standard deviation. GraphPad Prism was employed to analyze the results. Statistical differences between different groups were compared by one-way analysis of variance (one way ANOVA) followed by Tukey test for multiple comparisons and Student’s t-test for single comparisons. And the difference was considered to be statistically significant when p < 0.05.

RESULTS

Effect of phyllodulcin on cell viability

The effects of phyllodulcin on PC12 cells viability were determined by MTT assay. As shown in

Fig. 2, after treatment by OGD/R, cell viability was markedly reduced (45.55% ± 3.05% (p < 0.001)). When exposed to different concentrations of phyllodulcin, the cell viability was significantly increased in a dosage-dependent manner compared with the OGD/R group (54.66% ± 5.49% (p < 0.05), 71.39% ± 4.40% (p < 0.001) and 81.75% ± 10.76% (p < 0.001)), which indicates phyllodulcin can attenuate the injury induced by OGD/R.

Effect of phyllodulcin on extracellular LDH

To further confirm the effect of phyllodulcin on the PC12 cells viability, the activity of LDH in the extracellular matrix was determined. After induction by OGD/R, the activity of LDH in the culture medium of PC12 cells increased to 81.75 ±

10.76 ( $p < 0.001$ ) compared to the control group ( $37.55 \pm 4.15$  U/L) (Fig. 3). But in the presence of phyllodulcin, the activity of the extracellular LDH was inhibited as  $70.39 \pm 4.40$  U/L ( $p < 0.05$ ),  $59.45 \pm 5.88$  U/L ( $p < 0.01$ ) and  $54.66 \pm 5.49$  U/L ( $p < 0.01$ ) according to the different concentrations. These results demonstrate phyllodulcin can prevent the death of PC12 cells affected by OGD/R.

#### Effect of phyllodulcin on ROS production

OGD/R can elevate the level of ROS in PC12 cells ( $150.00 \pm 4.27$ ,  $p < 0.001$ ) by comparison with fluorescence intensity of control group ( $45.55 \pm 3.05$ ) (Fig. 4). But the fluorescence intensity decreased to  $111.75 \pm 10.76$  ( $p < 0.001$ ),  $81.72 \pm 9.13$  ( $p < 0.001$ ) and  $54.66 \pm 5.49$  ( $p < 0.001$ ) following the treatment with phyllodulcin (0.1, 1 and 10  $\mu$ M). These results gave the evidence for that phyllodulcin can reduce the production of ROS.

#### Effect of phyllodulcin on intracellular calcium

After treatment with OGD/R, the concentration of intracellular calcium was increased through the fluorescence intensity ( $800.00 \pm 42.67$ ,  $p < 0.01$ ) by contrast with the control group ( $455.54 \pm 30.50$ ) (Fig. 5). But when exposure to different concentrations of phyllodulcin, significant reduction of fluorescence intensity ( $717.52 \pm 76.99$  ( $p < 0.05$ ),  $663.90 \pm 44.04$  ( $p < 0.001$ ) and  $566.64 \pm 61.18$  ( $p < 0.001$ )) was detected, which demonstrates phyllodulcin can lessen the increased level of intracellular calcium in PC12 cells damaged by OGD/R.

#### Effect of phyllodulcin on MMP

The MMP was determined by the fluorescence probe rhodamine-123 to assess the depolarization of mitochondria for rhodamine-123 is proportional to MMP. As shown in Fig. 6, following the OGD/R, the MMP in PC12 cells was obviously reduced to  $35.55\% \pm 3.05\%$  ( $p < 0.001$ ). In contrast, when different concentrations of phyllodulcin at 0.1, 1 and 10  $\mu$ M appeared, the MMP in PC12 cells exhibited recovery with different extents as  $44.66\% \pm 5.49\%$  ( $p < 0.01$ ),  $63.42\% \pm 8.63\%$  ( $p < 0.001$ ) and  $71.06\% \pm 4.58\%$  ( $p < 0.001$ ). These results present the fact that phyllodulcin can ameliorate the dysfunction of mitochondria injured by OGD/R in PC12 cells.

#### Effect of phyllodulcin on Caspase-3, Bcl-2 and Bax

The loss of MMP has been observed in PC12 cells injured by OGD/R, so the expression of Caspase-3, Bcl-2 and Bax were analyzed by West-

ern blot to evaluate the anti-apoptotic effect of phyllodulcin. As a result, the expression of cleaved-Caspase-3 and Bax was up-regulated after OGD/R. Accordingly, Bcl-2 was down-regulated (Fig. 7a). When different concentrations of phyllodulcin appeared in the culture system, the expression of cleaved Caspase-3 and Bax was down-regulated as well as Bcl-2 was up-regulated with different extents. As a criterion to measure apoptosis, the ratio between Bcl-2 and Bax derived from the densitometric analysis displayed phyllodulcin could inhibit apoptosis of PC12 cells exposed to OGD/R, since the ratio for Bcl-2/Bax was increased following the increment of phyllodulcin (Fig. 7b). The colorimetric assay further validated the effect of phyllodulcin on the activity of Caspase-3. From the Fig. 7c, the absorbance in PC12 cells treated by OGD/R ( $0.90 \pm 0.06$ ,  $p < 0.001$ ) was larger than that of control group ( $0.36 \pm 0.09$ ). With the addition of different phyllodulcin, the values were significantly reduced as  $0.82 \pm 0.05$  ( $p < 0.05$ ),  $0.64 \pm 0.07$  ( $p < 0.001$ ) and  $0.55 \pm 0.05$  ( $p < 0.001$ ), respectively. This result demonstrates phyllodulcin can inhibit the activity of Caspase-3.

## DISCUSSION

Cerebral ischemia-reperfusion injury is results from the OGD and restoration, which can induce neuronal apoptosis (5). PC12 cells treated by OGD/R can mimic cerebral ischemia-reperfusion injury and be used to evaluate the potential effects of bioactive substances on that injury (10-12). Herein we assessed the protective effects of phyllodulcin on PC12 cells treated by OGD/R *in vitro*.

After exposure to OGD/R, the viability of PC12 cells was decreased, which demonstrated the cells' were injured. In the presence of phyllodulcin, the cells viability was increased and the injury was attenuated. LDH is a stable enzyme in the cytoplasm for energy metabolism. It will release into the culture medium when the plasma membrane is damaged. The activity of LDH in the culture supernatant indicates the viability of the cells. In this investigation, phyllodulcin can inhibit the leakage of LDH in the extracellular matrix induced by OGD/R.

Mitochondria is a crucial organelle affording energy metabolism and ROS production in living cells, and dysfunction of mitochondria is an early feature of ischemia in the nervous system (22). Ischemia-reperfusion can interrupt the homeostasis between the ROS generation and the detoxification and scavenging capacity of cellular antioxidant enzymes, which will lead to the oxidative stress in



mitochondria and exacerbate the ischemia insult (5,23). Calcium is a messenger in signal transduction and responsible for neurotransmitter release, modulation of membrane excitability and regulation of synaptic plasticity in the nervous system. The concentration of intracellular calcium is maintained through N-methyl-D-aspartic acid (NMDA) receptors and ion exchangers (24). Sustained elevated levels of calcium-induced by OGD/R may impair mitochondrial function and eventually lead to neuronal apoptosis (22). In addition, the overproduction of ROS can destroy the mitochondrial membrane through oxidative stress and result in the collapse of MMP (20). MMP is a sensitive indicator to characterize the function of mitochondria. Following the induction of apoptosis, the MMP is disrupted as one of the earliest intracellular events (21). Our results have shown the function of mitochondria in PC12 cells was disordered after OGD/R through the increasing ROS generation, elevated intracellular calcium level and decreasing MMP. While treated with phyllodulcin in advance, the dysfunction of mitochondria was significantly improved.

ROS-associated OGD/R can induce apoptosis through the mitochondrial pathway (5). Caspases are members of the cysteine-dependent aspartate proteases family and play an important role in cell apoptosis (25). Caspase-3 is one of the effector enzymes in apoptosis. Activation of Caspase-3 is involved in ischemia through cleavage (26). Bcl-2 and Bax are members of the Bcl-2 protein family and play a pivotal role in the mitochondrion-derived apoptosis. Bcl-2 exhibits an anti-apoptotic effect and can attenuate the activation of Caspase-3 while Bax shows a pro-apoptotic effect (27). In our investigation, the expression of activated Caspase-3 and Bax in PC12 cells was up-regulated and Bcl-2 was down-regulated after OGD/R, which demonstrates the apoptosis induced by OGD/R occurs. On the contrary, in the presence of phyllodulcin, the expression of activated Caspase-3 and Bax was down-regulated and Bcl-2 was up-regulated. The increased activity of Caspase-3 affected by OGD/R was inhibited after the treatment of phyllodulcin. Moreover, the densitometric analysis for the ratio between Bcl-2 and Bax also unravels the inhibitory effect of phyllodulcin on the apoptosis induced by OGD/R.

## CONCLUSION

We investigated the protective effects of phyllodulcin on the PC12 cells against the injury induced by OGD/R *in vitro*. Phyllodulcin can attenuate the PC12 cells damage through preventing oxidative

stress and inhibiting ROS-mediated mitochondrial apoptosis. This investigation gives promising evidences for the therapy of cerebral ischemia-reperfusion injury.

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## Conflict of interest

The authors declare no conflicts of interest.

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