

Original Article

β -Asarone protects PC12 cells against OGD/R-induced injury via attenuating Beclin-1-dependent autophagy

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Aim: To explore the effects of β -asarone from *Acorus Tatarinowii* Schott on autophagy in an ischemic stroke model of PC12 cells. **Methods:** The ischemic stroke model of PC12 cells was made by OGD/R (2 h oxygen-glucose deprivation followed by 24 h reperfusion). Drug administration was started 1 h before OGD and last for 3 h. Then the cells were incubated in the drug-free and full culture medium under normoxic conditions for 24 h. After the treatments, Beclin-1, intracellular free calcium concentration ($[Ca^{2+}]_i$) and mitochondrial membrane potential (MMP) were analyzed using flow cytometry. Cell viability was measured using MTT assay. Cell morphology was studied under inverted phase contrast microscope, and autophagosomes were observed under transmission electron microscope. **Results:** Pretreatment with β -asarone (20, 30, or 45 μ g/mL) or the calcium channel antagonist nimodipine (10 μ mol/L) significantly increased the cell viability and MMP, and decreased Beclin-1 expression and $[Ca^{2+}]_i$ in OGD/R-treated PC12 cells. Under inverted phase contrast microscope, pretreatment with β -asarone or nimodipine dramatically increase the number of cells and improved the cellular morphology. Autophagosomes were found in OGD/R-treated PC12 cells as well as in drug plus OGD/R-treated PC12 cells. **Conclusion:** β -Asarone protects PC12 cells against OGD/R-induced injury partly due to attenuating Beclin-1-dependent autophagy caused by decreasing $[Ca^{2+}]_i$ and increasing MMP.

Keywords: β -asarone; PC12 cells; oxygen-glucose deprivation/reperfusion; autophagy; Beclin-1; $[Ca^{2+}]_i$; MMP; nimodipine

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Introduction

Oxygen-glucose deprivation and reperfusion (OGD/R) leads to neuronal insult. Autophagy, a process of self-eating, is usually induced by starvation, ischemia and hypoxia, growth factor deficiency, etc. It helps to maintain cell homeostasis^[1–3], but excessive autophagy may also leads to autophagic neuron death and apoptosis^[4–6]. β -asarone, one of chief constituents from *Acorus Tatarinowii* Schott, can easily pass through the blood brain barrier^[7], and shows various neuroprotective effects such as protecting neuron against apoptosis^[8–11]. However, the effect of β -asarone on autophagy in a model of ischemic stroke *in vitro* is rarely reported. PC12 cell line is derived from a pheochromocytoma of the rat adrenal medulla. It can acquire neuron-like properties when exposed to nerve growth factor (NGF)^[12]. Therefore, it is widely used as an *in vitro* model of cerebral ischemia/reperfusion^[13, 14]. Intracellular free calcium concentration ($[Ca^{2+}]_i$) and mitochondrial membrane

potential (MMP) are important indicators to reflect cell injury. It is reported that autophagy can be induced by increased $[Ca^{2+}]_i$ and decreased MMP^[15, 16]. Beclin-1, an autophagy related gene, is an essential indicator of autophagy. Flow cytometry is adopted as an important quantitative analysis, but Beclin-1 analysis by flow cytometry was merely reported until we established a method of quantitative analysis of Beclin-1^[17]. In this research, we studied the protective mechanism of β -asarone in OGD/R treated PC12 cells by evaluation of Beclin-1, $[Ca^{2+}]_i$, MMP and cell viability and observation of autophagosomes and cellular morphology. Control group treated with nimodipine (a calcium antagonist) was established to explore whether β -asarone has effects on $[Ca^{2+}]_i$ and MMP so as to reduce autophagy.

Materials and methods

β -asarone preparation

β -asarone (cis forms of 2,4,5-trimethoxy-1-propenylbenzene) is a fat-soluble substance with a small molecular weight. It was extracted from *Acorus Tatarinowii* Schott according to the procedure that we have reported^[18]. β -asarone, the purity of

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which was up to 99.55%, was confirmed by gas chromatography-mass (GC-MS), infrared spectrum (IR), and nuclear magnetic resonance (NMR) detection.

Cell cultures

PC12 cells (Culture Collection of Chinese Academy of Science, Shanghai, China) were seeded in 25-cm² polystyrene flasks (Corning Costar Corp, USA) with 4.5 g/mL glucose in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), containing 5% heat-inactivated foetal bovine serum (Gibco, USA) and 5% horse serum (Gibco, USA). The cells were incubated under an atmosphere of 95% air and 5% CO₂. Culture medium was replaced every 48 h.

Glucose deprivation and hypoxia/reperfusion

PC12 cells were washed with phosphate buffer solution (PBS) for one time and incubated in Earle's balanced salt solution (116 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L MgSO₄, 1 mmol/L NaH₂PO₄, 0.9 mmol/L CaCl₂, and 10 mg/L phenol red). And then the cells were incubated in a hypoxia chamber (Thermo scientific, USA) with a compact gas oxygen controller to maintain oxygen concentration at 1% by injecting a gas mixture of 94% N₂ and 5% CO₂ for 2 h. After hypoxia, the cells were transferred back to full culture medium with oxygen for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions.

Drugs

PC12 cells were incubated with full culture medium containing β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) under normoxic conditions for 1 h before hypoxia. The full culture medium containing drug was discarded. The cells were rinsed once with PBS, and incubated with Earle's balanced salt solution containing β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) for 2 h of hypoxia. The Earle's balanced salt solution was discarded, and then PC12 cells were incubated with full culture medium free of drug under normoxic conditions for 24 h.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for cell viability

PC12 cells were seeded into 96-well plastic plates with 0.1 mL at the density of 3×10⁴ cells/mL. Then after growth for 48 h, the cells were treated with OGD/R as described above. After 24 h reperfusion, the MTT colorimetric assay was performed. It contained 10 samples in normal control group, OGD/R treated group and OGD/R+drug treated groups respectively. 110 μL of MTT (10 mg/mL) dissolved in full culture medium was added to each well. Plates were incubated at 37°C in normoxia for 4 h. 150 μL of Dimethyl sulfoxide (DMSO) was added to each well for 10 min to dissolve the dark blue crystals and the absorbance was read at 570 nm on a microplate reader (Thermo scientific, USA).

Flow cytometric analysis of Beclin-1 expression

PC12 cells were seeded into 24-well plastic plates with 1 mL at

the density of 1×10⁵ cells/mL. It contained 10 samples in normal control group, OGD/R treated group and OGD/R+drug treated groups, respectively. PC12 cells were harvested by trypsinization [0.25% trypsin/2.6 mmol/L ethylenediaminetetraacetic acid (EDTA), Gibco, USA] and incubated in 2 mL 2% (v/v) bovine serum albumin (BSA)/PBS for 30 min. Permeabilization of the cells was done using fixation and permeabilization (Invitrogen, Beijing, China), according to the manufacturer instructions. Then the cells were incubated with mouse anti-human Beclin-1 monoclonal antibody (100 μL, 2:100 dilution, Santa Cruz Biotechnology, catalog No sc-48381, USA) at room temperature for 30 min. After washing with 2% BSA/PBS once, cells were incubated with PE-conjugated goat anti-mouse IgG1 antibody (0.005 μg/μL, Mutiscience, catalog No gam0041, China) in the darkness at room temperature for 30 min. The control cells were incubated with the secondary antibody alone. After washing once with 2% BSA/PBS, the fluorescence of 10000 cells was analyzed by flow cytometer (Beckman Coulter, Florida, American, 488 nm) using the EXPO32 data analysis system (Beckman Coulter, Florida, USA).

Flow cytometric analysis of MMP and [Ca²⁺]_i

PC12 cells were seeded in 24-well plastic plates with 1 mL at the density of 1×10⁵ cells/mL. Then after growth for 48 h, the cells were treated with OGD/R as described above. Normal control group, OGD/R treated group and OGD/R+drug groups contained 10 samples, respectively. After 24 h reperfusion, cells were harvested, washed with 2 mL PBS once, centrifuged for 5 min at 1000 r/min (TDL-5-A Low Speed Centrifuge, Shanghai, China) and pelleted. The cells were resuspended in 0.5 mL PBS. For MMP detection, cell suspensions were incubated with Rhodamine123 (Rho123) (final concentration 10 μg/mL, ALEXIS, USA, catalog No alx-610-018-m005) in the darkness at 37°C for 30 min. For [Ca²⁺]_i detection, cell suspensions were incubated with Fura 3-acetoxymethyl ester (Fluo-3am) (final concentration 5 μmol/L, BIOTIUM, USA, catalog No 50013) in the darkness at 37°C for 40 min. Then cell suspensions were centrifuged at 1000 r/min for 5 min. After washing once in 2 mL PBS, the cells were resuspended in 0.5 mL PBS. The fluorescence of 10000 cells was analyzed by flow cytometry (488 nm). The mean fluorescence intensity of Rho123 represented the state of depolarization of MMP. The mean fluorescence intensity of Fluo-3 was used as the indication of [Ca²⁺]_i quantity.

Inverted phase contrast microscope for observation of cellular morphology

After normoxic, hypoxic or hypoxic+drug treatment (OGD 2 h+R 24 h), cellular morphology were observed under inverted phase contrast microscope (×200, Olympus, Japan).

Transmission electron microscope for observation of autophagy

After normoxic, hypoxic or hypoxic+drug treatment (OGD 2 h+R 24 h), the cells were trypsinized and pelleted. The cells were suspended and fixed with 2% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) at 4°C for 2 h and postfixed with 1%

osmium tetroxide in 0.1 mol/L PBS (pH 7.4) at 4 °C for 2 h. After washing twice with PBS, cells were subsequently washed with 50% acetone for 10 min, with 70% acetone for 10 min, twice with 80% acetone for 10 min, twice with 90% acetone for 10 min, twice with 100% acetone for 10 min and embedded in Epon 812 resin. The blocks were cut into ultrathin sections by a ultramicrotome and stained with uranyl acetate and lead citrate. The ultrastructure of the cells was then observed under a transmission electron microscope (H-600, HITACHI, Japan).

Statistical analysis

Measurement data were expressed as mean±standard deviation (Mean±SD). Statistical significance was determined by independent-samples *t*-test. A value of $P<0.05$ was considered significant. All statistical analyses were performed with version SPSS 13.0 statistical software.

Results

Effect of β -asarone on cell viability and MMP in OGD/R treated PC12 cells

Cell viability [optical density value (OD value)] and MMP were 0.79 ± 0.04 , 542.5 ± 26.6 in normal control cells. They were significantly decreased in OGD/R treated cells (0.46 ± 0.02 in OD value, 335.2 ± 19.3 in MMP, $P<0.001$). After treatment with β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$), or nimodipine (10 $\mu\text{mol}/\text{L}$), respectively, cell viability and MMP were dramatically increased. Cell viability was restored to near normal levels (0.79 ± 0.04 in OD value) at the dose of 30 $\mu\text{g}/\text{mL}$ of β -asarone or 10 $\mu\text{mol}/\text{L}$ of nimodipine (0.75 ± 0.09 , 0.74 ± 0.04 in OD value, respectively, $P>0.05$) (Table 1, Figure 1).

Effect of β -asarone on Beclin-1 and $[\text{Ca}^{2+}]_i$ in OGD/R treated PC12 cells

Beclin-1 expression and $[\text{Ca}^{2+}]_i$ were $6.3\%\pm 0.8\%$, 124.7 ± 14.9 in

Table 1. Effect of β -asarone on cell viability and MMP in OGD/R treated PC12 cells.

Groups	<i>n</i>	Cell viability (OD value)
Normal control	10	0.79 ± 0.04
Model control	10	0.46 ± 0.02^c
Nimodipine (10 $\mu\text{mol}/\text{L}$)	10	0.74 ± 0.04^f
β -asarone (20 $\mu\text{g}/\text{mL}$)	10	0.64 ± 0.07^{cf}
β -asarone (30 $\mu\text{g}/\text{mL}$)	10	0.75 ± 0.09^f
β -asarone (45 $\mu\text{g}/\text{mL}$)	10	0.56 ± 0.08^{cf}

Normal control cells were grown in full culture medium under normoxic conditions. Model control cells were treated with OGD/R (2 h OGD+24 h R). Therapeutic cells were incubated with nimodipine (10 $\mu\text{mol}/\text{L}$) or β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) respectively for 3 h (1 h before OGD and 2 h OGD). They were incubated with full and drug-free culture medium for 24 h. β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) and nimodipine (10 $\mu\text{mol}/\text{L}$) both significantly increased cell viability and MMP in OGD/R treated cells. Mean±SD for 10 samples. $^cP<0.01$ vs normal control. $^fP<0.01$ vs OGD/R treated group.

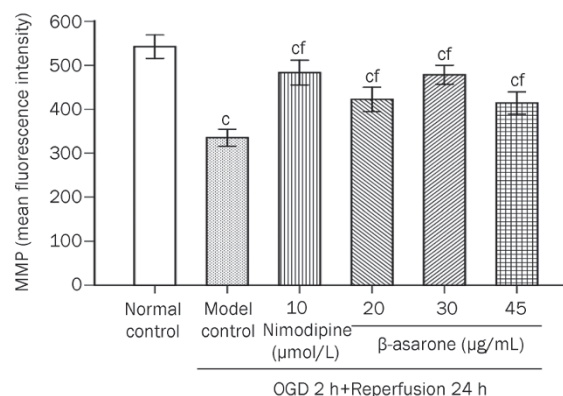


Figure 1. Effect of β -asarone on MMP in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 $\mu\text{mol}/\text{L}$) or β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, MMP was analyzed using flow cytometry. The mean fluorescence intensity of Rho123 represented the state of depolarization of MMP. Mean±SD for 10 samples. $^cP<0.01$ vs normal control group. $^fP<0.01$ vs model control group.

normal cultured cells respectively. They were dramatically increased after OGD/R treatment ($28.5\%\pm 2.3\%$ in Beclin-1, 294.9 ± 42.7 in $[\text{Ca}^{2+}]_i$, respectively, $P<0.001$). After incubation with β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) or nimodipine (10 $\mu\text{mol}/\text{L}$) respectively, Beclin-1 expression and $[\text{Ca}^{2+}]_i$ were both significantly reduced ($P<0.001$). As for β -asarone (30 $\mu\text{g}/\text{mL}$) group and nimodipine (10 $\mu\text{mol}/\text{L}$) group, Beclin-1 expression was reduced to $17.6\%\pm 2.6\%$ and $15.6\%\pm 2.5\%$, respectively, and $[\text{Ca}^{2+}]_i$ was decreased to 132.4 ± 25.5 and 139.0 ± 19.0 , respectively. For $[\text{Ca}^{2+}]_i$, there was no significant difference between β -asarone (30 $\mu\text{g}/\text{mL}$) group and normal control group or between nimodipine (10 $\mu\text{mol}/\text{L}$) group and normal control group ($P>0.05$) (Figure 2, 3).

Observation of cellular morphology

Cellular morphology was observed under inverted phase contrast microscope. The number of neurons showed a significant reduction. The PC12 cells exhibited round, slender and degenerated morphology after OGD/R treatment. However, the number of cells showed a dramatic increase. The cells exhibited improved cellular morphology after treatment with β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) or nimodipine (10 $\mu\text{mol}/\text{L}$), respectively. These observations were in accordance with the results of MTT assay (Figure 4).

Observation of ultrastructural morphology of autophagosome

Representative ultrastructural morphology of autophagy was observed under transmission electron microscopy. Normal morphology was observed in cells cultured under normal conditions (Figure 5A), autophagic morphology could be observed in cells treated with OGD/R, OGD/R+nimodipine

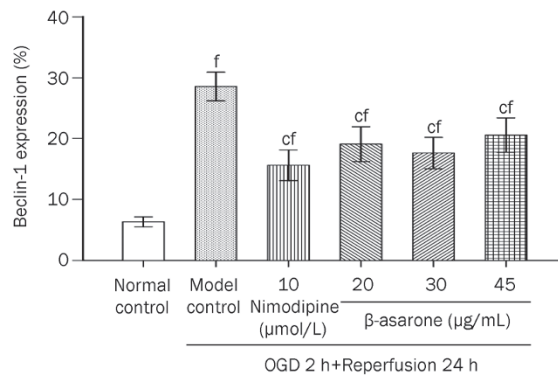


Figure 2. Effect of β -asarone on Beclin-1 expression in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 μ mol/L) or β -asarone (20, 30, or 45 μ g/mL) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, Beclin-1 expression was analyzed using flow cytometry. Beclin-1 was represented as a percentage of positive expression. Mean \pm SD for 10 samples. ^c P <0.01 vs normal control group. ^f P <0.01 vs model control group.

(10 μ mol/L) and OGD/R+ β -asarone (20, 30, and 45 μ g/mL) (Figure 5B–5F). The results indicated that OGD/R can generate autophagy, and β -asarone can attenuate OGD/R-induced autophagy in PC12 cells.

Discussion

β -asarone (cis forms of 2,4,5-trimethoxy-1-propenylbenzene) is a fat-soluble substance with a small molecular weight. It is extracted from *Acorus Tatarinowii* Schott. The absorption and elimination of β -asarone are rapid *in vivo*. β -asarone is easy to pass through blood brain barrier. Brain is an important organ of distribution of β -asarone. In the rat serum, elimination half-life and peak time are 54 and 12 min, respectively^[7]. The half lives of β -asarone in blood, hippocampus, cortex, brain stem, thalamus, and cerebellum of rabbits are 1.3801, 1.300, 1.937, 7.142, 2.832, and 8.149 h, respectively^[19]. β -asarone is excreted in urine, feces and bile, and the excretion efficiency is about 62% in urine, 22% in feces, and 16% in bile of rabbits. About 22% β -asarone is converted into α -asarone. Most β -asarone is excreted in 12 h^[20].

β -asarone has significant pharmacological effects on central nervous system. It has effects on multi-target genes in mouse brain^[8]. It can obviously increase the expression of c-fos in epilepsy rat brain^[9], and attenuate neuronal apoptosis induced by β -amyloid in rat hippocampus and in PC12 cells^[10, 11]. But it may also have side effects. It is reported that β -asarone could cause acute respiratory disturbance by inhibition of neurotransmission in the medullary respiratory neuronal network^[21]. In this study, we observed that β -asarone could aggravate OGD/R induced injury of the cells when they had been incubated with β -asarone for too long, such as 24 h.

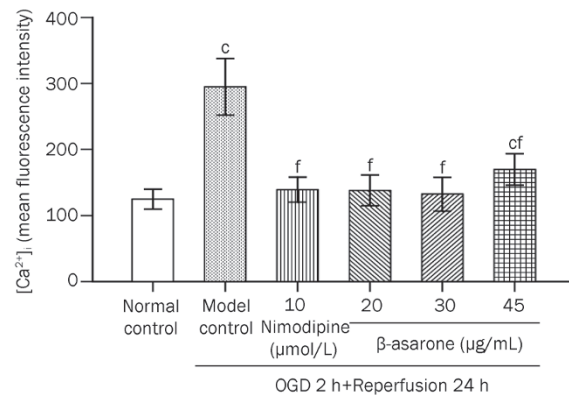


Figure 3. Effect of β -asarone on $[Ca^{2+}]_i$ in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 μ mol/L) or β -asarone (20, 30, or 45 μ g/mL) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, $[Ca^{2+}]_i$ was analyzed using flow cytometry. The mean fluorescent intensity of Fluo-3 was used as the indication of $[Ca^{2+}]_i$ quantity. Mean \pm SD for 10 samples. ^c P <0.01 vs normal control group. ^f P <0.01 vs model control group.

β -asarone can reduce neuronal apoptosis and protect neurons. However, its effect on autophagy in an ischemic stroke model of PC12 cells is rarely reported. Since PC12 cells possess the neuron-like properties, the protective effect of β -asarone on OGD/R induced injury in PC12 cells can demonstrate the possible protective mechanism of β -asarone on neurons following cerebral ischemia/reperfusion in a certain extent. Therefore, PC12 cells were established to an *in vitro* model of ischemia/reperfusion in this study. Autophagy is cellular process of degrading long-lived protein and organelles through the lysosomal pathway. It plays an important role in providing energy and turning over useless, superfluous and damaged organelles to maintain intracellular homeostasis. Activity of autophagy increases when cell injury, nutritional deficiencies, growth factors deficit or high energy demand occurs. However, excessive autophagy also leads to autophagic neuron death and apoptosis^[4–6]. Beclin-1, a key regulatory gene of autophagy, is first discovered in mammalian. It regulates localization of other autophagy-related proteins to autophagosome^[22, 23].

It is widely accepted that mitochondria are factories for energy production. And they are also the targets for autophagy. MMP is a sensitive indicator for evaluation of mitochondrial function. When mitochondrial membrane is damaged due to hypoxia, MMP decreases, which leads to ATP deficit and engulfment of damaged mitochondrial by the autophagosomes^[16, 24]. ATP deficit during ischemia and reperfusion leads to damage of energy-dependent ion pump on cellular membrane, concentration disorder of intracellular and extracellular ion, depolarization, and overload of intracellular Ca^{2+} which in turn leads to a decrease of MMP^[25]. Ca^{2+} partici-

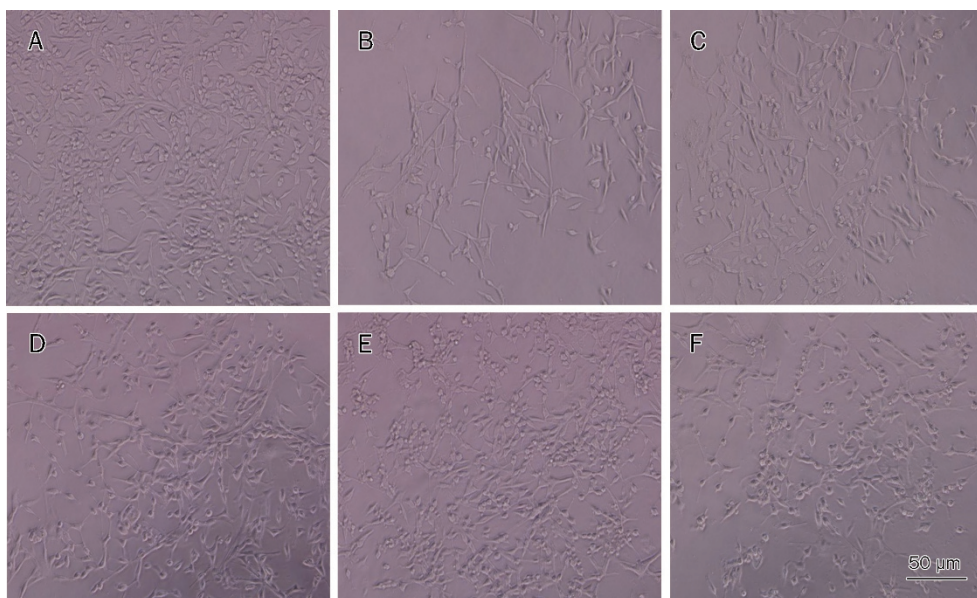


Figure 4. Cellular morphology was observed under inverted phase contrast microscope. Normal neuronal morphology was seen in the bright field images (A, $\times 200$). The number of cells showed a significant reduction. The cells exhibited round, slender and degenerated morphology after OGD/R (B, $\times 200$). The number of cells showed a dramatic increase. The cells exhibited improved cellular morphology after treatment with β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$) (D, E, F, $\times 200$) or nimodipine (10 $\mu\text{mol}/\text{L}$) (C, $\times 200$).

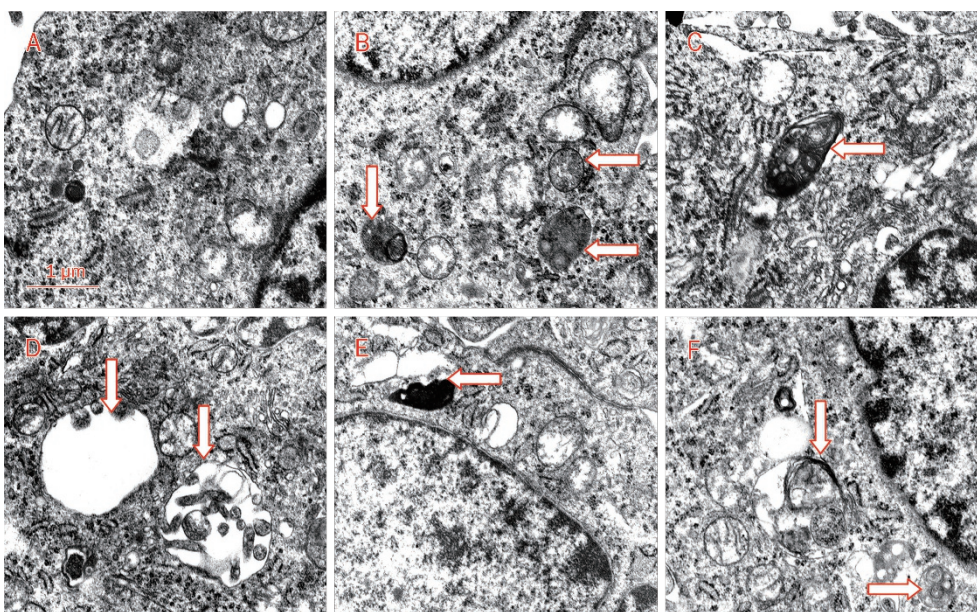


Figure 5. Representative ultrastructural morphology of autophagy. Normal morphology of cytoplasm, cell organelles in normal control group (A, $\times 20000$), characteristic ultrastructural morphology of autophagy in OGD/R treated group (B, $\times 17000$), β -asarone (20, 30, or 45 $\mu\text{g}/\text{mL}$, respectively) group (D, E, F, $\times 20000$), and nimodipine (10 $\mu\text{mol}/\text{L}$) group (C, $\times 20000$). Arrows indicate autophagosomes.

pates in formation of autophagosome as an important regulatory factor. It modulates localization of atg18 to autophagosomal membranes^[15]. Removal of intracellular calcium can inhibit autophagy. For example, thapsigargin inhibits release of sarcoplasmic reticulum calcium, which also reduces autophagic flux by 50%^[26]. Ca^{2+} leads to endoplasmic reticu-

lum misfolded proteins and mitochondria depolarization, which induces autophagy of general and specific organelles so as to reestablish cellular homeostasis^[27]. Taken together, MMP and Ca^{2+} are both closely related with autophagy.

In this study, we showed that β -asarone and nimodipine both significantly increased cell viability and MMP, and

improved cellular morphology in OGD/R treated PC12 cells but decreased Beclin-1 and $[Ca^{2+}]_i$. These results suggested that increased $[Ca^{2+}]_i$ and decreased MMP may induced Beclin-1 dependent autophagy, which is accordance with above listed studies. β -asarone can protect PC12 cells against OGD/R induced injury. It may attenuate Beclin-1 expression partly by decreasing $[Ca^{2+}]_i$ and increasing MMP as nimodipine does.

Acknowledgements

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Author contribution

Zhen-tao MO and Yong-qi FANG designed the research; Zhen-tao MO, Yu-ping HE, and Sheng ZHANG performed the research; Zhen-tao MO analyzed the data and wrote the paper.

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