

Original Article

Ischemic postconditioning alleviates ischemia/reperfusion-induced injury in SH-SY5Y cells: development of an in vitro model of ischemic postconditioning

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Abstract: Objective: Ischemic postconditioning (IPC) is shown to reduce ischemia/reperfusion-induced injury. However, the neuroprotective mechanisms of IPC are largely unclear, and animals or cultured neurons are being used to model the process of IPC, which is costly and unstable. This study was conducted to develop an in vitro IPC model of human neuroblastoma cells (SH-SY5Y cells) for studying the IPC mechanism. Methods: The SH-SY5Y cells were divided into 5 groups: the control group, the ischemia/reperfusion group, and the IPC1, 2, and 3 groups. The ischemia/reperfusion group was subjected to 12-h of oxygen glucose deprivation (OGD). After a 12-h OGD, the cells were cultured in 1 (IPC1) to 3 (IPC3) cycles of normal and OGD conditions, respectively. The changes in the SH-SY5Y cells were then evaluated using an inverted microscope, cell counting, Hoechst staining, flow cytometry, and Western blot analysis. Results: IPC ameliorated the morphology of the SH-SY5Y cells, increased the survival rate ($P < 0.05$) and the activity of superoxide dismutase activity ($P < 0.05$), and decreased the apoptosis rate and the expression of Bcl-2 associated protein X (Bax). The differences were significant between the control and the ischemia/reperfusion groups, and the ischemia/reperfusion group and the IPostC2 and 3 groups. 2 to 3 cycles of alternative normal and OGD cultures of 12 h-OGD treated SH-SY5Y cells could mimic the protection of IPC. The protective mechanisms of IPC in the cell models are likely achieved via anti-apoptotic and anti-oxidant mechanisms. Conclusions: This work demonstrates that an in vitro model of neuroblastoma cells can mimic the neuroprotection mechanisms observed in vivo and would facilitate the mechanistic research on IPC.

Keywords: Ischemic postconditioning, SH-SY5Y cells, *in vitro*, apoptosis, oxidative stress

Introduction

Ischemic stroke remains a troublesome public health problem worldwide [1]. It is characterized by a high incidence, a high disability rate, and high mortality. Although significant progress has been made in the prevention and supportive care of ischemic stroke, a great amount of effort to protect the brain from ischemic cell death has failed, except for the application of tissue plasminogen activator (tPA) [2]. However, because of the narrow therapeutic window (< 4.5 h) and safety concerns, less than 5% of patients are able to receive treatment with tPA [3], and most patients receive only supportive care, which is less effective.

On other hand, the brain has a well-developed capacity for self-preservation. Ischemic postconditioning (IPC) is a recently discovered endogenous process that mediates recovery from ischemia/reperfusion-induced injury. IPC generates a series of rapid intermittent interruptions of blood flow in the early phase of reperfusion [4, 5]. Since IPC can be applied after the ischemia of an organ, it has a greater potential for clinical application than ischemic preconditioning. A number of possible neuroprotective mechanisms by IPC have been proposed, including the inhibition of apoptosis, the reduction of oxygen radicals [6, 7], the activation of the reperfusion injury salvage kinase pathway [8], the closure of the mitochondrial

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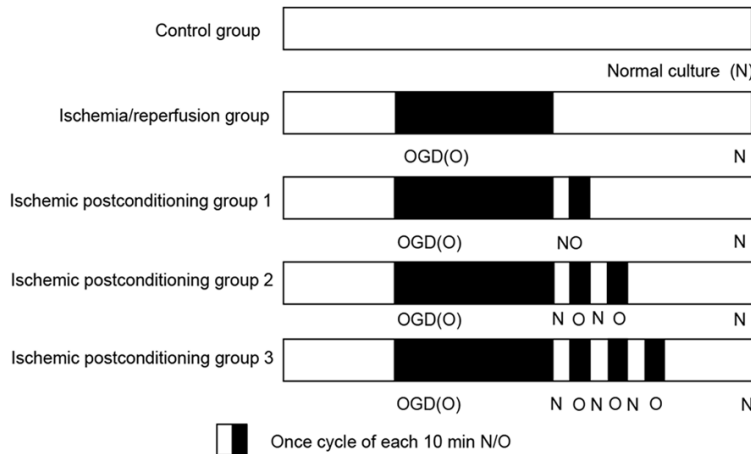


Figure 1. Diagram of the ischemia/reperfusion (OGD) and ischemic post-conditioning treatment of SH-SY5Y cells. Each normal and OGD culture lasts 10 min.

permeability transition pores [9], and the ATP-sensitive potassium channels [10, 11]. However, these mechanisms are still inadequate to fully explain the protective role of IPC for the brain [11]. Currently, animals or cultured cells are used as the main research models to study the processes and mechanism of IPC [8, 12-14]. In *in vitro* studies, IPC also shows a protective role [8, 13, 14]. Although intensive studies have been made, the mechanism of neuroprotection by IPC has not been sufficiently elucidated.

To facilitate research on the neuroprotective mechanism, we constructed an IPC model based on human neuroblastoma cells (SH-SY5Y cells), hoping this would offer new experimental methods and techniques for studying the mechanism underlying neurodegeneration and developing new strategies for better treatment of ischemic stroke.

Materials and methods

Cell culture

The SH-SY5Y neuroblastoma cell line was purchased from Typical Culture Collection, Chinese Academy of Science (Shanghai, China). The cells were cultured in a complete medium (Dulbecco's modified Eagle's medium-F12 (DMEM-F12), Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified incubator

at 5% CO₂. The medium was refreshed once every other day. When reaching 70-80% confluency, the cells were digested with 0.25% trypsin and sub-cultured.

Post conditioning

The SH-SY5Y cells were plated in 3.5 cm culture dishes at a density of 2×10^5 cells/dish and grown overnight before starting the experiments. The cells were divided into 5 groups: the control group, the ischemia/reperfusion group, and the IPC 1-3 groups. Cells cultured in 3151 mg/L glucose were used as a control.

For ischemia/reperfusion treatment, the cells were cultured in an oxygen glucose deprivation (OGD) medium in an air-tight chamber which is automatically monitored for oxygen content for 12 h in a humidified atmosphere of 0.5% O₂, 5% CO₂ and 94.5% N₂ at 37°C. The OGD medium was prepared by bubbling DMEM medium with 100% N₂ for 30 min. After a 12-h period of culture in OGD, which was used to mimic the ischemic process, the cells were cultured alternatively in normal and OGD media for 1, 2, or 3 cycles, each lasting 10 min (IPC1, 2, and 3) as shown in **Figure 1**. The culture conditions were the same as described above. After that, the cells were cultivated normally for 12 h.

Microscopy

The SH-SY5Y cells were seeded in 3.5 cm culture dishes at 2×10^5 cells/dish and subjected to IPC treatments as described above. The cell morphology was observed using an inverted microscope (Olympus, Japan).

Cell proliferation assay

Cell viability was determined calorimetrically using a Cell Counting Kit-8 (Dojindo Laboratories, Japan) according to the manufacturer's protocols. After incubation for 2 h, the OD value of the 96-well plates were read using an enzyme-linked immunosorbent assay (ELISA) reader (Victor 3 1420 Multiple Counter, Perkin Elmer) at 450 nm to calculate the survival rate of the cells.

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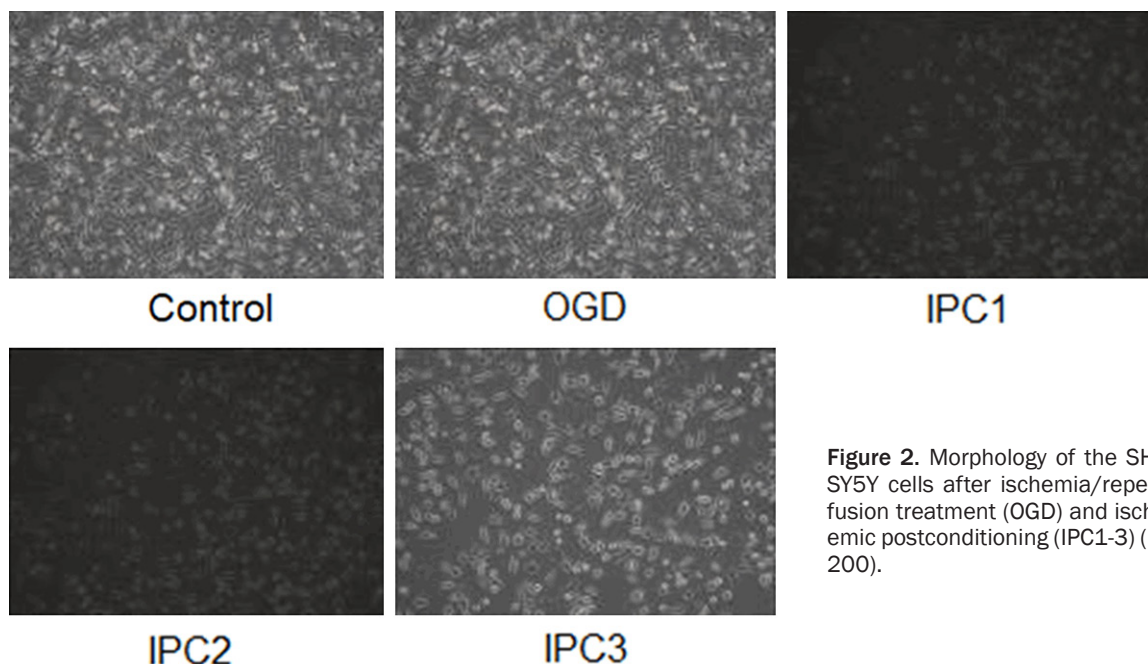


Figure 2. Morphology of the SH-SY5Y cells after ischemia/reperfusion treatment (OGD) and ischemic postconditioning (IPC1-3) ($\times 200$).

Apoptosis assay

The SH-SY5Y cells were seeded in 3.5 cm culture dishes and stained with Hoechst 33342 (0.1 mg/ml) for 10 min at 37°C in dark. Hoechst 33342-stained nuclei were observed using a fluorescence microscope (Olympus, Japan) at the 521 nm emission wavelength. The apoptotic cells were evaluated using an Annexin V/FITC kit (BestBio, Shanghai, China). The cells were stained according to the manufacturer's protocols and analyzed using a flow cytometer (EPICS® ALTRA™, Beckman Coulter, USA).

Determination of superoxide dismutase (SOD) activity

SOD activity was measured using the xanthine oxidase method according to the manufacturer's protocol (Jiancheng Bioengineering Inc, Nanjing, China). The absorbance was determined at 550 nm using an ELISA reader. The proteins were quantified using a Bradford method assay kit (Beyotime Biotech, Jiangsu, China).

Western blotting

Western blot analysis was performed to measure the apoptosis-related proteins. Cell lysates prepared in a RIPA lysis buffer (Beyotime, Beijing, China) were mixed with a protein loading buffer and separated on 10% SDS poly-

acrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, USA) and blocked in a Western blot blocking buffer (Beyotime) before immunoblotting for 1 h at room temperature. The membranes were probed with antibodies against Bcl-2 associated protein X (Bax) (at 1:1000 dilution, Santa Cruz Biotechnology, California, USA) and β -actin (at 1:500 dilution, Santa Cruz Biotechnology) at 4°C overnight and with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at 1:8000 dilution at room temperature for 1 h. The peroxidase reaction was visualized using the enhanced chemiluminescence (ECL) method (Beyotime), and imaged using GeneTools software.

Statistical analysis

All data were presented as the means \pm standard deviation of six replications. The means were compared using a one-way ANOVA test. A value of $P < 0.05$ was considered statistically significant. The statistical analyses were performed using SPSS 13.0 software.

Results

Morphologic changes of SH-SY5Y cells

As shown in **Figure 2**, the SH-SY5Y cells grew well in the DMEM-F12 with tightened connections between the cells and showed a slender

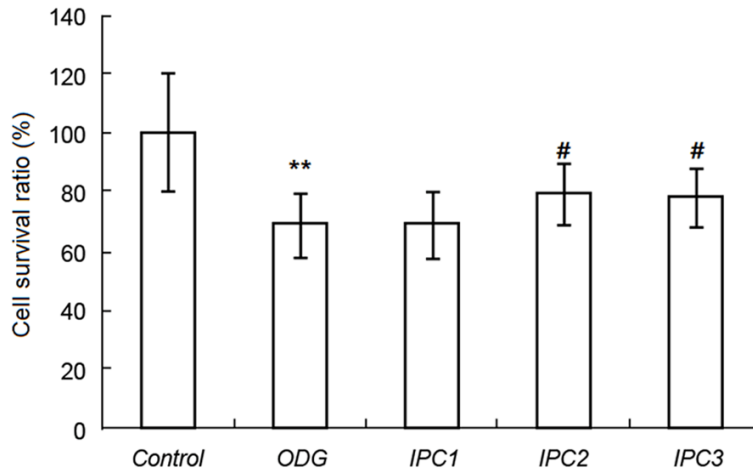


Figure 3. The survival ratio of SH-SY5Y cells after ischemia/reperfusion treatment (ODG) and 1 to 3 cycles of ischemic postconditioning (IPC1-3). **Denotes $P < 0.01$ vs control and #denotes $P < 0.05$ vs ODG.

and smooth morphology. On the other hand, the number of living cells was remarkably reduced after the ischemia/reperfusion treatment and the cells were wrinkled and round in shape with sparse cell to cell connections. Compared with the ischemia/reperfusion group, there were more living cells after the IPC treatments, and the cells, particularly in the IPC2 and IPC3 groups, showed a morphology similar to the morphology in the control group.

Survival rate of SH-SY5Y cells

After the ischemia/reperfusion treatment, the survival rate of the SH-SY5Y cells dropped significantly to $69.7 \pm 2.47\%$ of the control group ($P < 0.01$). The average survival rate was $69.5 \pm 2.53\%$ in IPC1, which is statistically similar to the rate in the ischemia/reperfusion group (ODG) ($P > 0.05$). However, the survival rates in the IPC2 and 3 groups were $79.9 \pm 7.04\%$ and $78.7 \pm 7.03\%$, respectively, which were significantly higher than that the ischemia/reperfusion group (ODG) ($P < 0.01$). The rates were not different between the IPC2 and IPC3 groups ($P > 0.05$) (**Figure 3**).

Hoechst 33342 staining

As shown in **Figure 4**, after the Hoechst 33342 staining, the nuclei of the SH-SY5Y cells in the control group were light blue under an inverted fluorescence microscope (**Figure 4A**). Some nuclei of the SH-SY5Y cells after the ischemia/reperfusion treatment were stained bright blue

or fragmented (**Figure 4B**), indicating that apoptosis occurred in the SH-SY5Y cells. The nuclei in the IPC groups were bright blue, but the number of stained nuclei decreased compared with the increased cycle of ischemia/reperfusion, particularly in the IPC2 and IPC3 groups (**Figure 4C-E**).

Apoptosis

To quantitatively assess the apoptosis, flow cytometry was used. The results showed that, compared with the control group, the number of living cells was significantly reduced,

and the early apoptotic and necrotic cells increased in the ischemia/reperfusion group. On other hand, the IPC treatments significantly reduced the early apoptotic and necrotic cells from nearly 40% to about 10% (**Figure 5A and 5B**).

SOD activity

The SOD activity in the SH-SY5Y cells decreased from 22.3 ± 1.34 U/mg protein in the control cells to 12.0 ± 2.13 U/mg protein by 46.3% in the ischemia/reperfusion group (ODG) ($P < 0.01$). The SOD activity of the IPC1, IPC2, and IPC3 groups increased by 45.2%, 76.5%, and 32.5%, respectively, compared with the ischemia/reperfusion group ($P < 0.01$) (**Figure 6**).

Expression of Bax

As shown in **Figure 7**, the expression of the apoptosis-associated protein Bax increased significantly in the ischemia/reperfusion group compared to the control group ($P < 0.01$). After 2 or 3 cycles of IPC, the levels of Bax decreased significantly compared with the ischemia/reperfusion group ($P < 0.05$).

Discussion

The present study showed that the alternative culture method for 2 or 3 cycles in normal and ODG media (10 min each) can ameliorate the ischemia/reperfusion-induced injury in SH-SY5Y cells, indicating that such a scheme could

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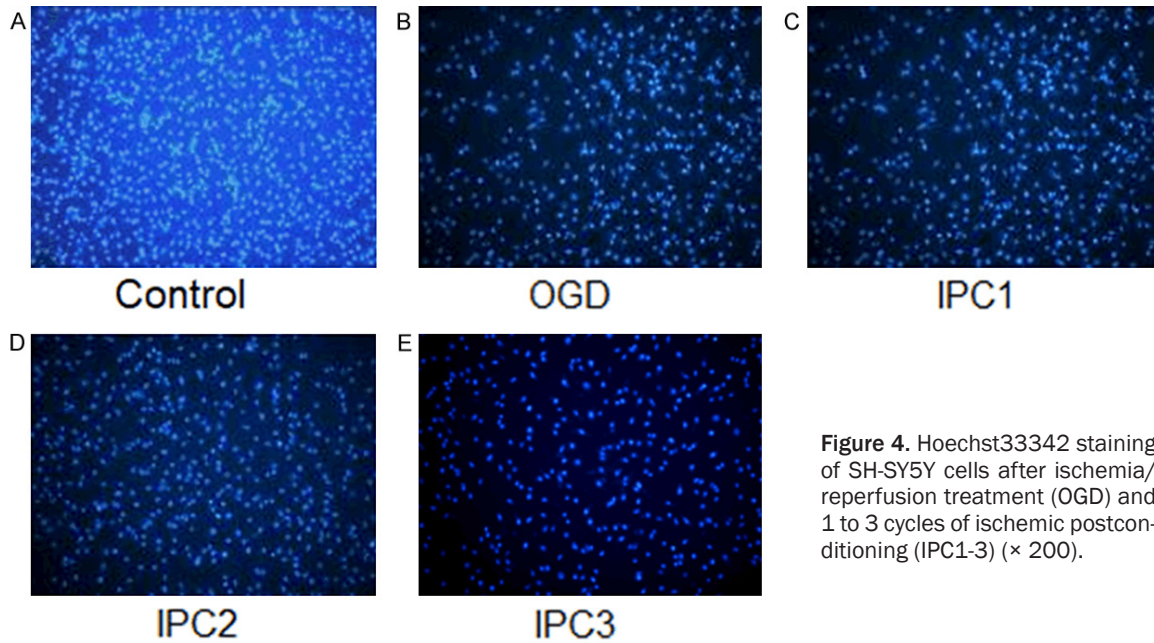


Figure 4. Hoechst33342 staining of SH-SY5Y cells after ischemia/reperfusion treatment (OGD) and 1 to 3 cycles of ischemic postconditioning (IPC1-3) ($\times 200$).

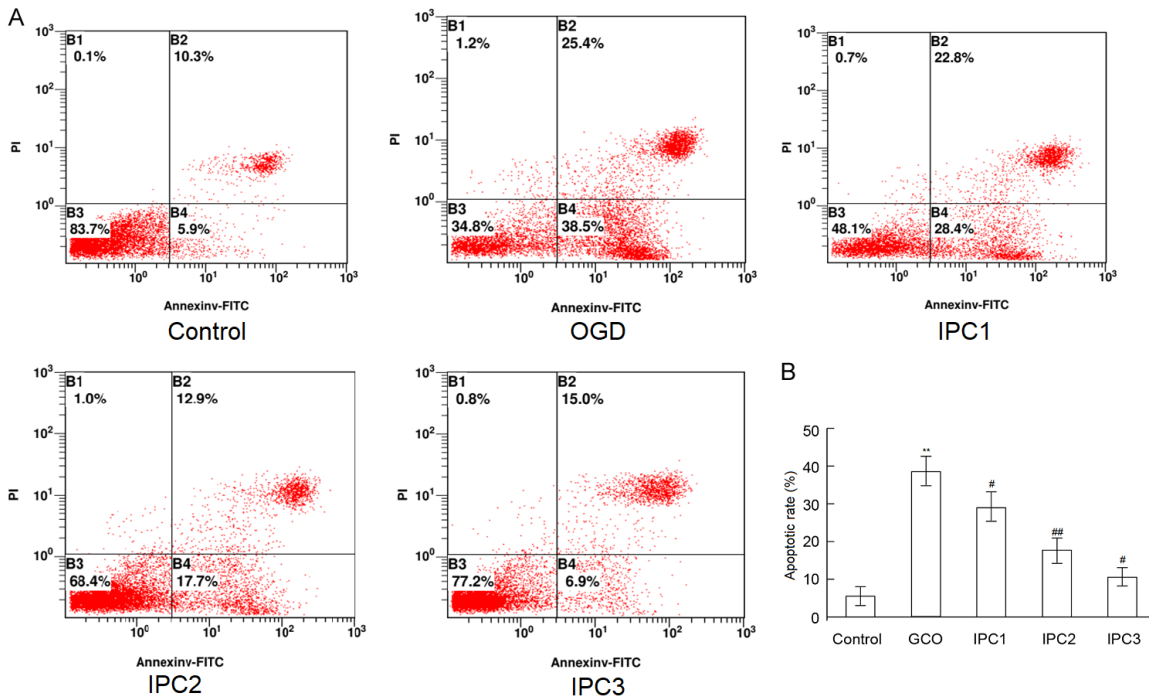


Figure 5. Flow cytometry results of SH-SY5Y cells after ischemia/reperfusion treatment (OGD) and 1 to 3 cycles of ischemic postconditioning (IPC1-3) ($\times 200$). A. Scatter plots, the lower left quadrant shows living cells as FITC⁻/PI⁻; the upper right quadrant shows dead cells for the FITC⁺/PI⁺; the lower right quadrant shows early apoptotic cells, FITC⁺/PI⁻. B. Apoptotic rate. **Denotes $P < 0.01$ vs control and #, ###denotes $P < 0.05$ and < 0.01 vs OGD, respectively.

be used to mimic the neuroprotection generated by IPC. The possible mechanism is that IPC reduces the apoptosis and oxidative stress of the SH-SY5Y cells.

Currently, animals or cultured cells are used to mimic IPC processes in humans. Zhao et al. found that IPC with a series of mechanical interruptions of reperfusion significantly reduc-

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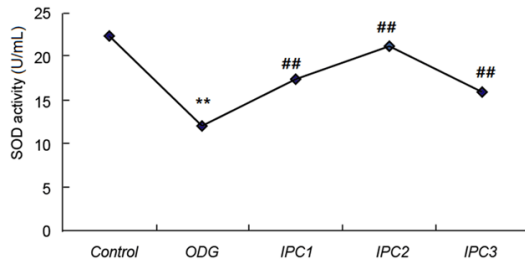


Figure 6. The changes of SOD activity in the SH-SY5Y cells after ischemia/reperfusion treatment (ODG) and 1 to 3 cycles of ischemic postconditioning (IPC1-3). **Denotes $P < 0.01$ vs control and ##denotes $P < 0.01$ vs OGD.

es ischemic damage after focal ischemia in rats [12]. They found that rapid postconditioning reduces the infarct size by 80%, 51%, and 17%, respectively, after a 15, 30, or 60 min common cerebral artery (CCA) occlusion combined with permanent double middle cerebral artery (MCA) occlusion. Since then, a number of studies also found that rapid ischemic postconditioning protects cerebral ischemic rats [8, 13, 14]. In vitro, IPC also shows a protective role. Studies found that IPC with OGD reduced neuronal death in non-neuronal cultures such as cortical cultures [13] and hippocampal slice cultures [14]. Although intensive studies have been carried out, the mechanism underlying neuroprotection by IPC has not been sufficiently elucidated.

To study the mechanism, animal experiments have many limitations, such as availability, cost, ethics, and uncontrolled variables. On the other hand, in vitro studies allow a better control of the experimental variables and are less costly. However, most of the in vitro studies used non-neuronal cultures, which cannot mimic the physiological response like neuronal cultures can, and it has been difficult to continuously culture neurons due to the culturing's low success rate and to natural aging. In this study, we chose SH-SY5Y cells to construct an in vitro model of IPC. The SH-SY5Y cell line is derived from human neuroblastoma and has is similar in its morphology and physiological and biochemical characteristics to neurons. It proliferates well in vitro and can be cultured continuously and stably. Therefore, it is the ideal choice for the in vitro study of the nervous system.

At the cellular level, hypoxia may be generated by culturing cells in low-glucose, hypoxia, hypox-

ia or low-glucose conditions. In this study, the incubator was filled with nitrogen, and glucose was removed from the medium to generate the hypoxic condition. Different concentrations of glucose cause varying degrees of tissue damage in hypoxia [15]. Elke et al. found that anoxia was not lethal to SH-SY5Y cells, and 98% of the cells were still viable after 32 h anoxia culture. However, significant changes occur when the OGD duration is ≥ 12 hours, and only 46% of the cells survive after 32 h [16]. We found that the damage to SH-SY5Y cells caused by anoxia or glucose deprivation is negligible (data not shown). The morphology and number of living SH-SY5Y cells changed significantly after the cells were exposed to OGD for 12 h. The cells were wrinkled and became round, suggesting that damage had occurred in the cells. Consistent with this, the survival rate of OGD-treated cells decreased and apoptosis increased significantly, further confirming that 12 h-ODG treatment can mimic ischemia/reperfusion to induce cellular injury in SH-SY5Y cells.

Furthermore, we used an alternative and repeated OGD/normal culture to allow the cells to uptake glucose and serum gradually after the ischemia/reperfusion treatment. Compared with the single normal/ODG culture method, such a culture scheme might reduce cell loss due to repeated washings with the PBS buffer and mimic the in vivo recovery process during blood flow after ischemia. As shown in **Figure 4**, after 2 or 3 cycles of such an alternative culture, the morphology of the SH-SY5Y cells became less altered. Consistent with the morphology observations, the survival rates in the IPC2 and IPC3 groups were higher compared with the cells before IPC. It is worth noting that the IPC1 treatment did not significantly improve the morphology or survival of the SH-SY5Y cells, suggesting that one cycle of IPC is inadequate to generate protection. Also, it would be interesting to know if an increase in the number of IPC cycles would increase the protection further.

The molecular mechanism underlying IPC-induced neuroprotection has not been fully elucidated yet. Following cerebral ischemia/reperfusion, apoptosis occurs in neurons [17]. One possibility is that IPC protects the brain from ischemia/reperfusion injury by inhibiting the expression of apoptotic molecules in the mitochondrial pathways and by activating endogenous protective molecules [7]. The results from

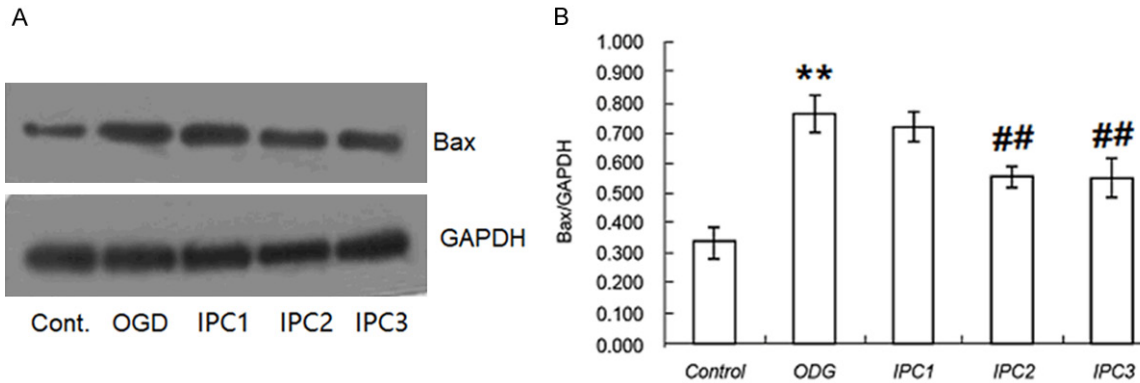


Figure 7. The expression of Bax in SH-SY5Y cells after ischemia/reperfusion treatment (ODG) and 1 to 3 cycles of ischemic postconditioning (IPC1-3). A. Representative Western blot. B. Relative expression level. **Denotes $P < 0.01$ vs control and ##denotes $P < 0.01$ vs OGD.

the animal experiments showed that multiple signaling pathways are involved in the anti-apoptotic mechanisms of IPC. For example, IPC was shown to upregulate the expression of Bcl-2 and downregulate the expression of cytochrome c released to the cytosol, the amount of Bax translocated to the mitochondria, and caspase-3 activity [18, 19]. In addition, Yuan et al. reported that IPC protects the brain from ischemia/reperfusion injury by attenuating endoplasmic reticulum stress-induced apoptosis through the PI3K-Akt pathway [20]. In this study, we found that there is increased apoptosis after ischemia/reperfusion treatment and decreased apoptosis after IPC2 and 3 treatments. Bax plays a central role in the mitochondria-dependent apoptotic pathway. In healthy cells, Bax is essentially cytosolic and inactive. Following a death signal, the protein is translocated to the outer mitochondrial membrane, where it promotes the permeabilization of the mitochondrial membrane that enhances the release of different apoptogenic factors [10]. We found that Bax was up-regulated after ischemia/reperfusion treatment and down-regulated after IPC2 and 3 treatments, suggesting that IPC reduces the apoptosis of SH-SY5Y cells from ischemia/reperfusion-induced injury via the Bax-related pathways.

IPC also protects the ischemic organ by blocking the overproduction of reactive oxygen species and reducing lipid peroxidation [4, 7]. It was shown that IPC treatments reduce oxidative stress by decreasing the expression and activity of SOD to protect the brains in rats [7, 18]. The level of SOD, as the enzyme for scavenging oxygen free radicals, can reflect the

damage from oxygen free radicals indirectly. In this study, increased SOD activity in SH-SY5Y cells following IPC treatment was observed, suggesting that lipid peroxidation and the generation of superoxide anions might be reduced.

The extremely short therapeutic window of rapid postconditioning, which spans a few seconds to minutes after reperfusion, may hinder its clinical translation. IPC performed hours to days after a stroke is regarded as delayed postconditioning [21]. Burda et al. found that when delayed postconditioning is conducted 2 days after reperfusion in rat models, there is about a 94% reduction in neuronal death in the hippocampus measured 7 days after global ischemia [22], a finding confirmed by other research groups [23-26]. Since delayed postconditioning can greatly improve the clinical potential of IPC, whether delayed postconditioning can provide neuroprotection in SH-SY5Y cells needs to be addressed in the future.

Conclusion

Our study demonstrates that 12 h-ODG treatment can mimic ischemia/reperfusion to generate injury in cultured SH-SY5Y cells and 2-3 cycles of IPC using the alternative and repeated culture method in normal and OGD media to generate protection for the cells. The possible mechanisms of IPC-generated protection include the reduction of apoptosis and oxidative stress. The in vitro model of IPC developed in this study would be useful for investigating the mechanisms of IPC-generated protection. Needless to say, further study is needed to optimize the parameters involved in the OGD-IPC procedures to facilitate its application in mechanistic studies.

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Disclosure of conflict of interest

None.

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