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·Original Article·

# Sulforaphane protects primary cultures of cortical neurons against injury induced by oxygen-glucose deprivation/reoxygenation *via* anti-apoptosis

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**Abstract: Objective** To determine whether sulforaphane (SFN) protects neurons against injury caused by oxygen-glucose deprivation/reoxygenation (OGD/R) and, if so, to investigate the possible mechanisms. **Methods** Primary cultures of neurons were prepared from the cerebral cortex of 1-day-old Sprague-Dawley rats. On days 5–6 *in vitro*, the neurons were exposed to OGD for 1 h, followed by reoxygenation for 24 h. Cells were treated with 0, 0.1, 0.2, 0.5, 1, 2.5, or 5 μmol/L SFN, with or without 10 μmol/L LY294002, a PI3K-specific inhibitor, during OGD/R (a total of 25 h). After 24-h reoxygenation, MTT was used to assess viability and injury was assessed by Hoechst 33258/propidium iodide (PI) staining; immunofluorescence staining and Western blot were performed to detect molecular events associated with apoptosis. **Results** The MTT assay showed that 1 μmol/L SFN significantly increased viability, and Hoechst 33258/PI staining showed that the numbers of injured neurons were reduced significantly in the SFN group. Furthermore, immunofluorescence staining and Western blot showed that SFN increased Bcl-2 and decreased cleaved caspase-3 levels. Moreover, LY294002 inhibited the phosphorylated-Akt expression evoked by SFN, decreased Bcl-2 expression and increased cleaved caspase-3 expression. **Conclusion** SFN protects neurons against injury from OGD/R and this effect may be partly associated with an anti-apoptosis pathway.

**Keywords:** sulforaphane; oxygen-glucose deprivation; apoptosis; neuroprotection

## 1 Introduction

Hypoxic-ischemic brain injury is one of the main causes of disability and death worldwide. The pathophysiology involves multiple biochemical mechanisms such as the generation of reactive oxygen species, glutamate-mediated excitotoxicity, inflammation and apoptosis[1]. One of the central mechanisms of hypoxic-ischemic brain injury is neuronal loss caused by apoptosis[2,3]. The Bcl-2 family and caspases play key roles in the apoptotic cascade[4]. A primary cell survival pathway, the PI3K/Akt pathway, has been shown to promote cell survival and inhibit apoptosis by phosphorylating and inhibiting apoptosis-induced proteins[5,6].

Sulforaphane (SFN), a naturally-occurring isothiocyanate, is found in cruciferous vegetables such as broccoli. It has been shown to be neuroprotective in models of hypoxic-ischemic brain injury[1,7,8]. SFN has various pharmacological actions, such as anti-inflammatory[9-11], antioxidant[12,13], anti-apoptotic[14], and anti-cancer[15,16] effects. Moreover, a previous study showed that dietary use of SFN can decrease aging-related degenerative changes[17]. These

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findings indicate that SFN could serve as a valid pharmacotherapy for the treatment of diseases in the central nervous system. Although anti-oxidation has been indicated as a possible mechanism underlying the neuroprotection of immature neurons by SFN, its anti-apoptotic effects have not been elucidated. The present study further explored whether SFN protects neurons against injury caused by oxygen-glucose deprivation/reoxygenation (OGD/R) and investigated possible mechanisms.

## 2 Materials and methods

**2.1 Primary culture of cortical neurons** All animal protocols were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University, China. Cortical neurons were isolated as described previously[18,19] with slight modifications. Briefly, samples of cerebral cortex were obtained from 1-day-old Sprague-Dawley rats and collected into cold D-Hanks solution. The tissues were minced and incubated in 0.25% trypsin (Beyotime, China) for 20 min at 37ºC. Enzymatic digestion was terminated by mixing Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The whole-cell suspension was filtered through 200-mesh nylon. The filtrate was centrifuged at 1 000 g for 8 min, then the cell sediment was resuspended in DMEM containing 10% FBS and 1% penicillin/streptomycin. The cells were plated onto poly-*L*-lysine-coated 24-well plates (Sigma; St. Louis, MO) at $4 \times 10^5$ cells/well. The cells were maintained at 37ºC in a humidified incubator (Thermo3111, Waltham, MA) containing 5% $CO_2$ and 95% air. After 24 h, the medium was changed to fresh neurobasal medium (Gibco) supplemented with 2% B27 (Gibco) and 1% penicillin/streptomycin, then refreshed every 3 days throughout the study. Under these conditions, this serum-free culture contained ~90% neurons as determined by NeuN and Hoechst 3258 staining. Experiments were performed at 5–6 days *in vitro*.

**2.2 OGD/R application** OGD was performed according to a previously described method[20,21]. In brief, the culture medium was removed and cells were washed twice with glucose-free DMEM (Gibco). To initiate OGD, glucose-free DMEM was added to cultures which were immediately transferred to a chamber (Thermo3131, Waltham, MA) with an atmosphere of 94% $N_2$, 1% $O_2$ and 5% $CO_2$ at 37ºC. OGD was terminated after 1 h by replacing the glucose-free DMEM with neurobasal medium supplemented with 2% B27 and the cultures were further incubated under 95% air and 5% $CO_2$ for 24 h at 37ºC. Cells in the control group were treated identically except that they were not exposed to OGD.

**2.3 Drug treatment** To determine the effects of SFN (Calbiochem, Cambridge, MA), cultured neurons were treated with 0.1, 0.2, 0.5, 1, 2.5, or 5 μmol/L SFN for 25 h during OGD/R. SFN was dissolved in dimethyl sulfoxide (DMSO) and the final concentration in the medium was 0.001%. LY294002 (10 μmol/L) was added to the neurons with or without SFN for 25 h during OGD/R. The control and OGD/R groups were given equal volumes of medium.

**2.4 Cell viability assay** Viability was assessed by the MTT (Sigma) assay. The assay is based on the ability of living cells to convert dissolved yellow MTT into insoluble purple formazan[22]. After OGD/R, MTT was added to a final concentration of 0.5 mg/mL and the cells were maintained in a humidified incubator under 5% $CO_2$ and 95% air for 4 h at 37ºC. After incubation, the medium was removed and 150 μL DMSO was added to dissolve the formazan product into a solution which was read at 490 nm using a microplate reader (Thermo1500, Waltham, MA). The absorbance of the formazan formed in control cells was taken as 100% viability.

**2.5 Hoechst and propidium iodide (PI) staining** Cell death was measured with a Hoechst 33258 and PI kit according to the manufacturer's instructions (Beyotime). Briefly, after OGD/R, cells cultured on coverslips were incubated with 10 μg/mL PI for 20 min at room temperature (RT). The cells were fixed in 4% paraformaldehyde for 20 min at RT, and rinsed three times in phosphate-buffered saline (PBS). Cells were incubated with Hoechst 33258 for 30 min at RT. After the cells were rinsed, images were captured using a light microscope (Olympus BX51, Tokyo, Japan). The dead/dying cells and total number of cells were counted in six high-power fields.

**2.6 Fluorescence immunocytochemistry** Fluorescence immunocytochemistry was performed on cells cultured on coverslips. The cells were washed twice and fixed in 4% paraformaldehyde for 20 min at RT, and treated with 3% $H_2O_2$ for 20 min at 37ºC to eliminate endogenous peroxidases. After washing, cells were blocked with normal goat serum for 30 min at 37ºC. Mouse anti-NeuN, rabbit anti-Bcl-2 or rabbit anti-cleaved caspase-3 was used as the primary antibody. Cells were incubated with primary antibody (anti-NeuN, 1:50, Millipore, Billerica, MA; anti-Bcl-2, 1:50, Santa Cruz Biotechndogy, Santa Cruz, CA; anti-cleaved caspase-3, 1:50, Cell Signaling Technology, Boston, MA) at 4ºC overnight. After washing, cells were incubated with TRITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG (1:100, Santa Cruz) for 20 min at 37ºC. Nuclei were stained with Hoechst 33258 for 30 min at RT. Images were captured using the Olympus microscope. The positive cells and total number of neurons were counted in six high-power fields.

**2.7 Western blot** Cells were collected and centrifuged at 12 000 g for 10 min at 4ºC. The supernatants were collected and total protein measured using a BCA protein assay kit (Beyotime). Total cellular proteins were electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel, and then transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h at RT and incubated with primary antibody (anti-Bcl-2, 1:200, Santa Cruz; anti-cleaved caspase-3, 1:200, Cell Signaling Technology; anti-p-Akt and-Akt, 1:1 000, Cell Signaling Technology; anti-β-actin, 1:1 000, Santa Cruz) at 4ºC overnight. After washing with PBS, membranes were incubated with goat anti-rabbit (1:1 000, Santa Cruz) as secondary antibody for 1 h at RT. Blots were developed with an ECL Western blot detection kit (Beyotime), then scanned with an imaging densitometer (Bio-Rad, Hercules, CA) and quantified by Quantity One 1-D analysis software. β-actin was used as an internal control.

**2.8 Statistical analysis** All data are presented as mean ± SEM. Statistical differences among the various groups were assessed with one-way ANOVA followed by a *post hoc* test. Comparisons between two groups were assessed by the unpaired *t*-test. Data were analyzed using SPSS 17.0 software. $P$ <0.05 was considered as statistically significant.

## 3 Results

**3.1 SFN increases cell viability on exposure to OGD/R** NeuN and Hoechst 3258 staining showed that the cultured cells were ~90% neurons (Fig. 1). MTT assays showed that maximal neuroprotection occurred at 1 μmol/L SFN (Fig. 2A), so we chose this dose for subsequent experiments. Viability was markedly decreased in the OGD/R group (37.5 ± 9.4%, $P$ <0.01 *vs* control 100%). SFN increased the viability (54.6 ± 8.8%, $P$ <0.01 *vs* OGD/R; $P$ <0.05 *vs* SFN+LY 40.8 ± 7.9%; $P$ <0.01 *vs* LY 36.9 ± 7.5%) (Fig. 2B). We found that LY294002 alone had no adverse or protective effect ($P$ >0.05 *vs* OGD/R). Therefore, LY294002 alone was not used in subsequent experiments.

**3.2 SFN decreases cell injury caused by OGD/R** Cell death was assessed by Hoechst33258/PI staining; Hoechst

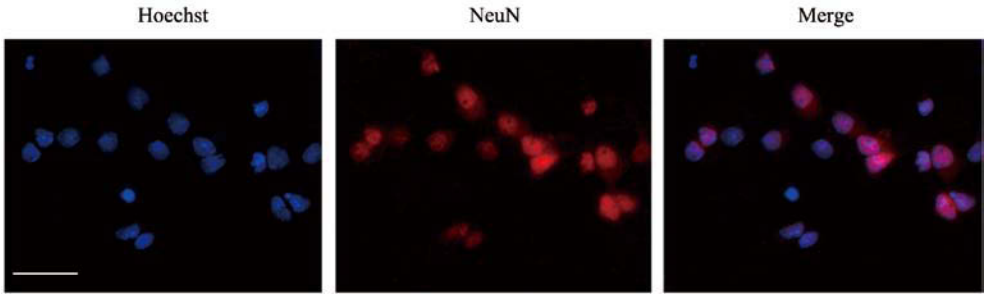


**Fig. 1. Photomicrographs of immunofluorescence in rat primary cortical neurons. Neurons were labeled with anti-NeuN (red) under normal conditions. The nuclei were stained with Hoechst 33258 (blue). Scale bar, 50 μm.**

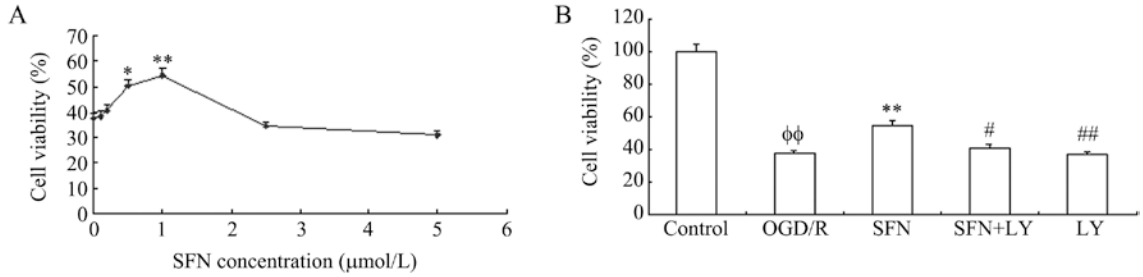


**Fig. 2. Effects of sulforaphane (SFN) on cell viability after OGD for 1 h and reoxygenation for 24 h. A: Viability of neurons treated with 0, 0.1, 0.2, 0.5, 1, 2.5, or 5 μmol/L SFN for 25 h during OGD/R. $^{*}P$ <0.05, $^{**}P$ < 0.01 *versus* 0 μmol/L SFN. B: Viability in control, OGD/R alone, OGD/R+SFN, OGD/R+SFN+LY, and OGD/R+LY groups. Data are normalized to control (100%). $^{\phi\phi}P$ <0.01 *versus* control; $^{**}P$ <0.01 *versus* OGD/R alone; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *versus* OGD/R+SFN; $n$ = 10.**

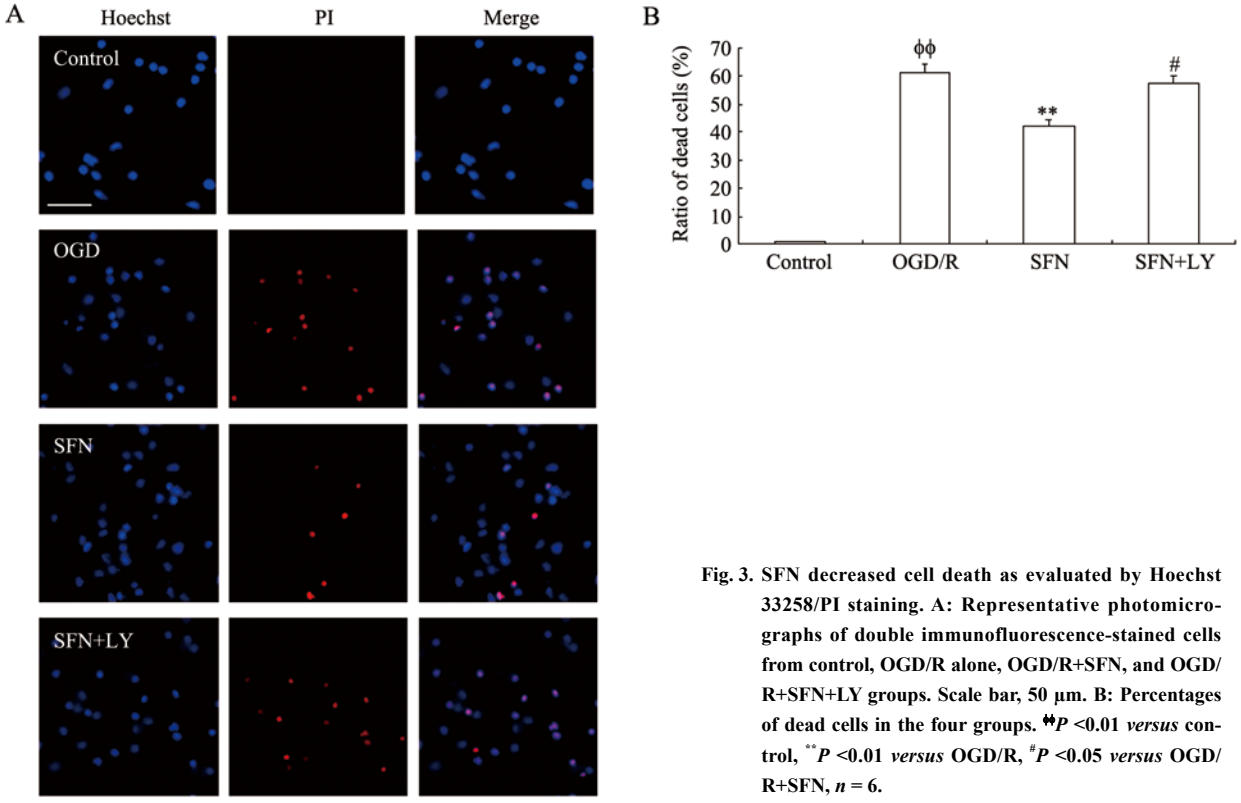


**Fig. 3. SFN decreased cell death as evaluated by Hoechst 33258/PI staining. A: Representative photomicrographs of double immunofluorescence-stained cells from control, OGD/R alone, OGD/R+SFN, and OGD/R+SFN+LY groups. Scale bar, 50 μm. B: Percentages of dead cells in the four groups. $^{\phi\phi}P$ <0.01 *versus* control, $^{**}P$ <0.01 *versus* OGD/R, $^{\#}P$ <0.05 *versus* OGD/R+SFN, $n$ = 6.**

33258 stained all nuclei, and PI-positive cells with condensed blue nuclei were considered to be dead/dying cells (Fig. 3A). The percentage of dead cells was considerably increased by exposure to OGD/R (61.5 ± 9.3%, $P$ <0.01 *vs* control 1.0 ± 0.5%), while SFN decreased the percentage (42.3 ± 7.8%, $P$ <0.01 *vs* OGD/R, $P$ <0.05 *vs* SFN+LY 57.7 ± 8.7%) (Fig. 3B).

**3.3 SFN decreases cleaved caspase-3 and increases Bcl-2 levels after OGD/R** We next investigated the mechanisms by which SFN acts against apoptosis, by analyzing the expression levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein cleaved caspase-3. After 24-h reoxygenation, Bcl-2 was increased in the SFN group according to the immunofluorescence staining (Fig. 4A, C;

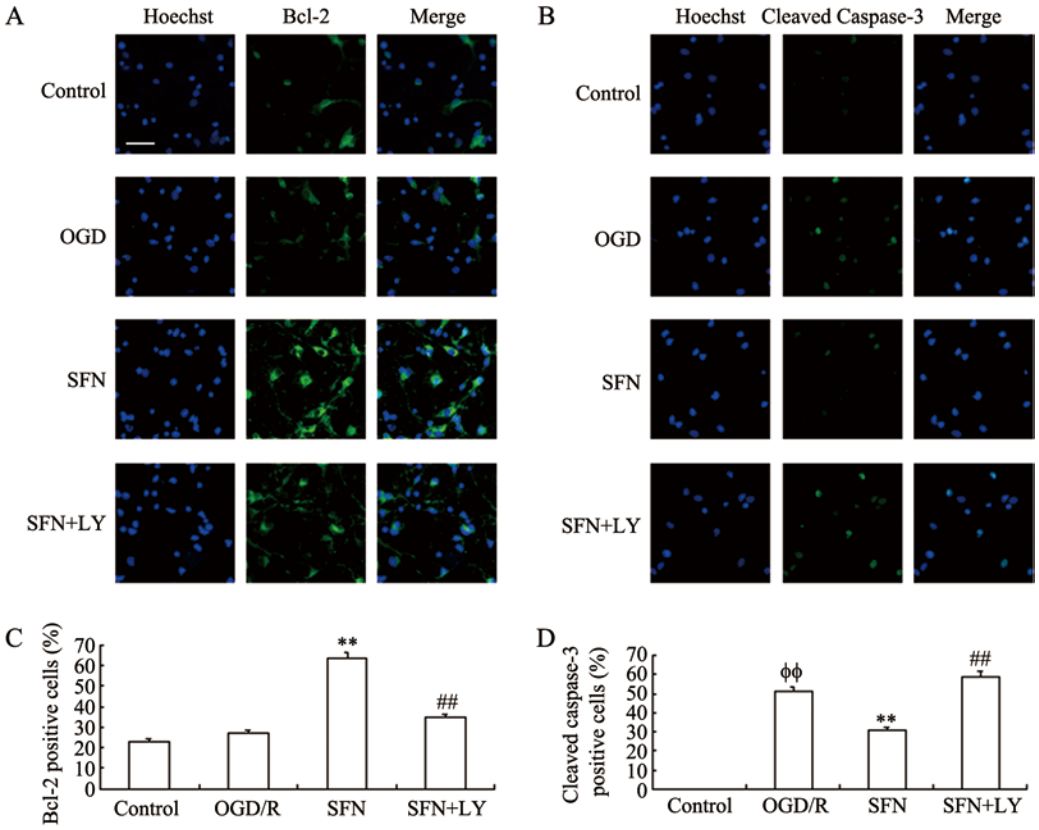


**Fig. 4. Effects of SFN on the expression of Bcl-2 and cleaved caspase-3 after OGD/R, as detected by fluorescence immunocytochemistry staining. A and B: Photomicrographs of representative double immunofluorescence-stained cells from control, OGD/R alone, OGD/R+SFN, and OGD/R+SFN+LY groups. Scale bar, 50 μm. C and D: Percentages of Bcl-2-positive (C) and cleaved caspase-3-positive (D) cells in the four groups. $^{\phi\phi}P$ <0.01 *versus* control, $^{**}P$ <0.01 *versus* OGD/R, $^{\#\#}P$ <0.01 *versus* OGD/R+SFN, $n$ = 6.**

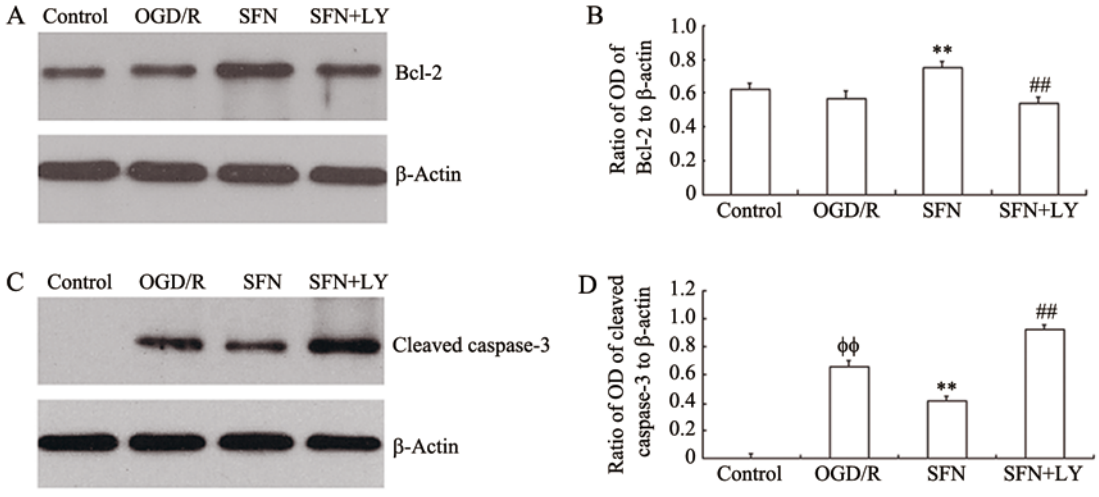


**Fig. 5. Effects of SFN on the levels of Bcl-2 and cleaved caspase-3 after OGD/R detected by Western blot. A and C: Representative Western blots of Bcl-2 and cleaved caspase-3 proteins. B and D: Quantification of Western blots for the Bcl-2 and cleaved caspase-3 compared with β-actin in control, OGD/R alone, OGD/R+SFN, and OGD/R+SFN+LY groups. Data are expressed as mean optical density (OD) ratio of Bcl-2 and cleaved caspase-3 to β-actin. $^{\phi\phi}P$ <0.01 *versus* control, $^{**}P$ <0.01 *versus* OGD/R, $^{\#\#}P$ <0.01 *versus* OGD/R+SFN, $n$ = 6.**

63.3 ± 7.6%, $P$ <0.01 *vs* OGD/R 26.9 ± 8.5%; $P$ < 0.01 *vs* SFN+LY 34.6 ± 7.9%) and Western blot (Fig. 5A, B; optical density, 0.75 ± 0.08, $P$ <0.01 *vs* OGD/R 0.57 ± 0.07; $P$ <0.01 *vs* SFN+LY 0.54 ± 0.09). Moreover, the level of cleaved caspase-3 increased remarkably in the OGD/R group, but SFN decreased it according to immunofluorescence staining (Fig. 4B, D; 31.2 ± 8.3%, $P$ <0.01 *vs* OGD/R 51.1 ± 9.6%; $P$ <0.01 *vs* SFN+LY 59.1 ± 7.8%) and Western blot (Fig. 5C, D; optical density, 0.41 ± 0.06, $P$ <0.01 *vs* OGD/R 0.67 ± 0.09; $P$ <0.01 *vs* SFN+LY 0.92 ± 0.04).

**3.4 Effect of PI3K/Akt pathway in SFN-mediated anti-apoptosis** We then explored whether SFN prevents apoptosis by activating the PI3K/Akt pathway. The MTT assay showed that viability decreased in the group treated with LY294002 and SFN (Fig. 2B; 40.8 ± 7.9%, $P$ <0.05 *vs* SFN 54.6 ± 8.8%). The MTT results were further confirmed by Hoechst 33258/PI staining (Fig. 3A, B), which showed that LY294002 with SFN increased the number of dead cells (57.7 ± 8.7%, $P$ <0.05 *vs* SFN 42.3 ± 7.8%). We also found that LY294002 with SFN decreased the expression of Bcl-2 and increased the expression of cleaved caspase-3 compared with the SFN group by immunofluorescence staining (Fig. 4) and Western blot analysis (Fig. 5). We then assessed the signaling molecules Akt and p-Akt by Western blot, and found that p-Akt was increased in the SFN group (optical density, 0.70 ± 0.10, $P$ <0.01 *vs* OGD/R 0.50 ± 0.07; $P$ <0.01 *vs* SFN+LY 0.35 ± 0.09). However, p-Akt was significantly inhibited by LY294002. In addition, we found that the total Akt expression did not differ among the groups (Control 0.86 ± 0.11, OGD/R 0.99 ± 0.05, SFN 0.92 ± 0.08, SFN+LY 0.88 ± 0.10; $P$ >0.05) (Fig. 6). These observations indicated that SFN prevented hypoxic-ischemic brain injury and this neuroprotective effect could be related to the activation of the PI3K/Akt pathway.

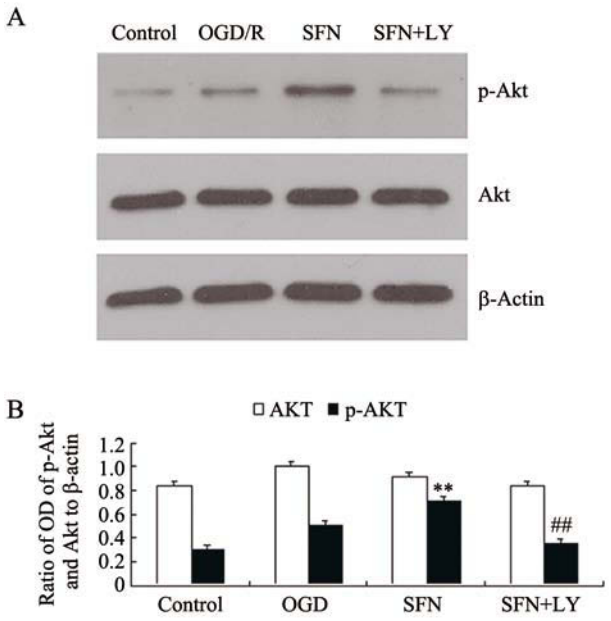


**Fig. 6. Effects of SFN on the PI3K/Akt pathway after OGD/R. A: Representative Western blots of Akt and p-Akt proteins. B: Quantification of Western blots for Akt and p-Akt compared with β-actin in control, OGD/R alone, OGD/R+SFN, and OGD/R+SFN+LY groups. Data are expressed as mean optical density (OD) ratio of p-Akt and Akt to β-actin. $^{**}P$ <0.01 *versus* OGD/R, $^{\#\#}P$ <0.01 *versus* OGD/R+SFN, $n$ = 6.**

## 4 Discussion

In the present study, we found that treatment with SFN was neuroprotective in OGD/R and explored its mechanism of action. SFN at 1 μmol/L significantly enhanced cell viability 24 h after OGD. Meanwhile, SFN remarkably increased Bcl-2 and p-Akt and decreased cleaved caspase-3 levels compared with the OGD/R group. We demonstrated that SFN protected neurons from hypoxic-ischemic injury and this effect was at least partly associated with anti-apoptosis.

SFN is an isothiocyanate present in cruciferous vegetables. It has been studied mainly because of its anti-carcinogenic and anti-oxidative effects. Recent studies *in vivo* and *in vitro* have demonstrated that SFN has neuroprotective effects against cerebral ischemic injury. It protects the brain against hypoxic-ischemic injury by inducing Nrf2-dependent phase 2 enzyme[8] and the blood-brain barrier after brain injury by increasing the expression of Nrf2-driven genes *in vivo*[23]. It also protects immature hippocampal neurons by activating the ARE/Nrf2 pathway *in vitro*[13]. However, the mechanism of SFN-mediated protection against ischemic brain injury needs further clarification.

To further clarify the relationship between apoptosis

and the effect of SFN in primary cultured cortical neurons exposed to OGD/R, we tested the effect of SFN on molecular events related to apoptosis. Bcl-2 family members are the arbiters of the mitochondrial apoptotic pathway and decide whether a cell lives or dies[24]. This family includes anti-apoptotic and pro-apoptotic genes[25]. Bcl-2, an inhibitor of apoptosis, prevents neuronal apoptosis by blocking the destruction of mitochondria and the subsequent release of cytochrome *c* and caspase activation[26]. Under the condition of hypoxic-ischemic brain injury, the outer membrane of mitochondria becomes permeable[27], which leads to the release of cytochrome *c* and further promotes the activation of caspase-3, a key mediator of apoptosis[28-31]. It is activated by cleavage, and active caspase-3 leads to DNA fragmentation and eventually apoptosis[32,33]. We found that 1 μmol/L SFN increased the level of anti-apoptotic Bcl-2 and decreased the level of cleaved caspase-3. These results suggested that one of the mechanisms of action of SFN against injury by OGD/R could be partly related to its anti-apoptotic effects.

It is well known that activation of the PI3K/Akt pathway is important for neuronal survival. Recent studies found that the PI3K/Akt pathway is involved in the upregulation of HIF-1α which plays important roles in neuroprotection after hypoxic-ischemic injury[34]. Decreased Akt activity caused by ischemia or hypoxia can lead to neuronal death[35]. In contrast, increased Akt activity can contribute to neuronal survival. Therefore, we investigated the role of the PI3K/Akt pathway in SFN-mediated anti-apoptosis after OGD/R. p-Akt is necessary for Akt activation. To further explore the neuroprotective mechanism of SFN, we assessed the expression of p-Akt. The results showed that SFN significantly increased the expression of p-Akt, but this was significantly inhibited by LY294002. These results suggest that SFN provides neuronal protection by activating the PI3K/Akt pathway.

In summary, our study demonstrated that SFN was neuroprotective against injury caused by OGD/R and this effect could be mediated by anti-apoptosis. These results suggest that SFN may be a promising agent against hypoxic-ischemic brain injury.

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