Shenfu Injection protects PC12 cells from oxygen-glucose deprivation by the NOS/NO pathway

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Abstract. Objective:To study whether the protective effect of Shenfu injection on PC12 cells damaged by Oxygen-glucose deprivation (OGD) involves NOS/NO pathway. Methods: PC12 cells co-incubated with a final concentration of 20.00mmol/L Na2S2O4 for 24 hours was used as an OGD induced damaged in vitro model of vascular dementia (VaD) . 50.00 μL/mL, 75.00 μL/mL, 100.00 μL/mL, 125.00 μL/mL, 150.00 μL/mL, 175.00μL/mL and 200.00μL/mL of SFI were co-incubated with 20.00 mmol•L-1 Na2S2O4-induced PC12 cells for 24 hour, 48 hour and 72 hour, respectively. Cell viability was determined by MTT assay to determine the appropriate concentration and timing of SFI. Griess method was used to detect NO content in the cell culture supernatant of the control group, OGD model group, 125.00 μL/mL, 150.00 μL/mL,and 175.00 μL/mL SFI group. QPCR was used to detect the expressions of iNOS and nNOS mRNA , and western blot was used to detect the protein expressions of iNOS and nNOS in these groups. Result: Compared with the OGD group, 50.00 μL/mL, 75.00 μL/mL, 100.00 μL/mL, 125.00 μL/mL, 150.00 μL/mL, 175.00 μL/mL and 200.00 μL/mL SFI can attenuate the inhibitory effect of Na2S2O4 on PC12 cells in a time and dose-dependent manner (P<0.05). Among them, the survival rate of PC12 cells in the 150.00μL/mL dose group was the highest, reaching 79.750 ±0.230. For the subsequent experiments, 125.00μL/mL, 150.00μL/mL, and 175μL/mL doses of SFI were elected to co-incubated with Na2S2O4-induced damaged PC12 cells for 24 hours. Compared with the control group, NO level in the supernatant of PC12 cells induced by Na2S2O4 were significantly increased. SFI decreased NO level in culture supernatant of PC12 cell induced by Na2S2O4 in a dose-dependent manner. Compared with the control group, the mRNA and protein expressions of iNOS and nNOS in PC12 cells was significantly increased after 24 hour induction by Na2S2O4 (P<0.05). Compared with the OGD group, the mRNA and protein expressions of iNOS and nNOS in the 125μL/mL, 150μL/mL and 175μL/mL SFI dose groups were significantly lower (P<0.05). Conclusion: These findings suggest that the protective effect of SFI on PC12 cells damaged by Na2S2O4 may be related to its down-regulation of iNOS and nNOS gene and protein expression, thereby reducing the excessive production of NO in PC12 cells.

Keywords: Vascular dementia; Shenfu injection; Nitric oxide.

1. Introduction

Vascular dementia (VaD) is a brain dysfunction caused by various ischemic, hemorrhagic cerebrovascular diseases and acute or chronic hypoxic cerebrovascular diseases. Under the cerebral ischemia and/or hypoxia, nerve cells often undergo necrosis, leading to cognitive dysfunction in the body[1]. During brain ischemia and/or hypoxia, it exists a local or systemic pathological physiological changes such as oxidative stress, inflammatory responses, and apoptosis, triggering multiple signaling pathways in the brain nervous system. Whether it is long-term chronic cerebral ischemia and hypoxia or repeated strokes, they can all damage the structure and function of the brain nervous system to varying degrees[2].

Nitric oxide (NO), as the most important endothelially-derived vasodilator factor, is closely associated with the neurovascular unit. Nitric oxide (NO) is produced by nitric oxide synthase (NOS) from L-arginine. NOS has three subtypes: neuronal nNOS, endothelial cell eNOS and inducible iNOS[3].nNOS exists in the form of dimers in nerve cells and is influenced by Ca2+; while iNOS is not affected by Ca2+, and it is mainly expressed in astrocytes, macrophages and nerve cells[3]. When brain subjected to ischemia and hypoxia, the expressions of nNOS and iNOS in brain nerve cells significantly increase, which produces a large amount of nitric oxide (NO) to induce neurotoxicity and leads to apoptosis of nerve cells in brain[4]. The mRNA expression of iNOS in brain tissue significantly increases with persistent severe ischemia and hypoxia, and thereby producing excessive toxic substances such as nitric oxide (NO)[5]. nNOS usually produces a small amount of nitric oxide (NO), however, cerebral ischemia and hypoxia produced a large amount of excitatory amino acids which activates the N-methyl-D-aspartate receptor to result a significant increase in intracellular Ca2+, then nNOS is activated to generate and release a large amount of nitric oxide (NO)[3]. These excessive nitric oxide (NO) damages the neural tissue, thereby triggering the neurodegenerative diseases such as central nervous inflammation, dementia and Parkinson's disease.

Shenfu injection(SFI) derived from Shenfu Decoction, its main components are ginsenosides and aconitine-like alkaloids[6]. In recent years, Shenfu Injection has become increasingly widespread in clinic. It is effectively used in the treatment of vascular diseases, such as heart failure, arrhythmia, shock, ischemia-reperfusion injury(IRI), myocardial infarction, thromboangiitis obliterans (TAO), thrombosis of deep vein in the lower extremities, etc.[7]. Further studies have shown that SFI show a good therapeutic effect on the intellectual impairments in mild to moderate vascular dementia (VaD), such as learning ability deficiency, memory decline and judgment impairment caused by long-term chronic cerebral ischemia or post-cerebral stroke, cerebral infarction, and ischemic hypoxic encephalopathy, etc. Up to now, research progresses in VaD treatment with SFI mainly focuses on clinical case reports and apoptotic signaling pathways[7-10].

PC12 cells are a commonly used neural cell line, mainly extracted from the adrenal chromaffin cells of the brown rat [11]. Usually, PC12 cells are employed to make a in vitro models of Parkinson's disease and Alzheimer's disease, etc[12]. So far, there have been few reports on using PC12 cells as an in vitro model for VaD. In this study, using oxygen-glucose deprivation (OGD) induced PC12 cells as an in vitro model of VaD, we firstly investigated whether the treatment mechanism of SFI for VaD involves the NOS/NO signaling pathway, it provides an experimental and theoretical basis for the clinical application of traditional Chinese medicine in the prevention and treatment of VaD.

2. Materials and Methods

2.1 Drugs and Reagents

Shenfu Injection is produced by Sanjiu Pharmaceutical Co., Ltd. in China ; Na2S2O4 is from Sigma Company in the United States; MTT, DMSO, DMEM,Tris, 0.25% trypsin are from Beijing Sollevab Technology Co., Ltd.; Fetal bovine serum is from Wuhan Purity Biotechnology Co., Ltd.; GoScriptTM Reverse Transcription Mix is produced by Promega Company; TB GreenTM Premix Ex TaqTM II is a product of TaKaRa Company; goat anti-rabbit IgG (H+L) is from Beijing Zhongshan Biotechnology Co., Ltd.; rabbit anti-nNOS is a product of Cell Signaling Company; rabbit anti-iNOS is provided by the United States Santa Cruz Company; rabbit anti-GAPDH is provided by the Origene Company in United States .

1.2 Cell Culture and drug treatments

PC12 cells were provided by the Shanghai Cell Bank of the Chinese Academy of Sciences. PC12 cells were cultured in a high-glucose DMEM medium supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100mg/mL streptomycin in a humidified atmosphere under 5% CO2 at 37℃. The cell passage was within 10 passages from when the cell line was purchased. For all

experiments, PC12 cells were grown to 80–85% confluence, and then, stimulated with 20.00 mM Na2S2O4 for 24 h to induce its oxygen-glucose deprivation (OGD) as an in vitro model of VaD. Subsequently, different concentrations ((50.0, 75.0, 100.0, 125.0, 150.0, 175.0, 200.0 μL/mL) of SFI were added to each well for another 24 h,, 48 h, 72 h and phosphate-buffered saline (PBS) was used as vehicle control in all experiments.

2.2 Cell viability assay

Cell viability was assessed by a modified MTT assayas described in our previous study[13]. Cells (4×104 cells/mL) were seeded in 96-well plates and incubated for 24 h. Then, the cells were exposed to 20.00 mM Na2S2O4 or PBS or 20.00 mM Na2S2O4 together with different concentrations of SFI for another 24 h, 48 h, and 72 h. Thereafter, 10 μL of 5 mg/mL MTT in PBS was added to each well, and cells were incubated for a further 4 h at 37°C. The medium was then replaced by 100 μL of DMSO to dissolve the formed precipitate. The optical density was measured in a microplate reader (Model 680, Bio-Rad, USA) at 490 nm. Viability (%) = (OD of experimental group－OD of blank control group) / (OD of control group－OD of blank control group) × 100%.

2.3 Measurement of NO Formation

NO formation was estimated by measuring the stable endproduct of NO in aqueous solutions, nitrite (NO2-), by using Griess reagent (0.1% N-(1-naphthyl)- ethylenediamine and 1% sulfanilamide in H3PO4) [31] . The nitrite concentration was determined using a curve calibrated on sodium nitrite standard.

2.4 Quantitative PCR

RNA was extracted from cell samples using TRIzol reagent, and the synthesis of complementary DNA (cDNA) for qPCR analysis was performed using a kit with subsequent melting curve analysis, according to the manufacturer's protocol. The PCR reaction mixture (forward primer, 0.4 μL; reverse primer, 0.4 μL; cDNA, 2 μL; ddH2O, 7 μL; CXR, 0.2 μL; GoTaq qPCR Master Mix, 2×, 10 μL) was prepared using GoTaq®qPCR Master Mix.

The PCR conditions were as follows: pre-denaturation at 95 ℃ for 1 min, denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 20 s, and extension at 72 ℃ for 1 min. Relative gene expression was determined by the 2−ΔΔCt method (where Ct = threshold cycle) using β-actin as a reference gene. Quantitative real-time PCR primer sequences were as follows (Table 1):

Tab1. The primer sepuences of nNOS and iNOS for RT-qPCR

Genes	Primer sepuences	PCR product (bp)
nNOS	Sense ： 5’-GCACAGAAGTGAGGGTATGC-3’ Antisense：5’-ACAAGGTCCGATTCAACAGC-3’	77 bp
iNOS	Sense ： 5’-CAGTATTATGGCTCCTTCAA-3’ Antisense : 5’-TTCCTGTTGTTTCTATTTCCT-3’	88 bp
GAPDH	Sense ： 5’-TGCACCACCAACTGCTTAG-3’ Antisense ： 5’-GATGCAGGGATGATGTTC-3’	250 bp

2.5 Western blot analysis

Proteins from cells subjected to every treatment were extracted in a lysis buffer. After lysis on ice for 30 min, cell lysates were then clarified by centrifugation at 12,000 rpm at 4°C for 10 min. Protein concentrations were determined by BCA assay. Immunoblot analysis of protein expressions of nNOS, iNOS and GAPDH was performed as described in our previous report[14]. Briefly, 30 μg of protein extracts were separated by 12% SDS–polyacrylamide gels; then, proteins were transferred onto PVDF membranes that were purchased from Millipore (Massachusetts, USA). The membranes were blocked with 5% nonfat milk powder in Tris-buffered saline/0.1% Tween-20 (TBST) for 2 h at room temperature and then incubated overnight with the primary antibody

(nNOS,1:1000; iNOS,1:1000; GAPTH, 1:2000) at 4°C and anti-rabbit secondary antibody (1:5000) for 2 h at room temperature. After washing thrice, the immunoblots were detected by enhanced chemiluminescence (ECL) detection reagent. The relative band intensity of each protein was normalized for GAPDH and measured using microscope image-analysis software (Image-Pro Plus, U.S.A.).

2.6 Statistical analysis

Values were expressed as mean±SD, analyzed using one-way analysis of variance(ANOVA) and Student's t-tests in the program SPSS19.0(USA). P-values of less than 0.05 were considered to be significant.

3. Results

3.1 Effect of Shenfu injection on the viability of PC12 cells induced by Oxygen-Glycogen Deprivation (OGD)

As shown in Table 2, after incubated 24, 48, 72h with 20mM Na2S2O4, PC12 cells viability in OGD groups showed a lower survival rate compared to control groups, PC12 cells activities in SFI treatment groups were all significantly increased compared to that in the OGD groups ($P < 0.05$). Because the highest survival rate was displayed in PC12 cells induced by 150 μL/mL of SFI and 20mM Na2S2O4 after co-incubation for 24 hours, the final concentration of SFI was set at 125.00 μL/mL, 150.00 μL/mL, and 175.00 μL/mL, and dedicated incubation time set at 24 hours in the following experiments.

Tab.2 Cell survival rates of PC12 cells after co-incubation with different concentrations of SFI and 20mM Na2S2O4 for 24 , 48 and 72 hours($\bar{x} \pm s$,n=6)

Group	24 hour	48 hour	72 hour
Control group	100.230 ± 0.980	99.520 ± 0.670	99.480 ± 1.050
OGD group	25.010 ± 0.110 ###	1.040 ± 0.990 ###	0.800 ± 0.010 ###
50.0 μL/mL group	27.440 ± 0.130 ***	1.240 ± 0.140 *	1.080 ± 0.070 ***
75.0 μL/mL group	45.670 ± 0.160 ***	1.720 ± 0.050 ***	1.200 ± 0.040 ***
100.0μL/mL group	46.490 ± 0.760 ***	2.380 ± 0.700 ***	1.270 ±0.020 ***
125.0 μL/mL group	59.810 ± 0.280 ***	2.760 ± 0.080 ***	1.490 ± 0.030 ***
150.0 μL/mL group	79.750 ± 0.230 ***	3.390 ± 0.040 ***	1.880 ± 0.020 ***
175.0 μL/mL group	57.410 ± 0.370 ***	2.560 ± 0.120 ***	1.300 ± 0.030 ***
200.0 μL/mL group	49.390 ± 0.240***	2.310 ± 0.100 ***	1.040 ± 0.060 ***

P<0.001, compare to control; *P<0.05; ***P<0.001 compare to OGD group.

3.2 Effect of SFI on nitric oxide (NO) level in the supernatant of PC12 cells subjected to oxygen-glucose deprivation (OGD)

After PC12 cells induced by 20mM Na2S2O4 for 24 hours, nitric oxide (NO) level in the cell culture supernatant of OGD group was significantly higher than that in the control group ($P < 0.001$). All doses of SFI significantly reduced the level of nitric oxide (NO) in the culture supernatant of PC12 cells subjected to oxygen-glucose deprivation (OGD) injury ($P < 0.01$) (see Fig.1).

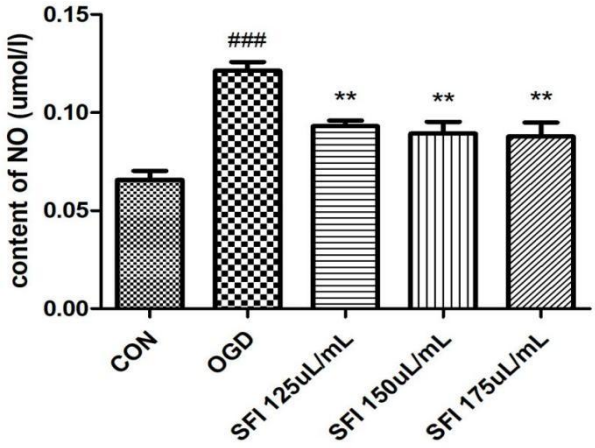

Fig.1. Effect of SFI on the supernatant NO level in PC12 cell in control group, OGD group and 125μL/mL, 150μL/mL, and 175μL/mL SFI groups ($\bar{x} \pm s$， n=6).
P<0.001, compare to control；**P<0.01 compare to OGD group.

3.3 Effect of SFI on the expression of nNOS gene and protein in PC12 cells subjected to oxygen-glucose deprivation (OGD) injury

The expression of nNOS gene and protein in PC12 cells of OGD group was significantly higher than that of the control group (P < 0.001). Compared with the OGD group, the expressions of nNOS gene and protein in PC12 cells in all SFI groups were significantly lower (P < 0.01) (see Figure 2).

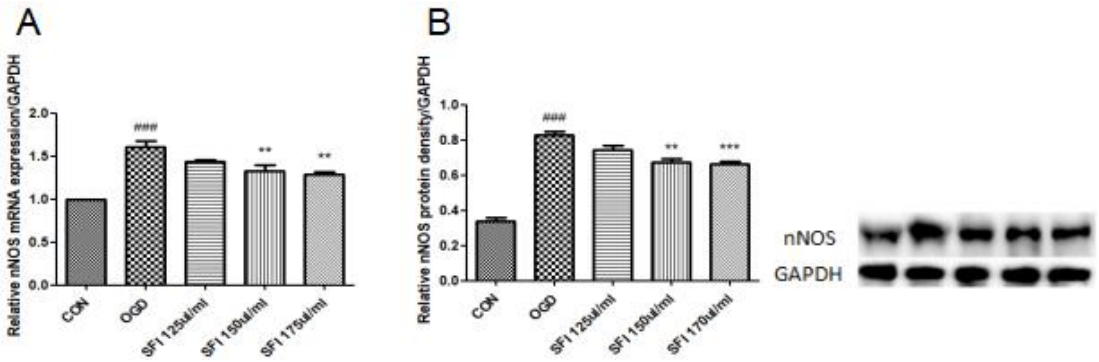

Fig.2. Effect of SFI on the nNOS gene and protein expressions of PC12 cells in control group, OGD group and 125μL/mL, 150μL/mL, and 175μL/mL SFI groups ($\bar{x} \pm s$,n=3).
(A) Effect of SFI on the nNOS gene expression in OGD damaged PC12 cells was evaluated by QPCR method. (B) Effect of SFI on the nNOS protein expression in OGD damaged PC12 cells was assessed by western blotting method.
P<0.001, compare to control；**P<0.01，***P<0.001 compare to OGD group.

3.4 Effect of SFI on the expressions of iNOS gene and protein in PC12 cells subjected to OGD treatment

Compared with the control group, the expression of iNOS gene and protein in PC12 cells of the OGD group was significantly increased (P < 0.001); compared with the OGD group, the expression levels of iNOS gene and protein in OGD-induced injured PC12 cells of all SFI dose groups were significantly decreased (P < 0.05). Among them, the expression level of iNOS gene and protein in PC12 cells of the 150 μL/mL SFI group was the lowest (see Figure 3).

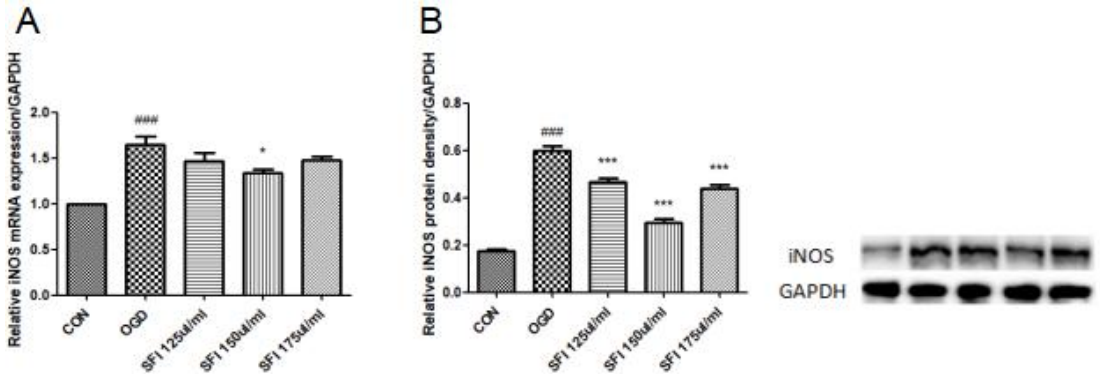

Fig.3. Effect of SFI on the iNOS gene and protein expressions of PC12 cells in control group, OGD group and 125μL/mL, 150μL/mL, and 175μL/mL SFI groups.($\bar{x} \pm s$,n=3). (A)Effect of SFI on the mRNA expression of iNOS in OGD damaged PC12 cells was evaluated by QPCR method. (B) Effect of SFI on the protein expression of iNOS in oxygen-glucose deprivation damaged PC12 cells was assessed by western blotting.method .

$^{###}$P<0.001, compare to control; *P<0.05, ***P<0.001 compare to OGD group.

4. Discussion

PC12 cells are derived from rat adrenal pheochromocytomas and are a type of neuronal precursor cell line with differentiation potential and sympathetic neuron-like characteristics[15, 16]. This type of cell is commonly used in studies such as neuropharmacology and neurotoxicology to make in vitro models [17-19].The oxygen in the cell culture medium can be rapidly and completely exhausted but the cell membranes not damaged by disodium sulfite (Na2S2O4)[20], and the combined use of Na2S2O4 and low-sugar culture medium can perfectly simulate the ischemic environment of cells in vivo[21, 22], thus inducing damage to PC12 cells using Na2S2O4 as an OGD model is currently the most widely used in vitro cell model for studying cerebral ischemia and hypoxia[23-25].

Using OGD induced PC12 cells as an in vitro VaD model, Dong et al. found that retinoic acid can inhibit the PC12 cells apoptosis resulted from OGD by increasing the expression of anti-apoptotic proteins[24]. Zhao et al found that OGD damaged PC12 cells,whereas ligustrazine inhibited the imbalance of brain-derived neurotrophic factor (BDNF), monocyte chemoattractant protein 1 (MCP1) and homocysteine, thereby reducing PC12 cells damage from OGD[26]. Using OGD/R induced PC12 cells as an in vitro ischemic-hypoxic model, Qi et al found that the main cause of tissue damage was the significant increase in ROS during I/R. Transcinnamaldehyde has a good therapeutic effect on ischemic injury because it can reduce the generation of reactive oxygen species to increase the cell vitality[25]. At present, there are no reports about the effect of SFI on OGD induced PC12 cells. In this study, Na2S2O4 induced PC12 cells was used as OGD model. The protective effect of SFI on OGD-induced PC12 cells was investigated, and the mechanism of its treatment for VaD was also studied. It was found that when PC12 cells co-incubated with various doses of SFI and Na2S2O4 for 24 hours, 48 hours, and 72 hours, SFI could promote the growth of PC12 cells damaged by OGD. It was confirmed that SFI has a protective effect on PC12 cells injuried by OGD. These findings suggest that SFI has potential therapeutic effects on VaD.

NO has a dual effect on cerebral ischemia. In the initial few hours of cerebral tissue ischemia, NO can protect brain tissue. However, cerebral ischemia reperfusion cause the excessive production of NO, it will trigger toxic effects on brain tissue. During cerebral ischemia reperfusion period, NO undergoes peroxidation reactions, generating a large amount of peronitrate, which damages the lipid membranes, nucleic acids and proteins of brain tissue, ultimately inducing neurotoxic effects[27, 28]. Our experimental results revealed that PC12 cells incubated with 20 mM Na2S2O4 for 24 hours in the OGD group induced damage to it, which accompanied by NO significantly increase in the supernatant of PC12 cells. These results suggests that excessive NO produced in OGD-induced

PC12 cells may damage the cells and inhibit their survival rate. SFI reduce the content of NO in the supernatant of OGD-induced PC12 cells, indicating that SFI can maintain an appropriate NO level and thereby protect PC12 cells from OGD treatment.

NOS is the rate-limiting enzyme for NO synthesis. In brain tissue, the NOS activation leading to high levels of NO production is not only related to ischemic brain injury, but also has a significant impact on the formation process of dementia[29]. Studies have shown that NOS activity and NO level significantly increase in the VaD mouse, Kowloon Worm decreases NOS activity and NO level in brain tissue, thereby minimizing its damage to brain tissue[30]. Our preliminary experiments also revealed similar findings that compared with the sham group, the pathological damage in the CA1 region of the hippocampus of VaD group mice was significantly more severe, and the expression of nNOS protein was also significantly increased. However, up to now, there have been few reports indicating that the SFI may affect the cognitive dysfunction of VaD mice by regulating nNOS. We first reported that the pathological damage in the CA1 region of the hippocampus in the SFI group was significantly less compared to the VaD group, and the expression of nNOS protein was significantly lower[31]. nNOS is mainly distributed in neurons and glial cells, and it participates in regulating synaptic plasticity, neural circuits, learning ability and memory ability in the central nervous system[29]. Zhang et al indicates that nNOS activity in PC12 cells significantly increased 12 hours after OGD treatment, nNOS catalyzes the excessive NO production, which triggers a cascade reaction to mediate neuronal excitotoxicity[32]. It suggests that OGD may induce the damage to PC12 cells through the nNOS/NO signaling pathway. In present experiment, we also detected that the expression of the nNOS gene and protein were significantly upregulated in PC12 cells of OGD group, and the content of NO in the cell supernatant was significantly increased. Moreover, we firstly demonstrated that SFI significantly down-regulated the expression of the nNOS gene and protein in PC12 cells subjected to OGD. These results indicate that SFI may exert a protective effect on PC12 cells subjected to OGD by down-regulating the expression of nNOS genes and proteins to reduce the production of NO in these cells.

Jing et al found that the activities of iNOS and nNOS in the plasma and hippocampal tissues of in the VaD rats, as well as the level of NO, were significantly higher compared to those in the sham group; at the same time, the expressions of NOS genes and proteins in the hippocampus of the VaD group were significantly upregulated[33]. Our previous experiments also observed that the learning and memory abilities of mice in the VaD group were reduced, and the expression of iNOS protein in the CA1 area of the hippocampus was significantly increased[31]. To date, there have been few literature suggesting that SFI may improve the cognitive dysfunction of VaD mice via regulating iNOS. In the previous study, we first reported that the learning and memory abilities of mice in the SF group were improved compared to the VaD group, and the expression of iNOS protein in the CA1 area of the hippocampus was significantly decreased[31]. In this experiment, an OGD-induced PC12 cell was established to simulate the damage caused by in vivo hypoxia-ischemia to nerve cells as an in vitro VaD model. The results showed that the expression of iNOS mRNA and protein in PC12 cells of the OGD group was significantly increased, and NO level in the supernatant of OGD-induced PC12 cells was also increased. This result is consistent with the literature, indicating that the upregulation of iNOS expression in OGD-induced PC12 cells leads to excessive NO production, which decreased the survival rate of PC12 cells. At present, we discovered for the first time that low, medium and high dosage of SFI could significantly down-regulate the expressions of the iNOS gene and protein to reduce the level of NO in the supernatant of PC12 cells subjected to OGD. This finding suggests that SFI can maintain a moderate NO production by down-regulating the expression of iNOS, thereby reducing the damage to PC12 cells induced by OGD.

In conclusion, reducing the expression of iNOS and nNOS genes and proteins cells and thereby decreasing the excessive production of NO in PC12 may be one of the protective mechanisms of SFI against Na2S2O4-induced damage to PC12 cells. It suggests that the NOS/NO signal pathway may be involved in the pathogenesis of VaD, and SFI may prevent and treat VaD through

regulating the NOS/NO signal pathway. These findings have further enriched the pathological mechanism of VaD, providing experimental and theoretical basis for the prevention and treatment of VaD by traditional Chinese medicine.

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