Original Paper

Sonic Hedgehog Signaling Mediates Resveratrol to Increase Proliferation of Neural Stem Cells After Oxygen-Glucose Deprivation/Reoxygenation Injury *in Vitro*

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Key Words

Resveratrol • Neural stem cells • Sonic hedgehog signaling • Proliferation • Cyclopamine

Abstract

Background/Aims: There is interest in drugs and rehabilitation methods to enhance neurogenesis and improve neurological function after brain injury or degeneration. Resveratrol may enhance hippocampal neurogenesis and improve hippocampal atrophy in chronic fatigue mice and prenatally stressed rats. However, its effect and mechanism of neurogenesis after stroke is less well understood. Sonic hedgehog (Shh) signaling is crucial for neurogenesis in the embryonic and adult brain, but relatively little is known about the role of Shh signaling in resveratrol-enhanced neurogenesis after stroke. **Methods:** Neural stem cells (NSCs) before oxygen-glucose deprivation/reoxygenation (OGD/R) *in vitro* were pretreated with resveratrol with or without cyclopamine. Survival and proliferation of NSCs was assessed by the CCK8 assay and BrdU immunocytochemical staining. The expressions and activity of signaling proteins and mRNAs were detected by immunocytochemistry, Western blotting, and RT-PCR analysis. **Results:** Resveratrol significantly increased NSCs survival and proliferation in a concentration-dependent manner after OGD/R injury *in vitro*. At the same time, the expression of Patched-1, Smoothed (Smo), and Gli-1 proteins and mRNAs was upregulated, and Gli-1 entered the nucleus, which was inhibited by cyclopamine, a Smo inhibitor. **Conclusion:** Shh signaling mediates resveratrol to increase NSCs proliferation after OGD/R injury *in vitro*.

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Introduction

Neural stem cells (NSCs) present in the brain and spinal cord of developing and mature mammals bring hope for self-repair or regeneration after injury or degeneration of the central nervous system. Quiescent in the physiological condition, NSCs of mature mammalian neurogenic areas could be activated in the pathological conditions. The activated NSCs can proliferate, differentiate, migrate, and integrate into the target area to improve neurological function [1, 2]. However, these abilities are remarkably poor. Furthermore, the transplantation of exogenous NSCs is restricted because sources are limited, immunological rejection can occur, and there are ethical considerations. Therefore, there is great interest in the identification of drugs and rehabilitation methods for activating, enhancing, and regulating the regeneration, migration, differentiation, and integration for endogenous NSCs [3, 4].

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin that is found in dietary sources such as grapes, mulberries, *Polygonum cuspidatum*, *Semen cassiae*, peanuts, and red wine [5]. Studies have shown that resveratrol has anti-oxidant, anti-cancer, anti-inflammatory, anti-aging, and lipid-lowering properties [6, 7]. In addition, it has been reported that resveratrol exhibits neuroprotective effects in Alzheimer disease, Parkinson disease, and ischemic cerebral stroke [8-10]. We have previously observed that resveratrol pretreatment can attenuate cerebral ischemic injury by up-regulating the expression of transcription factor Nrf-2 and HO-1 to improve neurological function [11]. Resveratrol has also been shown to reduce neuronal apoptosis by up-regulating the expression of Bcl-2 and down-regulating the expression of Bax in the hippocampus after focal cerebral ischemia in rats [12]. Moreover, resveratrol enhanced hippocampal neurogenesis and improved hippocampal atrophy in chronic fatigue mice and prenatally stressed rats [13, 14]. However, the effect of resveratrol on neurogenesis after cerebral ischemic damage is largely unknown.

The Hedgehog (Hh) signaling pathway plays an important role in embryonic development, and adult stem cell function. Sonic hedgehog (Shh) is one of three ligands for Hh signaling in mammals [15]. When secreted glycoprotein Shh binds the patched (Ptc) receptor on the cell surface, inhibition of Smoothened (Smo) is relieved. Activated Smo triggers the activation of the Gli transcription factor, which regulates cell patterning, proliferation, migration, and differentiation during development. In mammals, there are three Gli transcription factors, Gli-1, Gli-2, and Gli-3. Gli-1 is a constitutive activator, but Gli-2 and Gli-3 have a C-terminal transcriptional activator domain and an N-terminal transcriptional repressor domain. Shh signaling is required for patterning of the central nervous system, the maintenance, proliferation, and specialization of neural progenitor cells (NPCs)/NSCs, and axonal growth and guidance in the embryonic and adult brain [16, 17]. After stroke, the activated Shh signaling pathway is involved in enhancing NPCs proliferation in the subventricular zone, promoting neurogenesis, oligodendrogenesis, and axonal remodeling in the peri-infarct area, and improvement of functional recovery in rats [18]. However, relatively little is known about the role of Shh signaling in resveratrol-enhanced proliferation after stroke.

We hypothesized that Shh signaling mediates resveratrol-increased proliferation of NSCs after oxygen-glucose deprivation/ reoxygenation (OGD/R) injury of NSCs *in vitro*. We definitively demonstrated that after OGD/R injury of NSCs *in vitro*, resveratrol pretreatment significantly increased survival and proliferation of NSCs in a concentration-dependent manner, and strengthened the activation of the Shh signaling pathway. Moreover, Shh signaling was essential for resveratrol-increased proliferation of NSCs.

Materials and Methods

Isolation and cultivation of NSCs

All experimental procedures were carried out with the approval of the Animal Experimental Committee of Chongqing Medical University, Chongqing, China, and complied with relevant guidelines and

laws. Six neonatal 1-3 day old male and female Sprague-Dawley (SD) rats, provided by the Experimental Animal Center of Chongqing Medical University, Chongqing, China, were used for primary NSCs cultivation, as previously described with slight modifications [19-21]. Briefly, the cerebral cortices of the neonatal rats were minced and incubated at 37°C for 30 min in a solution of 0.125% trypsin (HyClone, USA). The tissue was washed in media containing 10% fetal bovine serum (FBS; HyClone), and then passed through a 200 mesh filter screen. The extracted cells were resuspended in complete medium containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, USA), 2% B27 (Gibco), 20 ng/mL recombinant rat basic fibroblast growth factor (bFGF), and 20 ng/mL recombinant rat epidermal growth factor (EGF) (both from Peprotech, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Beyotime Institute of Biotechnology, China). The cell suspension was filtered through a 400 mesh filter screen, plated in 50 cm² glass culture flasks (3-5×10⁵ cells/mL), and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Fresh complete medium was replaced every 2-3 days, and the cells were subcultured once every 7 days. Experiments were performed using third passage cells. Cells were plated onto poly-L-Lysine (PLL; Beyotime Institute of Biotechnology)-coated plastic culture plates or dishes containing complete medium.

OGD/R model

OGD/R of cortical NSCs was performed to mimic cerebral artery occlusion and reperfusion injury, according to previously described methods [22, 23] with slight modifications. In brief, after washing three times with D'Hanks solution (Hyclone), NSCs were maintained with D'Hanks solution in a humidified anaerobic incubator (Thermo 3111, Thermo Fisher Scientific Inc., USA) containing 94% N₂, 1% O₂, and 5% CO₂ at 37°C for 150 min. For reoxygenation, the D'Hanks solution was replaced with complete medium and the NSCs were incubated in a humidified normoxic atmosphere containing 5% CO₂ for 24 h.

Drug treatment

To examine whether resveratrol reduced injury and promoted the proliferation of NSCs, four groups were studied: 1) normal group, NSCs were cultured in NSCs culture medium without OGD/R; 2) control group, NSCs were treated by OGD/R only; 3) vehicle group, NSCs were treated with NSCs medium containing ethanol (volume fraction 1.3%) for 24 h before OGD/R; 4) resveratrol pretreatment group. Cells were maintained in complete medium containing different concentrations (1 μmol/L, 5 μmol/L, and 20 μmol/L) of resveratrol (purity 99%, Sigma, USA) for 24 h before OGD/R. The best effect was observed with a concentration of 5 μmol/L resveratrol, and thus 5 μmol/L resveratrol was used for further study.

To investigate whether the Shh signaling pathway plays a role in the effect of resveratrol on NSCs proliferation, five groups were studied: 1) normal group, NSCs were cultured in complete medium without OGD/R; 2) control group, NSCs were treated with OGD/R only; 3) 5 μmol/L resveratrol (Res 5) group, NSCs were maintained in complete medium containing 5 μmol/L resveratrol for 24 h before OGD/R; 4) 5 μmol/L cyclopamine (Cyc) group, NSCs were maintained in complete medium containing 5 μmol/L cyclopamine (Cayman Chemical, USA) for 24 h before OGD/R; and 5) 5 μmol/L resveratrol+cyclopamine (Res 5+Cyc) group, NSCs were maintained in complete medium containing 5 μmol/L resveratrol and 5 μmol/L cyclopamine for 24 h before OGD/R.

CCK-8 cell viability assay

The CCK-8 assay was used to quantitatively assess cell survival. Briefly, third passage NSCs (approximately 5,000 cells/well) were seeded in poly-L-lysine-coated 96-well plates with six replicates in each group, and subjected to the various treatments described previously. CCK-8 solution (10 μL/100 μL) was added to each culture well, and NSCs were incubated for 4 h at 37°C. Absorbance at 450 nm was measured with a microplate reader (Thermo Labsystems, Vantaa, Finland). Each experiment was repeated for four times.

5-Bromodeoxyuridine labeling of proliferative NSCs

To evaluate the proliferation of NSCs, cells in the different treatment groups were incubated in 24-well plates with three replicates in each group with 10 μmol/L 5-bromodeoxyuridine (BrdU; Beijing Suolaibao Technology, Beijing, China) for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. An immunocytochemical assay was used to determine the incorporation. Experiments were done by triplicate.

Immunocytochemistry

Cells were seeded in 24-well plates with three replicates in each group. After fixing with 4% paraformaldehyde for 30 min at room temperature, cells were washed three times with PBS and treated with 0.1% TritonX-100 for 30 min at room temperature for permeabilization. NSCs incorporating BrdU were washed three times in PBS, and incubated in 2 N hydrochloric acid for 30 min at 37°C. Cells were washed in PBS and blocked with 10% normal goat serum (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) for 30 min at room temperature. Subsequently, the NSCs were incubated overnight at 4°C with the following primary antibodies: mouse polyclonal anti-nestin antibody (1:100; Santa Cruz Biotechnology, USA); mouse monoclonal anti-BrdU antibody (1:200; Cell Signaling Technology, USA); rabbit polyclonal anti-Gli-1 antibody (1:50; Santa Cruz Biotechnology). After washing in PBS, cells were reacted with the appropriate secondary antibodies as follows: FITC-conjugated goat anti-mouse or goat anti-rabbit IgG or rhodamine (TRITC)-conjugated goat anti-mouse IgG (1:100; Beijing Zhongshan Golden Bridge Biotechnology) for 1 h at 37°C. The primary antibodies were replaced with PBS in negative controls. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI; both from Beyotime Institute of Biotechnology) for 3-5 min in the dark. Finally, the cells were examined by TH4-200 fluorescence microscopy (Olympus, Tokyo, Japan). Experiments were done by triplicate.

RT-PCR analysis

Total RNA was extracted from cultured NSCs using Trizol (TaKaRa, Dalian, China), according to the manufacturer's instructions. For RNA extraction, cells were seeded at 1×10^6 cells/well in six-well plates with two replicates in each group. The reverse transcription reaction was performed with a First-Strand cDNA Synthesis Kit (TaKaRa), and a total of 35 cycles were performed for the amplification. The forward (F) and reverse (R) primer sequences (Sangon Biotech Co., Ltd., Shanghai, China) were: Ptc-1 (135 bp), F: 5'-AACCACAGGGCTATGCTC-3', R: 5'-CAGGACGGCAAAGAAGTA-3'; Smo (193 bp), F: 5'-TCTCGGGCAAGACATCCT-3', R: 5'-TAGCCTCCCACAATAAGCA-3'; Gli-1 (195 bp), F: 5'-GCCAATCACAATCAGTCTCC-3', R: 5'-TGCTCCTAACCTGCCAC-3'; GAPDH (289 bp) as an internal reference, F: 5'-GTGCTGAGTATGTCGTGGAG-3', R: 5'-GTCTTCTGAGTGGCAGTGAT-3'. PCR products were separated on 3% agarose gels using 5 μ L of amplification products. Bands were visualized using a Bio-Rad instrument (Bio-Rad Laboratories, Richmond, CA, USA), and measured using Quantity One software version 4.6.2 (Bio-Rad Laboratories). The amplification products gray ratio of objective gene to internal gene represented the mRNA transcriptional level. Experiments were done by triplicate.

Western blot analysis

For analysis of cellular protein levels, cells were seeded at 1×10^6 cells/well in six-well plates with two replicates in each group. Cells were rinsed twice with ice-cold PBS, lysed in RIPA lysis buffer containing 1% PMSF (Beyotime Institute of Biotechnology), and incubated on ice for 30 min. The protein was collected by centrifugation (12000 rpm, 10 min) at 4°C, and protein concentration was quantified by BCA assay (Beyotime Institute of Biotechnology). The protein samples were mixed with 5 $\times$ loading buffer (1:4), and boiled for 10 min. Next, equal protein quantity for every control and test sample (50 μ g protein per lane) was separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membrane. The membranes were blocked with 5% non-fat milk in PBST (Tween 20: PBS = 1:2) for 2 h at room temperature, and the following primary antibodies diluted with 5% non-fat milk in PBST were used and cells incubated overnight at 4°C: polyclonal goat anti-Ptc-1 (1:100) and polyclonal rabbit anti-Gli-1 (1:100) (both from Santa Cruz Biotechnology); rabbit anti-Smo (1:200; Abcam, USA); mouse anti- β -actin (1:1000; Beyotime Institute of Biotechnology) as an internal control. Subsequently, the membranes were washed three times with PBST after rewarming for 1 h at 37°C, and then incubated with the following secondary antibodies at a 1:1000 dilution in PBST containing 5% non-fat milk at 37°C for 1 h: horseradish peroxidase (HRP)-labeled goat anti-mouse or goat anti-rabbit IgG or HRP-labeled rabbit anti-goat IgG (both from Beijing Zhongshan Golden Bridge Biotechnology). Specific bands were detected by an enhanced chemiluminescence (ECL) reagent after rinsing three times with PBST. The Bio-Rad instrument and Quantity One software version 4.6.2 (Bio-Rad Laboratories) which were used for semi-quantitative analysis. The gray ratio of test protein to internal protein represented the test protein relative expression level. Experiments were done by triplicate.

Statistical analysis

Quantitative data were presented as mean $\pm$ standard deviation, and represent the results from three independent experiments. One-way ANOVA and Tukey's post-hoc analyses were used to determine the differences between groups. Statistical analysis was performed using SPSS 17.0 for Windows. A value of $P < 0.05$ was considered statistically significant.

Results

High expression of nestin protein in NSCs

Nestin is a neural stem cell marker. A high expression of nestin protein in cells with adherent monolayer cultivation was seen using an immunofluorescence assay (Fig. 1). The result indicates that the cells are NSCs.

Resveratrol reduces injury and increases proliferation of NSCs in a concentration-dependent manner after OGD/R injury in vitro

The CCK-8 assay was used to evaluate the optimal concentration effect of resveratrol pretreatment on NSC viability after OGD/R. Various concentrations of resveratrol (1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$, 20 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) were examined in a preliminary experiment, and concentrations of 1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, 20 $\mu\text{mol/L}$ were selected for further study.

As shown in Figure 2, NSC viability was significantly reduced in the control (0.450 ± 0.020), vehicle (0.447 ± 0.017), and 1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, 20 $\mu\text{mol/L}$ resveratrol (0.483 ± 0.021 , 0.553 ± 0.034 , 0.480 ± 0.028 , respectively) groups compared with the normal (0.817 ± 0.044) group. There was no significant difference between the control and vehicle group. However, NSC viability in the resveratrol groups was significantly higher than in the control and vehicle groups, and the highest viability was in the 5 $\mu\text{mol/L}$ resveratrol group. These results show that resveratrol can attenuate OGD/R injury of NSCs.

To investigate whether resveratrol can increase proliferation of NSCs after OGD/R injury *in vitro*, proliferated NSCs were labeled with BrdU, a thymidine analog that incorporates into dividing cells during the DNA synthesis phase. Immunocytochemical staining BrdU-positive cells were proliferative cells. As shown in Figure 3, there were few BrdU-positive

Fig. 1. High expression of nestin protein in NSCs with adherent cultivation by immunofluorescence. NSCs were immunostained with antibodies to nestin. A-C: Cells were strongly positive for nestin (green). D-F: The nestin antibody was replaced with PBS to serve as a negative control (NC). Nuclei (B, E) were labeled with DAPI (blue). Scale bars = 100 μm .

Fig. 2. Resveratrol in a concentration-dependent manner increased NSC viability after OGD/R injury as determined by the CCK-8 assay. OD values were significantly decreased in the control (Ctrl), vehicle (Veh), 1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, 20 $\mu\text{mol/L}$ resveratrol (Res 1, Res 5, Res 20) and blank (only NSCs complete medium) groups as compared with the normal (Norm) group. There was no difference between the control and vehicle groups. The OD values of the resveratrol groups were significantly increased compared to the control and vehicle groups, and highest value was in the 5 $\mu\text{mol/L}$ resveratrol group. The results show that resveratrol can ameliorate OGD/R injury of NSCs. ▲ $P < 0.05$ versus Norm; * $P < 0.05$ vs. Ctrl and Veh; # $P < 0.05$ vs. Res 5; ANOVA, $n = 4$ each group.

cells in the normal group and there were significantly greater numbers in the control and resveratrol groups. Moreover, the number of BrdU-positive cells in the resveratrol groups was significantly higher than in the control group, and was greatest in the 5 $\mu\text{mol/L}$ resveratrol group. The percentage of BrdU-positive cells in the normal, control, and resveratrol groups was $6.3 \pm 0.4\%$, $14.1 \pm 0.7\%$, $29.2 \pm 1.8\%$, $42.1 \pm 3.1\%$, and $18.6 \pm 2.2\%$, respectively. These results show that resveratrol can increase proliferation of NSCs after OGD/R injury *in vitro*.

Taken together, these results indicate that resveratrol can reduce injury and increase proliferation of NSCs in a concentration-dependent manner after OGD/R injury *in vitro*. Moreover, the best effective concentration of resveratrol is 5 $\mu\text{mol/L}$. Therefore, we selected 5 $\mu\text{mol/L}$ resveratrol for further study.

Shh signaling mediates resveratrol-increased proliferation of NSCs after OGD/R injury in vitro

We investigated the mechanism by which resveratrol increases the proliferation of NSCs. Shh signaling plays an important role in proliferation and specialization of NPCs/NSCs. Shh signaling pathway components in mammals include the Shh ligand, Ptc and Smo receptors, and Gli transcription factors. Therefore, we used cyclopamine, a Smo inhibitor, to study the role of Shh signaling in the resveratrol-increased proliferation of NSCs.

First, we examined the effects of resveratrol and cyclopamine on NSCs viability with the CCK-8 assay. As shown in Figure 4, the viability of cells in the control group (0.443 ± 0.015) was significantly decreased compared with the normal group (0.810 ± 0.042). After pretreated with resveratrol, cell viability (0.542 ± 0.027) was increased significantly as compared with the control group. However, the effect of resveratrol on NSC viability was significantly inhibited when cells were cultured with cyclopamine (0.371 ± 0.013) alone or with cyclopamine combined with resveratrol (0.480 ± 0.017). Moreover, in the cyclopamine alone group cell viability was minimal. These results demonstrate that blocking Shh signaling with cyclopamine eliminates the increased cell viability observed after treatment with resveratrol.

Next, we evaluated the effects of resveratrol and cyclopamine on NSCs proliferation with the BrdU assay (Fig. 5). The percentage of BrdU-positive cells in the resveratrol group ($40.5 \pm 0.9\%$) group was markedly increased compared with the normal ($7.7 \pm 1.0\%$) and control ($19.1 \pm 0.3\%$) groups. However, cyclopamine ($9.7 \pm 1.7\%$) reversed the effect

Fig. 3. Resveratrol in a concentration-dependent manner increased proliferation of NSCs after OGD/R injury determined with BrdU labelling (white arrows). NSCs were immunostained with antibodies to BrdU (red). BrdU-positive cells in the resveratrol groups (Res 1, Res 5, Res 20; G-O) were increased markedly compared with the normal (Norm, A-C) and control (Ctrl, D-F) groups, and peaked in the 5 $\mu\text{mol/L}$ resveratrol group. P: The percentage of BrdU-positive NSCs in each group. These results show that resveratrol can increase proliferation of NSCs after OGD/R injury *in vitro*. Nuclei were labeled with DAPI (blue). $\blacktriangle P < 0.05$ vs. Norm; $*P < 0.05$ vs. Ctrl; $\#P < 0.05$ vs. Res 5; ANOVA, $n = 3$ each group. Scale bars = 75 μm .

of resveratrol-increased BrdU-positive cell density. After co-administration of cyclopamine and resveratrol, the suppressive effect of cyclopamine was alleviated ($25.9 \pm 1.2\%$). These results indicate that cyclopamine blocks resveratrol-increased NSCs proliferation *in vitro* after OGD/R injury.

During neurogenesis or differentiation of mesenchymal stem cells, Smo is activated when Shh combines with its receptor, Ptc. This triggers the processing and activation of Gli transcription factors that enter the nucleus from the cytoplasm to control cell proliferation and differentiation [24, 25]. Therefore, we examined Gli-1 distribution with an

Fig. 4. Cyclopamine inhibits the effect of resveratrol-increased NSCs viability after OGD/R injury determined with the CCK-8 assay. The viability of cells in the control (Ctrl) group was significantly decreased compared with the normal (Norm) group. After pretreatment with 5 μ mol/L resveratrol (Res 5), cellular viability was significantly increased compared with the control group. The effect of resveratrol-increased NSC viability was significantly inhibited when cyclopamine (Cyc) alone or cyclopamine combined with resveratrol (Res 5+Cyc) was administered. In the cyclopamine alone group, cell viability was minimal. The blank group (only NSCs complete medium) was used to exclude the influence of NSCs complete medium. ▲ $P < 0.05$ vs. Norm; * $P < 0.05$ vs. Ctrl; # $P < 0.05$ vs. Res 5; △ $P < 0.05$ vs. Res 5 +Cyc; ANOVA, $n = 4$ each group.

immunofluorescence assay, and the expression of Ptc-1, Smo, and Gli-1 proteins and mRNA with Western blotting and PCR. In the normal group, Gli-1 accumulated in cytoplasm (Fig. 6A). In the control group, Gli-1 partially transferred to the nuclei (Fig. 6B). In the resveratrol group, Gli-1 almost transferred to the nuclei from the cytoplasm (Fig. 6C). At the same time, the expression of Gli-1 proteins in the nuclei, and Smo and Ptc-1 proteins in the cytoplasm was remarkably increased after resveratrol pretreatment compared with the normal and control groups (Fig. 7A, 7B). The expression of Gli-1, Ptc-1, and Smo mRNA in the resveratrol group was also markedly up-regulated compared with the normal and control groups (Fig. 7E, F). These results suggest that the Shh signaling pathway is activated after OGD/R injury, and resveratrol pretreatment strengthens activation of the Shh signaling pathway.

When cyclopamine alone or cyclopamine combined with resveratrol was administered, the effects of resveratrol on Gli-1 nuclear translocation (Fig. 6D, E), and the upregulated expression of Gli-1, Ptc-1, and Smo proteins and mRNAs (Fig. 7C-D, G-H) was remarkably inhibited. These results indicate that resveratrol-increased proliferation of NSCs after OGD/R injury *in vitro* is involved in the activation of the Shh signaling pathway. In other words, the Shh signaling mediates resveratrol-increased proliferation of NSCs after OGD/R injury *in vitro*.

Discussion

This study demonstrated that resveratrol enhances the viability and promotes the proliferation of NSCs in a concentration-dependent manner after OGD/R injury *in vitro*, and the most effective concentration of resveratrol is 5 μ mol/L. Park et al. reported that resveratrol inhibited hippocampal neurogenesis of healthy mice (1–10 mg/kg body weight), and proliferation of NPCs in culture (20 and 50 μ mol/L) [26]. Leong et al. reported that 30–120 μ mol/L resveratrol inhibited the proliferation of embryonic cardiomyoblasts [27]. Moriya et al. found that resveratrol could enhance hippocampal neurogenesis, and improve hippocampal atrophy in chronic fatigue mice [13]. Harada showed that resveratrol increased hippocampal neurogenesis and enhanced cognitive function in wild-type mice [28]. Madhyastha et al. also found that resveratrol could ameliorate postnatal hippocampal neurogenesis in prenatally stressed rats [14]. Moreover, an increasing number of reports

Fig. 5. Cyclopamine inhibits the effect of resveratrol-increased NSCs proliferation after OGD/R injury determined with the BrdU assay. NSCs were immunostained with antibodies to BrdU (red). BrdU-positive cells in resveratrol group (Res 5, G-I) were markedly increased as compared with normal (Norm, A-C) and control (Ctrl, D-F) groups. However, cyclopamine (Cyc, J-L) completely reversed the effect of resveratrol-increased BrdU-positive cell density. After co-administration of cyclopamine and resveratrol (Res 5+Cyc, M-O), the suppressive effect of cyclopamine was remarkably alleviated. P: Percentage of BrdU-positive NSCs in each group. White arrows indicate proliferative cells, nuclei were labeled with DAPI (blue). $\Delta P < 0.05$ vs. Norm; $*P < 0.05$ vs. Ctrl; $\#P < 0.05$ vs. Res 5; $\Delta P < 0.05$ vs. Res 5+Cyc; ANOVA, $n = 3$ each group. Scale bars = 75 μ m.

are showing that resveratrol has a general range of activities that depend on many factors including the organism or the cell type being studied, the physiological or pathological state of the organism or cells, and the concentration of resveratrol. In our preliminary experiment, we observed that 50 and 100 μ mol/L resveratrol decreased NSC viability. Park and Leong reported that resveratrol at high concentrations (20 and 50 μ mol/L, 30-120 μ mol/L, respectively) inhibited proliferation of NPCs and embryonic cardiomyoblasts [26, 27]. Therefore, further studies should investigate whether resveratrol exhibits a dual

Fig. 6. Cyclopamine inhibits resveratrol-triggered nuclear translocation of Gli-1 in NSCs after OGD/R injury. NSCs were immunostained with antibodies to Gli-1 (A-E, green). F: In the negative control (NC) group, the Gli-1 antibody was replaced with PBS. Nuclei were labeled with PI (red). In the normal (Norm) group, Gli-1 accumulated in cytoplasm (A, arrows). After OGD/R injury (Ctrl), Gli-1 partially transferred to the nucleus (B, arrows). After resveratrol treatment (Res 5), Gli-1 almost transferred to the nucleus (C, arrows). However, cyclopamine alone (D, Cyc) or cyclopamine combined with resveratrol (E, Res 5+Cyc) inhibited the nuclear translocation of Gli-1. These results indicate Shh signaling is activated after OGD/R injury, and resveratrol increased the activation of Shh signaling. Scale bars = 100 μ m.

concentration effect for neurogenesis in different physiological or pathological states.

We previously demonstrated that resveratrol pretreatment for 7 days could significantly attenuate cerebral ischemic injury, and improve neurological function in rats after middle cerebral artery occlusion. This neuroprotective effect is likely due to up-regulated expression of transcription factor Nrf2 and HO-1 to ameliorate oxidative damage, and decreased caspase-3 protein expression [11]. Our previous finding suggested that the Nrf2/ARE signaling pathway plays an important role in resveratrol neuroprotection against the damage from cerebral ischemia. Other *in vivo* and *in vitro* studies also showed that resveratrol exerts a neuroprotective effect in focal cerebral ischemia and OGD/R injury [29-34]. Here, we extended our previous finding by showing that resveratrol pretreatment enhances proliferation after OGD/R injury of NSCs *in vitro*.

Both our study and previous reports indicate that resveratrol enhances proliferation after OGD/R injury of NSCs *in vitro*, but the exact mechanisms of resveratrol-promoted proliferation are not well understood.

In neurogenic regions of the mammalian brain, NSCs and NPCs are present throughout life. These cells proliferate, differentiate, migrate, and eventually integrate into neural networks to promote brain remodeling and improve neurological function in various pathological conditions. This neurogenic process is regulated by various signaling pathways in the local environment of the neurogenic regions, such as Notch, Wnt, and Shh [35-37]. Shh signaling is especially crucial for embryonic development and tissue differentiation [38, 39]. It is also required for maintaining the NSC niche in the neurogenic regions in the adult rodent brain [40]. After brain injury, activation of Shh signaling is involved in promoting the brain remodeling process including neurogenesis, oligodendrogenesis, and axonal remodeling [41, 42], but relatively little is known about its role in resveratrol-enhanced proliferation after stroke.

Fig. 7. Cyclopamine inhibits resveratrol-increased expression of Ptc-1, Smo, Gli-1 proteins and mRNAs in NSCs after OGD/R injury. Compared with the normal (Norm) group, Ptc-1, Smo, and Gli-1 protein and mRNA levels in the control (Ctrl) and resveratrol (Res 5) groups were significantly increased. Highest levels were observed in the resveratrol group (A, B, E, F). In contrast, cyclopamine (Cyc) alone or cyclopamine combined with resveratrol (Res 5+Cyc) abolished these effects, and the suppressive effect of cyclopamine alone was the greatest (C, D, G, H). M: Marker; $\Delta P < 0.05$ vs. Norm; $*P < 0.05$ vs. Ctrl; $\#P < 0.05$ vs. Res 5; $\Delta P < 0.05$ vs. Res 5+Cyc; ANOVA, $n = 3$ each group.

Here, we observed that Gli-1 partially transferred to the nuclei from the cytoplasm, and the expression of Gli-1 protein and mRNA in the nuclei, and Smo and Ptc-1 proteins and mRNAs in the cytoplasm was increased remarkably after OGD/R injury of NSCs. This suggests that the Shh signaling pathway is partially activated by OGD/R injury. After resveratrol pretreatment, Gli-1 almost transferred to the nuclei from the cytoplasm. Simultaneously, the expression of Gli-1 protein and mRNA in the nuclei, and Smo and Ptc-1 proteins and mRNAs in the cytoplasm was increased markedly compared with the control group. This indicates that resveratrol pretreatment reinforces the activation of the Shh signaling pathway. On the contrary, when cyclopamine, a Smo inhibitor, alone or cyclopamine combined with resveratrol

was administrated, the viability and proliferation of NSCs was notably inhibited. At the same time, nuclear translocation of Gli-1 and the expression of Gli-1, Ptc-1, and Smo proteins and mRNAs was also suppressed. Our previous report demonstrated that resveratrol could induce bone mesenchymal stem cells to differentiate into neuronal-like cells and activate the Shh signaling pathway. Moreover, Shh signaling is essential for resveratrol inducing neuronal-like differentiation of bone mesenchymal stem cells [25]. Taken together, the findings suggest that the Shh signaling pathway may play an important role in resveratrol-enhanced neurogenetic processes after OGD/R injury of NSCs.

We showed that resveratrol could increase the viability and proliferation of NSCs, and activate the Shh signaling pathway after OGD/R injury *in vitro*. Moreover, Shh signaling might be essential for resveratrol-increased proliferation of NSCs. However, it may be not the only mechanism by which resveratrol affects cell proliferation [11]. The findings of this study are important for understanding the mechanism of resveratrol-increased proliferation of NSCs. In the future, we will investigate whether Shh signaling mediates resveratrol-promoted brain remodeling processes, including neurogenesis, oligodendrogenesis, and axonal remodeling after stroke *in vivo*.

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Disclosure Statement

None.

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