Neuroprotective effects of neural stem cells pretreated with neuregulin1β on PC12 cells exposed to oxygen-glucose deprivation/reoxygenation

Qiu-Yue Zhai,1 Yun-Hua Ye2, Yu-Qian Ren1, Zhen-Hua Song1, Ke-Li Ge1, Bao-He Cheng1, Yun-Liang Guo1, *

Abstract

Studies on ischemia/reperfusion (I/R) injury suggest that exogenous neural stem cells (NSCs) are ideal candidates for stem cell therapy reperfusion injury. However, NSCs are difficult to obtain owing to ethical limitations. In addition, the survival, differentiation, and proliferation rates of transplanted exogenous NSCs are low, which limit their clinical application. Our previous study showed that neuregulin1β (NRG1β) alleviated cerebral I/R injury in rats. In this study, we aimed to induce human umbilical cord mesenchymal stem cells into NSCs and investigate the improvement effect and mechanism of NSCs pretreated with 10 nM NRG1β on PC12 cells injured by oxygen-glucose deprivation/reoxygenation (OGD/R). Our results found that 5 and 10 nM NRG1β promoted the generation and proliferation of NSCs. Co-culture of NSCs and PC12 cells under condition of OGD/R showed that pretreatment of NSCs with NRG1β improved the level of reactive oxygen species, malondialdehyde, glutathione, superoxide dismutase, nicotinamide adenine dinucleotide phosphate, and nuclear factor erythroid 2-related factor 2 (Nrf2) and mitochondrial damage in injured PC12 cells; these indexes are related to ferroptosis. Our data show that p53 and solute carrier family 7 member 11 (SLC7A11) play vital roles in ferroptosis caused by cerebral I/R injury. Our data show that the expression of p53 was increased and the level of glutathione peroxidase 4 (GPX4) was decreased after RNA interference-mediated knockdown of SLC7A11 in PC12 cells, but this change was alleviated after co-culturing NSCs with damaged PC12 cells. These findings suggest that NSCs pretreated with NRG1β exhibited neuroprotective effects on PC12 cells subjected to OGD/R through influencing the level of ferroptosis regulated by p53/SLC7A11/GPX4 pathway.

Key Words: ferroptosis; p53; SLC7A11; GPX4; human umbilical cord-mesenchymal stem cells; neural stem cells; neuregulin1β; neuroprotection; oxygen-glucose deprivation/reoxygenation; PC12 cell

Introduction

Ischemic stroke is a common type of cerebrovascular disease caused by the blockage of an artery supplying blood to the brain and one of the major causes of death and disability worldwide (Feigin et al., 2016; Campbell et al., 2019; Martinez-Coria et al., 2021; Zhang et al., 2021). Ischemic stroke limits the supply of oxygen and glucose to the brain, which is also the key cause of neuronal cell death in cerebral infarction. Restoring blood reperfusion as early as possible is crucial for sustaining neuronal viability (Carden and Granger, 2000). However, the reperfusion process triggers additional injury in the ischemic brain (referred to as cerebral ischemia/reperfusion (I/R) injury) that causes irreversible neuronal damage in the brain. Clarifying the mechanism underlying I/R injury and effective prevention strategies has been formidable challenges in stroke treatment (Carden and Granger, 2000; Daubail et al., 2016).

Stem cell therapy has emerged as an important strategy for treatment of cerebral I/R injury, and various stem cell sources have been explored in clinical trials (Hicks and Jolkkonen, 2009; Chen et al., 2022). Among the various stem cell sources, neural stem cells (NSCs) are considered an ideal cell type for the treatment of cerebral I/R injury and have been applied in practice (Liu et al., 2009; Tang et al., 2017). However, the use of NSCs has some limitations, such as the difficulty in obtaining human NSCs because of ethical issues and the low survival, differentiation and proliferation rates of NSCs, which have restricted their clinical application (Kokaia and Darsalia, 2011; Lindvall and Kokaia, 2011; Casarosa et al., 2014). Therefore, an effective method to obtain NSCs/neural progenitor cells (NPCs) and enhance the neurogenic potential of NSCs is urgently required.

The neurotrophic factor neuregulin1β (NRG1β) is a member of the neuregulin family that contains an epidermal growth factor-like motif and play a vital role in the nervous system through activating ErbB receptor tyrosine kinase (Buonanno and Fischbach, 2001). NRG1β regulates the proliferation, differentiation, and migration of neural cells (including NSCs, NPCs, glial cells, and neurons), as well as synaptogenesis and synaptic plasticity (Buonanno and Fischbach, 2001; Birnmeier, 2009). NRG1β is best known for its function in regulating central nervous system injury and repair (Corfas et al., 2004). Administration of NRG1β has been shown to exhibit neuroprotective effects on ischemic stroke in rats (Shyu et al., 2004; Guo et al., 2006; Li et al., 2007). NRG1β treatment also significantly attenuates rat cortical neuron and primary hippocampal neuron damage under oxygen-glucose deprivation (OGD)
The sections were placed on slides, deparaffinized, rehydrated, and stained with Alcian Blue solution (KeyGen BioTECH, Cat#: KGA354).

**Generation of NSCs from hUC-MSCs**

Neurospheres are free-floating spherical aggregates that contain a mixture of NSCs/NPCs that retain their capacity to differentiate in neurons or glial cells under appropriate environmental conditions (Lindvall et al., 2004; Mukai et al., 2016). The generation of NSCs from hUC-MSCs was performed following previously published protocols with slight modifications (Mukai et al., 2016; Zhao et al., 2016; Peng et al., 2019). Briefly, hUC-MSCs were harvested as above and seeded at a density of 1.5–2 × 10^5 cells/cm^2 in low-attachment 6-well plates (Corning, Cat#: 3471) with neurosphere-forming medium containing DMEM/nutrient mixture (Gibco, Cat#: 10563-014) supplemented with 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA, Cat#: AF-100-15-100), 20 ng/mL basic fibroblast growth factor (PeproTech, Cat#: AF-100-188-50), and 2% B27 supplement (Gibco, 12507-010). Cells were cultured at 37°C with 5% CO₂ for 7 days and were cultured every 4–6 days in culture to maintain the neurosphere formation. hUC-MSCs began to aggregate and gradually form sphere-like structures; the cells were frequently observed clumped together. Over several days in vitro culture, almost all hUC-MSCs formed neurosphere-like structures, and the size of the neurospheres increased from 3 to 7 days. The medium was changed and the diameters of the neurospheres were measured on the 7th day under a light microscope with 100× magnification (Nikon, Tokyo, Japan, TE2000U) in six random visual fields. Cells were incubated with 50 μL PBS and with 20 μL 5-MT (R&D, Minneapolis, MN, USA, Cat#: C396-HB) was added to the neurosphere medium during the induction process.

**Immunofluorescence staining**

Cells were fixed with 4% paraformaldehyde and washed with PBS. Cells were permeabilized with PBS with 0.5% Triton X-100 for 10 minutes and blocked with PBS with 3% PBZ for 30 minutes. The cells were then fixed with 4% paraformaldehyde and washed with PBS and examined with a fluorescent microscope. The immunofluorescence intensity was measured by ImageJ software (version 1.52p; National Institutes of Health, Bethesda, MD, USA) (Schindelin et al., 2012).

**Flow cytometry analysis**

When cells at passage 3 reached 80–90% confluence, the adherent cells were digested with 0.25% trypsin-ethylenediamine tetraacetic acid (Gibco, 25200056) and fixed in 80% ethyl alcohol. Cells were permeabilized with PBS with 0.5% Triton X-100 and then blocked. The cells were incubated with primary antibodies (listed in Table 1) overnight at 4°C, followed by incubation with goat anti-rabbit Alexa Fluor 488 (anti-rabbit Alexa Fluor 488; Life Technologies, Cat#: A-31570) and goat anti-mouse Alexa Fluor 594 (anti-mouse Alexa Fluor 594; Life Technologies, Cat#: A-31575) at 37°C for 1 hour. Hoechst 33342 (Beyotime, Cat#: 90030) was used for nuclei staining. Cells were visualized using a fluorescent microscope and the immunofluorescence intensity was measured by ImageJ software (version 1.52p). The experiments were performed in duplicate.

**Quantitative polymerase chain reaction (qPCR)**

Total RNA was extracted from cells using a Micro Sample RNA kit (SparkLaide, Jinan, China, Cat#: AC-1001) according to the manufacturer's instructions. The qPCR system (ThermoFisher, Shanghai, China, Cat#: A28567) was used for PCR reactions. The amplification conditions were as follows: 50°C for 15 minutes, 95°C for 30 seconds, 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The 5'-end of each primer is labeled with 5′-FAM and the 3'-end with 3′-TAMRA. The comparative Ct method was used to calculate the relative mRNA expression levels.

The real-time PCR data were expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey’s post hoc test. All experiments were performed in triplicate, and *p < 0.05 was considered statistically significant.

**Table 1**

| No. | Gene Symbol | Description |
|-----|-------------|-------------|
| 1   | Bax         | Pro-apoptotic |
| 2   | Bcl-2       | Anti-apoptotic |
| 3   | Caspase-3   | |

**Table 2**

| Condition       | 0.5% Triton X-100 | 1% Triton X-100 | 2% Triton X-100 | 3% Triton X-100 | 4% Triton X-100 |
|-----------------|-------------------|-----------------|-----------------|-----------------|-----------------|
| PDCA3 expression| 0.42 ± 0.02       | 0.48 ± 0.03     | 0.50 ± 0.04     | 0.52 ± 0.05     | 0.54 ± 0.06     |

**Flow cytometry analysis:**

Cells were washed with PBS and incubated with 50 μL PBS and with 20 μL 5-MT (R&D, Minneapolis, MN, USA, Cat#: C396-HB) was added to the neurosphere medium during the induction process.
GLUTATHIONE PEROXIDASE 4 (GPX4) and NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 (NRF2) were evaluated in the samples by western blotting. The protein expression of GPX4 and NRF2 was standardized against β-actin, and the signal intensity was normalized to the protein expression level of β-actin. The Western blot bands were visualized using the BioRad ChemiDoc system (BioRad, Hercules, CA, USA).

Figure 1: Western blot analysis of GPX4 and NRF2 expression in PC12 cells. The expression of GPX4 and NRF2 (upper panel) was normalized to β-actin (lower panel). The bands were visualized using the BioRad ChemiDoc system. *p < 0.05 compared to the control group.

**Table 1: Information of primary antibodies**

| Antibody | Supplier | Catalog No. | RRID No. | Species | Dilution | Applications |
|----------|----------|-------------|----------|---------|-----------|--------------|
| CD44     | Abcam (Cambridge, UK) | ab189524 | AB_2885107 | Mouse, rat, human | 1:200 | Immunocytochemistry |
| CD90     | Bios (Beijing, China) | bs-0778R | AB_1109787 | Human, mouse, rat | 1:1000 | Flow cytometry |
| CD105    | Abcam (Cambridge, UK) | ab231774 | AB_2905493 | Human | 1:1000 | Flow cytometry |
| CD34     | Abcam (Cambridge, UK) | ab18289 | AB_1640331 | Mouse, rat, human | 1:1000 | Flow cytometry |
| CD45     | Abcam (Cambridge, UK) | ab40763 | AB_726545 | Human | 1:1000 | Flow cytometry |
| HLR-DA   | Abcam (Cambridge, UK) | 9ab2511 | AB_1056365 | Human, non-human primate | 1:1000 | Flow cytometry |
| SOX2     | Proteintech (Wuhan, Hubei Province, China) | 11064-1-AP | AB_2195801 | Human, mouse, rat, zebrafish | 1:200 | Immunocytochemistry |
| PAX6     | ZENBIO (Chengdu, Sichuan Province, China) | A0208 | AB_2892644 | Human, mouse, rat | 1:1000 | Western blot |
| β-ACTIN  | Proteintech (Wuhan, Hubei Province, China) | 20536-1-AP | AB_10700003 | Human, mouse, rat | 1:1000 | Western blot |
| SLC7A11  | Abclonal (Wuhan, Hubei Province, China) | A15604 | AB_2763010 | Rat | 1:500 | Western blot |
| p53      | Abclonal (Wuhan, Hubei Province, China) | A3185 | AB_2764972 | Human, mouse, rat | 1:1000 | Western blot |
| Nestin   | STEMCELL (Seattle, WA, USA) | AF7006 | AB_2835314 | Human, mouse, rat | 1:500 | Western blot |

**Table 2: Primers used for quantitative polymerase chain reaction**

| Genes        | Genbanks | Forward primer sequences (5'–3') | Reverse primer sequences (5'–3') | Product length (bp) |
|--------------|----------|----------------------------------|----------------------------------|--------------------|
| β-ACTIN      | NM_001101.5 | CAT CCG CAA AGA CCT GTA CG       | CTT GGT TGC TGA TCA ACA TC       | 218                |
| NANOG        | JX105036.1 | CAG CCA AAT TCT CCT GCG AG       | CAC GTC TAC AGG TTG CAT GT       | 153                |
| 4-CTT        | NM_00185986.2 | GGT CCT AGT GTG CTG TTG TA      | GCG GGA GTG CAG TGC AGT GA       | 190                |
| SOX2         | NM_005106.4 | TGA TGG AGA CCG AGG TGA AG      | GAA GTT GCT TAG CCC CAG GA       | 165                |
| BMI1         | NM_001840.9 | TCC TTG TGT GCA TGG TCA TGA      | CAT CCG CAA AGA CCT GTA CG       | 288                |
| NESTIN       | NM_006617.2 | TCT TGT CCT CCA CCA CTG CTG      | GGT GGC TGA TGC CTA CGT CT        | 187                |
| NEUROD1      | NM_005250.5 | GAG AGC CAT GAA GGC TAA CG       | CTG AAG CAA GGA GAC GAC CAG GT   | 268                |
| PAX6         | AH014790.2 | GTC GTC TGT GTC AAG GGC         | TAC CAC GGA TGG CCC TG           | 189                |

**NeuroD1: Neurogenic differentiation 1; Oct4: octamer-binding transcription factor 4.**

**Figure 2: Western blot assay of GPX4 and NRF2 expression in PC12 cells.** The expression of GPX4 and NRF2 (upper panel) was normalized to β-actin (lower panel). The bands were visualized using the BioRad ChemiDoc system. *p < 0.05 compared to the control group.

**Detection of malondialdehyde (MDA), superoxide dismutase (SOD), GSH-oxidized glutathione (GSSG) and NADPH/NADP⁺ levels**

The levels of MDA, SOD, GSSG, and NADPH/NADP⁺ in PC12 cells were measured using MDA (Beyotime, Cat# S0031), SOD (Solarbio, Cat# BC0175), GSSG/GSH (Beyotime, Cat# S0053), and NADPH/NADP⁺ (Nanjing Jiancheng, Nanjing, China, Cat# A115-1-1) assay kits, respectively. The absorbance was measured using a spectrophotometer at 530 nm for MDA and SOD, and at 405 nm for GSSG/GSH and NADPH/NADP⁺.
Results

Culture and characterization of hUC-MSCs

UC tissues from healthy full-term pregnant women were isolated and cultured, and primary hUC-MSCs were generated as described in Methods. As shown in Figure 2A, hUC-MSCs were arranged in parallel or a spiral shape. Cells were generally passed every 3 days because of their rapid proliferation. Immunofluorescence analysis demonstrated that hUC-MSCs were immunopositive for characteristic markers CD44, CD90, and CD105 (Figure 2B). To further characterize the hUC-MSCs, passage 3 cells were examined human MSC surface markers CD105, CD90, CD44, CD34, CD45 and HLA-DR, as defined by the International Therapy of Cellular Therapy, with flow cytometry. Over 90% of the cells exhibited positive surface expression for CD105, CD90 and CD44; fewer cells were positive for CD34, CD45 and HLA-DR (Figure 2C). These data show that the obtained hUC-MSCs exhibited similar morphology and surface marker expression compared with MSCs. To investigate the differentiation potential of hUC-MSCs, cells were induced into adipocytes, osteoblasts, and chondrocytes. An accumulation of Oil Red O-stained lipid droplets was observed in different hUC-MSCs, indicating adipogenesis; osteogenesis was observed with significant calcium deposition determined by alizarin S staining in treated cells, and chondrogenesis was revealed by proteoglycan staining with Alcian blue and was proved by the presence of extracellular matrix formation (Figure 2D). These results showed that the hUC-MSCs had differentiation potential.

Effects of NRG1β on the generation of NSCs from hUC-MSCs

hUC-MSCs were induced into NSCs through the addition of growth factor, and NRG1β was added during induction in the pretreatment group, as described in Methods section. As shown in Figure 3A, neurospheres usually have a round shape, dense core, and clear outline; some cell clusters also show an irregular shape. On the 7th day after induction of hUC-MSCs into NSCs, NSCs were collected and identified by assessing the key markers associated with pluripotency and neural progenitor cells. As shown in Figure 3B, qPCR showed that NSCs expressed the neural progenitor cell markers NESTIN, SOX2, PAX6, and NEUROD1 as well as pluripotency markers NANOG, OCT4, and BM1. These genes were upregulated in the 5 and 10 nM NRG1β groups compared with the control group, with a larger difference in the 10 nM NRG1β group than that in the 5 nM NRG1β group ($P < 0.05$ or $P < 0.01$). Immunofluorescence analysis demonstrated that NSCs were also positive for SOX2 and Nestin, and the expressions of SOX2 and Nestin in the 10 nM NRG1β group were significantly higher than those in the 5 nM NRG1β and control groups (Figure 4A and B, $P < 0.01$ and $P < 0.05$). Together, these data suggested that NRG1β enhances the differentiation of hUC-MSCs into NSCs.

Figure 3 | NRG1β enhances the differentiation of hUC-MSCs to NSCs.

(A) hUC-MSCs aggregate to form neurospheres within 3 to 7 days in suspension. Scale bars: 100 μm. (B) qPCR analysis of the expression level of NSCs markers, NESTIN, NEUROD1, PAX6, and SOX2, and pluripotency markers: NANOG, OCT4, and BM1. Data were normalized to controls and are presented as mean ± SD. The experiment was repeated three times. *$P < 0.05$, **$P < 0.01$ (one-way analysis of variance followed by Dunnett’s post hoc test); hUC-MSCs: Human umbilical cord mesenchymal stem cells; NeuroD1: neurogenic differentiation 1; NRG1β: neuregulin1β; NSCs: neural stem cells; Oct4: octamer-binding transcription factor 4; qPCR: quantitative polymerase chain reaction.
Statistical results showed that the diameter of neurospheres was significantly increased in the 5 and 10 nM NRG1β groups on the 7th day compared with that in the control group (P < 0.05). The percentage of neurospheres over 80 μm in diameter in the 10 nM NRG1β group on the 7th day was higher than those in the control and 5 nM NRG1β groups (Figure 5A). We further performed EdU assays and the results showed that the percentages of EdU-positive cells in the 5 and 10 nM NRG1β groups were significantly higher than that in the control group, with a significantly greater increase in the 10 nM NRG1β group than that in the 5 nM NRG1β group (P < 0.01; Figure 5B). Together, these findings confirmed that NRG1β positively regulates the proliferation of NSCs, and 10 nM NRG1β exhibited superior effects on the generation and proliferation of NSCs compared with 5 nM NRG1β.

Figure 5 | NRG1β enhances the proliferation potential of NSCs. (A) Diameter and percentage of different neurospheres on the 7th day in the control, 5 nM, and 10 nM NRG1β groups; each dot represents one biological replicate. (B) Representative images of NSCs proliferation activity determined using EdU assay on the 7th day. Red indicates EdU-positive cells, and Hoechst 33342 (blue) was used for nuclei staining. The number of EdU-positive cells in the 5 and 10 nM NRG1β groups was higher than that in the control group. (C) Percentage of EdU-positive cells among total cells in different groups; the percentage of EdU-positive cells in the 5 and 10 nM NRG1β groups was higher than that in the control group. Data are expressed as mean ± SD. The experiment was repeated four times. *P < 0.05, **P < 0.01 (one-way analysis of variance followed by Dunnett’s post hoc test). EdU: 5-Ethynyl-2′-deoxyuridine; NRG1β: neuregulin1β; NSCs: neural stem cells.

Pretreatment of NSCs with 10 nM NRG1β leads to a strong neuroprotective effect on damaged PC12 cells. We next used OGD/R as an in vitro model for I/R injury. Cells were cultured in anaerobic conditions for 2, 4, 6, 8, 10, and 12 hours, followed by reoxygenation under normal conditions for 24, 48, and 72 hours (Figure 6A). Cell viability assays showed that cell viability dramatically declined with increasing OGD time. With reoxygenation treatment, the quality and viability of PC12 cells were also reduced (Figure 6A and B). From these results, we chose OGD for 6 hours and reoxygenation for 48 hours for subsequent experiments.

To examine the effects of NSCs alone or with NRG1β on cell viability during OGD/R, the Transwell system was used to achieve co-culture of PC12 and NSCs. Co-culture condition was given immediately on reoxygenation and continuous for 48 hours; we found that PC12 cells formed an extensive connection in NSCs and NSCs + 10 nM NRG1β groups, but round shape cells and adhesion decreased in the OGD/R group (Figure 6C). The combination treatment led to an improvement in cell viability, and the difference in the viability of the NSCs + 10 nM NRG1β group compared with the OGD/R group was significant (Figure 6D).

Owing to cerebral I/R injury caused ROS generation and as ferroptosis is characterized by the accumulation of lipid ROS, we next examined the level of ROS. The data indicated that compared with ROS level in the OGD/R group, the production of ROS was decreased in the NSCs + 10 nM NRG1β group (P < 0.05; Figure 6E and F). No differences were observed with other groups.

Previous studies reported that lipid peroxidation and oxidative stress may play a central role in regulating the process of ferroptosis. Next, we measured the level of MDA (an important product of lipid peroxidation (Conrad et al., 2018; Piloni et al., 2021; Yuan et al., 2021)) and the expression of important intracellular antioxidants including GSH/GSSG, SOD, NADPH/NAPD+, and Nrf2 in different groups. The results showed that OGD/R decreased the levels of GSH/GSSG (P < 0.05), SOD (P < 0.01), NADPH/NADP+ (P < 0.05), and Nrf2 (P < 0.01) in PC12 cells compared with results in the control group; co-culture with NSCs and NSCs + 10 nM NRG1β remarkably increased the contents of these indexes after OGD/R, with a more pronounced effect in the NSCs + 10 nM NRG1β group (P < 0.01; Figure 7A and B). MDA level was higher in the OGD/R group compared with the control group, but co-culture decreased the expression of MDA compared with expression in the OGD/R group (P < 0.05, **P < 0.01), NADPH/NADP+ and SLC7A11/GPX4, SLC7A11 was knocked down using siRNA-SLC7A11 (Figure 8A). Reduced SLC7A11 leads to decreased membrane density and reduction/vanishing of mitochondria in damaged PC12 cells; mitochondrial damage improved in the co-culture groups (Figure 8A).

Studies have shown that p53 suppresses the expression of SLC7A11 (which is the light chain subunit of System Xc−). Reduced SLC7A11 leads to decreased synthesis of GSH and inactivation of GPX4, thus reduces cystine uptake and triggers ferroptosis (Liu et al., 2022). Western blot assay demonstrated that p53 level in the PC12 cells co-cultured with NSCs (OGD/R + NSCs) group and PC12 cells co-cultured with NSCs with pretreated 10 nM NRG1β (OGD/R + NRG1β) was lower compared with levels in the OGD/R group (P < 0.01); no differences were observed between the OGD/R and OGD/R + 10 nM NRG1β groups. GPX4 and SLC7A11 levels were lower in the OGD/R group compared with the OGD/R + NSCs and OGD/R + NSCs + 10 nM NRG1β groups (P < 0.01; Figure 8B). These results suggested that the intervention group may play a protective role in reducing the level of ferroptosis in damaged PC12 cells through activating expression of p53, SLC7A11 and GPX4. To further determine whether the protective role of NSCs pretreated with NRG1β on damaged PC12 cells occurs via the activating pathway of p53/SLC7A11/GPX4, SLC7A11 was knocked down using siRNA-SLC7A11 (Figure 9A and B). Western blotting confirmed a significant decrease in SLC7A11 expression after SLC7A11 knockdown (P < 0.05). We found that the expression levels of p53, SLC7A11 and GPX4 in the NSCs + 10 nM NRG1β group were significantly reversed compared with the OGD/R + siRNA-SLC7A11 group (P < 0.01; Figure 9C). These findings suggested that NSCs + 10 nM NRG1β play an intervention role in reducing levels of ferroptosis in damaged PC12 cells through activating the p53/SLC7A11/GPX4 pathway.
Effects of NSCs + 10 nM NRG1β on ferroptosis caused by OGD/R in the presence of siRNA-SLC7A11.

(A) Fluorescence expression of negative control-FAM (NC-FAM) after transfection for 8 hours. (B) Western blotting was performed to detect protein expression in control, NC, and positive control (ACTIN) groups after transfection for 24 hours. (C) The expression of p53, SLC7A11 and GPX4 were detected by western blotting. Data are expressed as mean ± SD. The experiments were repeated at least three times. *P < 0.05, **P < 0.01 (one-way analysis of variance followed by Dunnett’s post hoc test). GPX4: Glutathione peroxidase 4; NC: negative control; NRG1β: neuregulin1β; NSCs: neural stem cells; OGD/R: oxygen-glucose deprivation/reoxygenation.

Discussion

Several therapies for cerebral I/R injury are available, including drugs and surgery, but these treatments have their limitations (Zhu et al., 2012). Cell therapy has become an emerging alternative treatment for cerebral I/R injury, especially therapies using stem cells (Lindvall and Kokaia, 2011; Scheibe et al., 2012; Liu et al., 2014b). Among various stem cell sources, NSCs are considered as an ideal candidate seed cell for stem cell-based treatment for cerebral I/R injury because they retain the ability to self-renew and can differentiate into the major cell types of the brain (Kelly et al., 2004; Boese et al., 2012). Nevertheless, technical difficulties associated with the isolation and propagation of NSCs and low rates of survival and neuronal differentiation limit their clinical application. It is therefore essential to find an alternative source of NSCs and strategies to increase the capacity of NSCs to adapt their fate and function in a changing pathological environment.

The issue of direct conversion of hUC-MSCs into NSCs/NPCs under certain conditions has not been addressed (Fu et al., 2008; Liu et al., 2014a). Improving the efficiency of exogenous NSCs in the treatment of reperfusion injury. Therefore, many studies have attempted to use different strategies to influence these functions by expressing specific genes via viral transfection, pretreating NSCs with inflammatory immune factors, and combining NSCs with cytokines to increase the therapeutic effect of transplanted cells (Zhao et al., 2019). These methods provide a basis for improving the effect of exogenous NSC therapy.

In this study, we isolated hUC-MSCs and cultured cells with serum-free medium supplemented with epidermal growth factor and basic fibroblast growth factor. Cells showed formation of spheroid structures, and sphere colonies generated large neurospheres. Our research indicated that NRG1β enhanced the differentiation of hUC-MSCs to NSCs, and the effect of 10 nM NRG1β was more effective than that of 5 nM NRG1β. The proliferation of NSCs was increased in the presence of 5 nM and 10 nM NRG1β. However, the mechanism of how NRG1β promotes the differentiation and proliferation of NSCs remains elusive. We hypothesized that increasing the proliferation capacity of NSCs led to the increase in the number of NSCs, and thus the differentiation efficiency of hUC-MSCs to NSCs was also improved. This finding provides a new strategy for using in vitro interventions to improve the therapeutic effects of NSCs after transplantation.
We further assessed the neuroprotective effects of NSCs pretreated with NRG1β on damaged PC12 cells induced by OGD/R. Our results showed that the levels of ROS, MDA, GSH/GSSG, SOD, NAPDH/NAPD+ and Nrf2 and the level of mitochondrial damage were all improved in damaged PC12 cells after intervention with NSCs pretreated with NRG1β. The neuroprotective effects of NSCs and NSCs pretreated with 10 nM NRG1β were stronger than those in NRG1β groups. Importantly, the intervention effect in NSCs pretreated with 10 nM NRG1β, in terms of ROS, MAD and Nrf2, was better than in the NSCs group.

Transplantation of NSCs has been shown to regulate the immune response through a paracrine regulation response: NSCs release neurotrophic factor, vascular endothelial growth factor and other specific factors, which inhibit or reduce the inflammatory response after stroke. Endogenous NSCs regulate the local inflammatory microenvironment after transplantation and promote angiogenesis and synaptic plasticity by secreting nerve growth factor, laminin, integrins, and thrombospondins (Lladó et al., 2004; Staquicini et al., 2009; Horie et al., 2011). We speculate that 10 nM NRG1β may enhance the potential of NSCs and make them more resilient to impaired microenvironments through secreting some molecules to better respond to the pathological environment.

Studies have indicated that ferroptosis mediates ischemic injury of neurons by regulating the p53/SLC7A11/GPX4 pathway (Lan et al., 2020; Lu et al., 2020). As a subunit of System Xc-, SLC7A11 plays a key role in the conversion of extracellular cysteine and intracellular glutamate, and the imbalance of glutamate further induces the inactivation of GPX4 (Cao and Dixon, 2016; He et al., 2021). GPX4 plays a pivotal role in the inhibition of lipid peroxidation and ROS recruitment, which ultimately leads to the occurrence of ferroptosis (Maiorino et al., 2018). SLC7A11 is a target of p53, which binds to p53-responsive elements in the promoter region of SLC7A11 and represses its expression, further decreasing the uptake of extracellular cysteine and the synthesis of intracellular GSH and activating ferroptosis (Murphy, 2016; Liu et al., 2020). Our results showed that while the expressions of SLC7A11 and GPX4 were decreased in response to OGD/R, the expressions were increased in the 10 nM NRG1β, NSCs, and NSCs + 10 nM NRG1β groups, especially in the NSCs + 10 nM NRG1β group. In addition, the activation of p53 was inhibited in co-culture groups compared with that in the OGD/R group. siRNA-mediated knockdown of SLC7A11 showed that the NSCs + 10 nM NRG1β intervention attenuated ferroptosis induced by OGD/R in PC12 cells. These results provide evidence of the neuroprotective effects of NSCs pretreated with NRG1β during OGD/R and indicated that ferroptosis may be mediated by the activation of p53/SLC7A11/GPX4 in the process.

In summary, our data indicate that pretreatment of NSCs with 10 nM NRG1β enhanced their neuroprotective effects during OGD/R. In addition, our results suggest that ferroptosis is involved in the pathogenesis of OGD/R, and the activation of p53/SLC7A11/GPX4 pathway may decrease the level of ferroptosis in damaged PC12 cells. These findings may provide important evidence showing that NSCs showed better therapy for cerebral I/R injury. However, the precise effects of NRG1β on the differentiation of NSCs derived from HUC-MSCs remain unclear, and whether the inner character of NSCs was affected by NRG1β should be examined. Additionally, further studies should investigate the effects of transplantation of stem cells pretreated with NRG1β into a cerebral I/R injury animal model to verify the neuroprotective effects and the related molecular mechanism.

Author contributions: Study design, literature search, data analysis, statistical analysis, and manuscript preparation: QYZ, YQD, YLG; data acquisition: YHY, ZHS, KLG; manuscript revision: BHC. All authors read and approved the final version of the manuscript.

Conflicts of interest: The authors declare that they have no competing interests.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons AttributionNonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

References
Birchmeier C (2009) ErbB receptors and the development of the nervous system. Exp Cell Res 315:611-618.
Boese AC, Le QN, Pham D, Hamblin MH, Lee JP (2018) Neural stem cell therapy for subacute and chronic ischemic stroke. Stem Cell Res Ther 9:154.
Buonanno A, Fischbach GD (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol 11:287-296.
Campbell BVC, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, Donnan GA (2019) Ischaemic stroke. Nat Rev Dis Primers 5:70.
Cao JY, Dixon SJ (2016) Mechanisms of ferroptosis. Cell Mol Life Sci 73:2195-2209.
Carden DL, Granger DN (2000) Pathophysiology of ischaemia-reperfusion injury. J Pathol 190:255-266.
Casara S, Bozzi Y, Conti L (2014) Neural stem cells: ready for therapeutic applications? Mol Cell Ther 2:31.
Chen X, Yu C, Kang R, Kroemer G, Tang D (2021) Cellular degradation systems in ferroptosis. Cell Death Differ 28:1135-1148.
Chen B, Zhang L, Liu Y, Sang P, Jin Y, Lin MY, Xiao LY, Xiong HZ (2022) Mechanism and different transplantation approaches of mesenchymal stem cells in repairing chronic wounds. Zhongguo Zuzhi Gongcheng Yanyi J 26:4896-4903.
Conrad M, Kagan VE, Byar H, Pagnussat GC, Head B, Traber MG, Stockwell BR (2018) Regulation of lipid peroxidation and ferroptosis in diverse species. Genes Dev 32:602-619.
Corfas G, Roy K, Buxbaum JD (2004) Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. Nat Neurosci 7:575-580.
Dauball B, Ricolli F, Thouant P, Vague C, Chavent A, Osseby GV, Hervieu-Begue M, Delpont B, Mangola B, Beijt Y, Giroud M (2016) Impact of mechanical thrombectomy on the organization of the management of acute ischemic stroke. Eur Neurol 75:41-47.
De Simone U, Caloni F, Gribaldo L, Coccini T (2017) Human co-culture model of neurons and astrocytes to test acute cytotoxicity of neurotoxic compounds. Int J Toxicol 36:463-477.
Feigin VL, Norrving B, George MG, Foltz JL, Roth GA, Mensah GA (2016) Prevention of stroke: a strategic global imperative. Nat Rev Neurol 12:501-512.
Fu L, Zhu L, Huang Y, Lee TD, Forman SJ, ShiH CC (2008) Derivation of neural stem cells from mesenchymal stemcells: evidence for a bipotential stem cell population. Stem Cells Dev 17:1109-1121.
Gu N, Ge K, Hao C, Ji Y, Li H, Guo Y (2017) Neuregulin1β effects on brain tissue via ERK5-dependent MAPK pathway in a rat model of cerebral ischemia-reperfusion injury. J Mol Neurosci 61:607-616.
Guang YF, Wu CY, Fang YG, Zeng YN, Luo ZY, Li Z, Li XW, Zhu XH, Mei L, Gao TM (2015) Neuregulin1 protects against ischemic brain injury by ErbB4 receptors by increasing GABAAergic transmission. Neuroscience 307:151-159.
Guo WP, Wang J, Li RX, Peng YW (2006) Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia. Brain Res 1087:180-185.
He J, Liu J, Huang Y, Tang X, Xiao H, Hu Z (2021) Oxidative stress, inflammation, and autophagy: potential targets of mesenchymal stem cells-based therapies in ischemic stroke. Front Neurosci 15:641157.
Hicks A, Jolkkonen J (2009) Challenges and possibilities of intravascular cell therapy in stroke. Acta Neurobiol Exp (Wars) 69:1-11.
Hong L, Yan L, Xin Z, Hao J, Liu W, Sang S, Liao S, Wang H, Yang X (2020) Protective effects of human umbilical cord mesenchymal stem cell-derived conditioned medium on ovarian damage. J Mol Cell Biol 12:372-385.
Horie N, Pereira MP, Nizuma K, Sun G, Keren-Gill H, Encarnacion A, Shamloo M, Hamilton SA, Jiang K, Huhn S, Palmer TD, Bliss TM, Steinberg GK (2011) Transplanted stem cell-secreted vascular endothelial growth factor factors poststroke recovery, inflammation, and vascular repair. Stem Cells 29:274-285.
Kang R, Kroemer G, Tang D (2019) The tumor suppressor protein p53 and the ferroptosis network. Free Radic Biol Med 133:162-168.
Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foc WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK (2004) Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A 101:11839-11844.
Kokaia Z, Darsalia V (2011) Neuronal stem cell-based therapy for ischemic stroke. Transl Stroke Res 2:272-278.
Lan B, Ge J, Cheng SW, Zheng XJ, Liao J, He C, Rao QJ, Wang GZ (2020) Extract of Naotalfang, a compound Chinese herbal medicine, protects neuron ferroptosis induced by acute cerebral ischemia in rats. J Integr Med 18:344-350.
Li Q, Li Z, Mei Y, Guo Y (2008) Neuregulin attenuated cerebral ischemia-Creperfusion injury via inhibiting apoptosis and upregulating aquaporin-4. Neurosci Lett 443:155-159.
Li Q, Zhang R, Ge YL, Mei YW, Guo YL (2009) Effects of neuregulin on expression of MMP-9 and NSE in brain of ischemia/reperfusion rat. J Mol Neurosci 38:207-215.

Li Y, Xu Z, Ford GD, Crosland DR, Cairobe T, Li Z, Ford BD (2007) Neuroprotection by neuregulin-1 in a rat model of permanent focal cerebral ischemia. Brain Res 1184:277-283.

Lindvall O, Kokaia Z (2011) Stem cell research in stroke: how far from the clinic? Stroke 42:2369-2375.

Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 10 Suppl:S42-50.

Liu J, Kou Z, Tian Y (2014a) Diffuse axonal injury after traumatic cerebral microbleeds: an evaluation of imaging techniques. Neural Regen Res 9:1222-1230.

Liu J, Zhang C, Wang J, Hu W, Feng Z (2020) The regulation of ferroptosis by tumor suppressor p53 and its pathway. Int J Mol Sci 21:8387.

Liu M, Kong XY, Yao Y, Wang XA, Yang W, Wu H, Li S, Ding JW, Yang J (2022) The critical role and molecular mechanisms of ferroptosis in antioxidant systems: a narrative review. Ann Transl Med 10:368.

Liu X, Ye R, Yan T, Yu SP, Wei L, Xu G, Fan X, Jiang Y, Steelter LA, Liu G, Chen J (2014b) Cell-based therapies for ischemic stroke: from basic science to bedside. Prog Neurobiol 115:92-115.

Liu YP, Lang BT, Baskaya MK, Dempsey RJ, Vemuganti R (2009) The potential of neural stem cells to repair stroke-induced brain damage. Acta Neuropathol 117:469-480.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lladó J, Haenggeli C, Maragakis NJ, Snyder EY, Rothstein JD (2004) Neuronal stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. Mol Cell Neurosci 27:322-331.

Lu J, Xu F, Lu H (2020) LncRNA PVT1 regulates ferroptosis through miR-214-mediated TFR1 and p53. Life Sci 260:118305.

Lu YM, Gao YP, Tao RR, Liao MH, Huang JY, Wu G, Han F, Li XM (2016) Calpain-dependent ErbB4 cleavage is involved in brain ischemia-induced neuronal death. Mol Neurobiol 53:2600-2609.

Maieronino M, Conrad M, Ursini F (2018) GPx4, lipid peroxidation, and cell death: process and function. Cell Death Differ 23:369-379.

Marin C, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S (2013) Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. Biomed Res Int 2013:916136.

Mukai T, Nagamura-Inoue T, Shimazu T, Moriyoshi K, Takahashi A, Tsunoda H, Yamaguchi S, Tojo A (2016) Neuropeptide formation enhances the neurogenic differentiation potential and migratory ability of umbilical cord-mesenchymal stem cells. Cytotherapy 18:229-241.

Murphy ME (2016) Ironing out how p53 regulates ferroptosis. Proc Natl Acad Sci U S A 113:12350-12352.