

Unveiling the Role of N-butylphthalide in Ischemic Stroke: Boosting Neural Stem Cell Proliferation via GSK-3 β /NRF2 Pathway

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Background: Significant brain damage and loss of function are frequent outcomes of ischemic stroke. After ischemic damage, neural stem cells (NSCs) exhibit considerable potential to promote neuroprotection and facilitate neural repair. N-butylphthalide (NBP) efficiently increases the viability of NSCs under hypoxic conditions. Given that the underlying mechanisms remain unknown, this study aims to investigate the protective effects of NBP on NSCs both *in vitro* and *in vivo*.

Methods: This study assessed the neuroprotective effects of NBP using a middle cerebral artery occlusion/reperfusion (MCAO/R) model in mice. Neurobehavioral performance of MCAO/R mice was evaluated using neurobehavioral tests, and brain infarct volumes were analyzed. Western blot analysis was used to measure the expression of Nestin, Ki-67, glycogen synthase kinase-3 β (GSK-3 β), phosphorylation-GSK-3 β (p-GSK-3 β), and nuclear factor erythroid 2-related factor 2 (NRF2) in brain tissues. *In vitro* experiments were conducted using NSCs under Oxygen-Glucose Deprivation/Reoxygenation (OGD/R) conditions, followed by treatment with NBP and GSK-3 β inhibitors to assess cell viability and the levels of the GSK-3 β /NRF2 signaling pathway.

Results: NBP significantly improved the neurobehavioral scores and reduced infarct volumes in MCAO/R mice ($p < 0.05$). It also increased the expression levels of Nestin and Ki-67, indicating enhanced proliferation of NSCs ($p < 0.05$). Additionally, NBP upregulated the expression levels of GSK-3 β and NRF2 in brain tissues ($p < 0.05$). *In vitro*, NBP promoted the viability of NSCs and proliferation under OGD/R conditions, with increased expression levels of Nestin and Ki-67 ($p < 0.05$). The effect of NBP was partially reversed by GSK-3 β inhibitors, indicating that NBP's effects are partially mediated by the GSK-3 β /NRF2 signaling pathway ($p < 0.05$).

Conclusion: By regulating the GSK-3 β /NRF2 signaling pathway, this study shows that NBP stimulates proliferation of NSCs and enhances neurofunctional recovery after ischemic stroke. These findings indicate that NBP not only promotes cellular survival and repair but also has the potential to improve overall neurological outcomes.

Keywords: ischemic stroke; neural stem cells; n-butylphthalide; glycogen synthase kinase-3 β ; neuroprotection

Introduction

Ischemic stroke is a frequent and serious neurological condition caused by cerebrovascular occlusion, which stops blood flow to certain brain tissues, resulting in ischemic damage and cell death, with 5.9 million deaths and 102 million disability-adjusted life years lost [1,2]. As one of the leading causes of death and disability worldwide, its incidence and mortality continue to rise, particularly in low- and middle-income countries [3]. The etiology of ischemic stroke is multifaceted, including atherosclerosis, thromboembolism, and cardioembolism, with long-term recurrence risks and resultant disabilities imposing substantial medical and socioeconomic burdens on patients and society [4]. Current treatments for ischemic stroke primarily focus on vascular recanalization, such as intravenous thrombolysis and mechanical thrombectomy, but their ef-

fectiveness is limited by the therapeutic time window [5]. Against this backdrop, neural stem cells (NSCs), owing to their capabilities for self-renewal and multipotent differentiation, have emerged as a promising focus for research [6]. A study has demonstrated that transplantation of NSCs can repair damaged brain tissues by differentiating into neurons [7]. Additionally, NSCs secrete neurotrophic factors that optimize the microenvironment and mitigate inflammatory responses [8]. Notably, activating endogenous NSCs has been proposed as a non-invasive strategy with unique clinical advantages [9]. A previous study showed that activating endogenous NSCs promotes neuroregeneration and repairs damaged neural tissue [10]. Therefore, elucidating the activation mechanisms and roles of NSCs in ischemic stroke is critical for improving therapeutic outcomes and developing innovative treatment strategies.

N-Butylphthalide (NBP) is a small-molecule compound extracted from celery seed oil that has garnered considerable attention due to its diverse pharmacological properties [11]. Research has shown that NBP exerts neuroprotective effects by improving cerebral hemodynamics, enhancing microcirculation, and optimizing cellular energy metabolism [11]. NBP has demonstrated remarkable clinical efficacy in treating ischemic stroke [12]. Previous studies have highlighted its ability to alleviate microcirculatory disturbances and metabolic dysfunction in ischemic stroke and to protect NSCs from hypoxic injury [13,14]. Moreover, NBP has been reported to enhance glycogen synthase kinase-3 β (GSK-3 β) signaling, a key player in promoting proliferation of NSCs during ischemic-reperfusion injury [15]. Importantly, the GSK-3 β /nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway has been proven to exert neuroprotective effects in ischemic stroke [16]. In this study, we first evaluated the effects of NBP on neurological recovery following ischemic stroke. Subsequently, we explored its regulatory role in the proliferation of NSCs and further validated the pivotal role of the GSK-3 β /NRF2 signaling pathway in mediating this effect. Collectively, our findings demonstrate that NBP promotes the proliferation of NSCs after ischemic stroke by activating the GSK-3 β /NRF2 pathway, providing a mechanistic basis for its therapeutic application in ischemic stroke.

Materials and Methods

Animal Ethics

Male C57BL/6 mice (n = 50, 8 weeks old, 20–25 g) were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China). Pregnant female C57BL/6 mice at embryonic day 12.5 (n = 10) were obtained from Beijing United Animal Biotechnology Co., Ltd. (Beijing, China). Every mouse was kept in a specialized pathogen-free (SPF) environment with a 12-h light/dark cycle, a regulated temperature (22 \pm 2 $^{\circ}$ C), and a humidity level of 55 \pm 5%. Food and water were freely available to the animals. All animal studies were approved by the Animal Welfare Ethics Committee of Hebei North University, which were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Approval No. HBNU20240223053). Before the trial, mice were given at least one week to acclimate to the laboratory environment.

Establishment of the Middle Cerebral Artery Occlusion/Reperfusion (MCAO/R) Model

The MCAO/R model was created as previously described [17]. In short, 1.5% isoflurane (1349003, Sigma-Aldrich, St. Louis, MO, USA) was inhaled into the animals to induce anesthesia. To reveal the left external carotid artery (ECA) and common carotid artery (CCA), a midline incision was generated on the neck. To obstruct the origin of the middle cerebral artery (MCA) and limit blood

flow to the striatum and cortex, a silicone-coated 6-0 nylon monofilament (Doccol, Sharon, MA, USA) was placed through the ECA's stump and into the left internal carotid artery (ICA). Following 60 min of occlusion, the incision was sutured and the monofilament was withdrawn to permit reperfusion for 12 h. The mice's body temperature was maintained at 37 \pm 0.5 $^{\circ}$ C throughout the MCAO/R procedure. The identical surgical procedure was performed on sham-operated mice, except that the monofilament was not inserted.

Animal Treatment

A two-step randomization procedure was employed: First, the 50 mice were randomly divided into two main groups: the Sham surgery group (n = 10) and the MCAO/R modeling group (n = 40). Second, after successful MCAO/R surgery (with no intraoperative mortality), the 40 mice in the MCAO/R group were randomly assigned to three subgroups: the MCAO/R (n = 14), MCAO/R+NBP-Low (NBP-L) (n = 13), and MCAO/R+NBP-High (NBP-H) (n = 13). Prior to ischemia, intraperitoneal injections of 10 mg/kg/day and 20 mg/kg/day of n-butylphthalide (NBP, HY-B0647, MCE, Monmouth Junction, NJ, USA) were administered to the MCAO/R+NBP-L and MCAO/R+NBP-H groups, respectively, for 7 consecutive days [18]. The Sham and MCAO/R groups received equal volumes of saline (ST341, Beyotime, Shanghai, China) on the same schedule. 24 h after the final injection (Day 8), mice in the MCAO/R, MCAO/R+NBP-L, and MCAO/R+NBP-H groups underwent transient MCAO for 60 min followed by reperfusion to induce ischemic injury. The Sham group underwent an identical surgical procedure without filament insertion. During the experimental phase, 2 animals in the MCAO/R group, 1 animal in the MCAO/R+NBP-L group, and 1 animal in the MCAO/R+NBP-H group died, leaving 10 mice in each of the MCAO/R, MCAO/R+NBP-L, and MCAO/R+NBP-H groups for subsequent analysis. Each mouse was tested on 1 day before and on 2 days after MCAO/R. All mice were sacrificed after the animal experiments by 1.5% isoflurane inhalation and cervical dislocation. Brain tissue samples were collected and kept at -80° C.

Neurological Function Assessment

Neurological function was evaluated using an 18-point Garcia neurological scoring system, which assessed responses to tactile stimuli, climbing ability, limb symmetry, forepaw extension, spontaneous activity, and body proprioception [19,20]. Lower ratings indicate more serious damage. Each test was videotaped and independently scored by two blinded observers who had undergone standardized training before the study to ensure consistent scoring criteria. Forelimb motor coordination was evaluated using the foot-fault test, which counted the number of times the forelimbs stumbled while moving across a grid. Each

Table 1. Antibodies used in Western blot analysis.

Antibody name	Molecular weight	Source	Catalog number	Supplier	Type
Nestin	230 kDa	Rabbit	#10959	CST	Primary antibody
Ki-67	358 kDa	Rabbit	ab16667	Abcam	Primary antibody
p-GSK-3 β	47 kDa	Rabbit	ab75745	Abcam	Primary antibody
GSK-3 β	47 kDa	Mouse	ab93926	Abcam	Primary antibody
NRF2	97 kDa	Rabbit	#12721	CST	Primary antibody
β -actin	42 kDa	Mouse	ab8226	Abcam	Primary antibody
Goat Anti-Rabbit IgG H&L (HRP)			ab6721	Abcam	Secondary antibody
Goat Anti-Mouse IgG H&L (HRP)			ab205719	Abcam	Secondary antibody

mouse was observed for five min or until fifty steps were completed with the affected forelimb. Two independent observers recorded the number of steps and forelimb slips. Out of all steps, the proportion of right forelimb foot defects (stroke-affected) was computed and reported. To validate the inter-observer reliability, both Cohen's Kappa and Intra-class Correlation Coefficient (ICC) were calculated. Cohen's Kappa of 0.85 and an ICC of 0.90 indicate excellent inter-observer agreement.

2,3,5-triphenyltetrazolium Chloride (TTC) Staining

Brains were sectioned into 2-mm coronal slices and incubated for 20 min (37 °C) in 2% TTC (MM1006, Maokang Bio, Shanghai, China). Infarct locations were photographed using a Nikon camera (DXM1200, Tokyo, Japan), and the infarct volume as a proportion of total brain volume was quantified in a blinded manner using ImageJ software (version 1.50i, NIH, Bethesda, MD, USA).

Western Blot Analysis

After homogenizing and lysing the ipsilateral brain tissues and NSCs in ice-cold radioimmunoprecipitation assay (RIPA) buffer (P0013, Beyotime, China), the supernatant was collected by centrifugation. Protein samples were separated via polyvinylidene difluoride membranes (PVDF; FFP24, Beyotime, China) after being separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; P0690, Beyotime, China). The membranes were blocked for 1 h with 5% non-fat milk, treated with primary antibodies for a whole night (4 °C), and then incubated for 1 h with secondary antibodies. Chemiluminescence signals were detected using an enhanced chemiluminescence (ECL) kit (KGC4902, Keygene, Shanghai, China) and imaged on a Bio-Rad gel imaging system (Hercules, CA, USA). ImageJ software was used to standardize band intensities to β -actin. The antibodies employed in this study are listed in Table 1.

Isolation and Identification of Neural Stem Cells (NSCs) From Mice

NSCs were extracted from female C57BL/6 mice (embryonic day 12.5) using previously documented procedures [21]. Briefly, the neocortical tissues were removed, and

digested for 30 min in 0.25% trypsin (SM-2003, Sigma-Aldrich, St. Louis, MO, USA), and the enzymatic activity was neutralized with soybean trypsin inhibitor (T6414, Sigma-Aldrich, USA). The tissues were washed twice with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; A4192001, ThermoFisher, Waltham, MA, USA) before mechanical dissociation. To obtain single-cell suspensions, the resulting cell suspension was passed through a 100 μ m cell strainer (BS-100-XBS, Biosharp, Beijing, China). The dissociated cells (1×10^5 cells/mL) were cultivated at 37 °C in a humidified incubator with 5% CO₂ after being seeded in DMEM/F12 supplemented with B-27 (17504044, ThermoFisher, USA), 20 ng/mL mouse epidermal growth factor (EGF; SRP3196, Sigma-Aldrich, USA), and 20 ng/mL mouse fibroblast growth factor-2 (FGF-2; HY-P7066, MedChemExpress, Monmouth Junction, NJ, USA). Neurosphere formation was observed after three days of culture. NSCs were kept in the medium mentioned above, and immunofluorescence was employed to assess Nestin expression. All cultures tested negative for mycoplasma contamination.

Immunofluorescence

For ten min, separated NSCs and neurospheres were preserved in 4% paraformaldehyde (P6148, Sigma-Aldrich, USA). The neurospheres were submerged in antigen retrieval solution (C1031, Solarbio, Beijing, China) for ten min (95 °C) in order to perform antigen retrieval. For 1 h, 5% bovine serum albumin (BSA; A1933, Sigma-Aldrich, USA) was used to inhibit permeability. A primary antibody against Nestin (ab81462, Abcam, Cambridge, UK) was incubated with neurospheres at 4 °C for a whole night. The neurospheres were incubated for 1 h with Alexa Fluor 488-conjugated goat anti-rat secondary antibody (A-1106, ThermoFisher, USA) following washing with phosphate-buffered saline (PBS; P4417, Sigma-Aldrich, USA). Similarly, NSCs were incubated overnight (4 °C) with primary antibodies against Nestin and Ki-67 (MA5-14520, ThermoFisher, USA). They were then incubated for 1 h at room temperature with secondary antibodies Alexa Fluor 488-conjugated goat anti-rat and Alexa Fluor 647-conjugated goat anti-rabbit (A27040, ThermoFisher, USA). 4',6-diamino-2-styryl alcohol (DAPI;

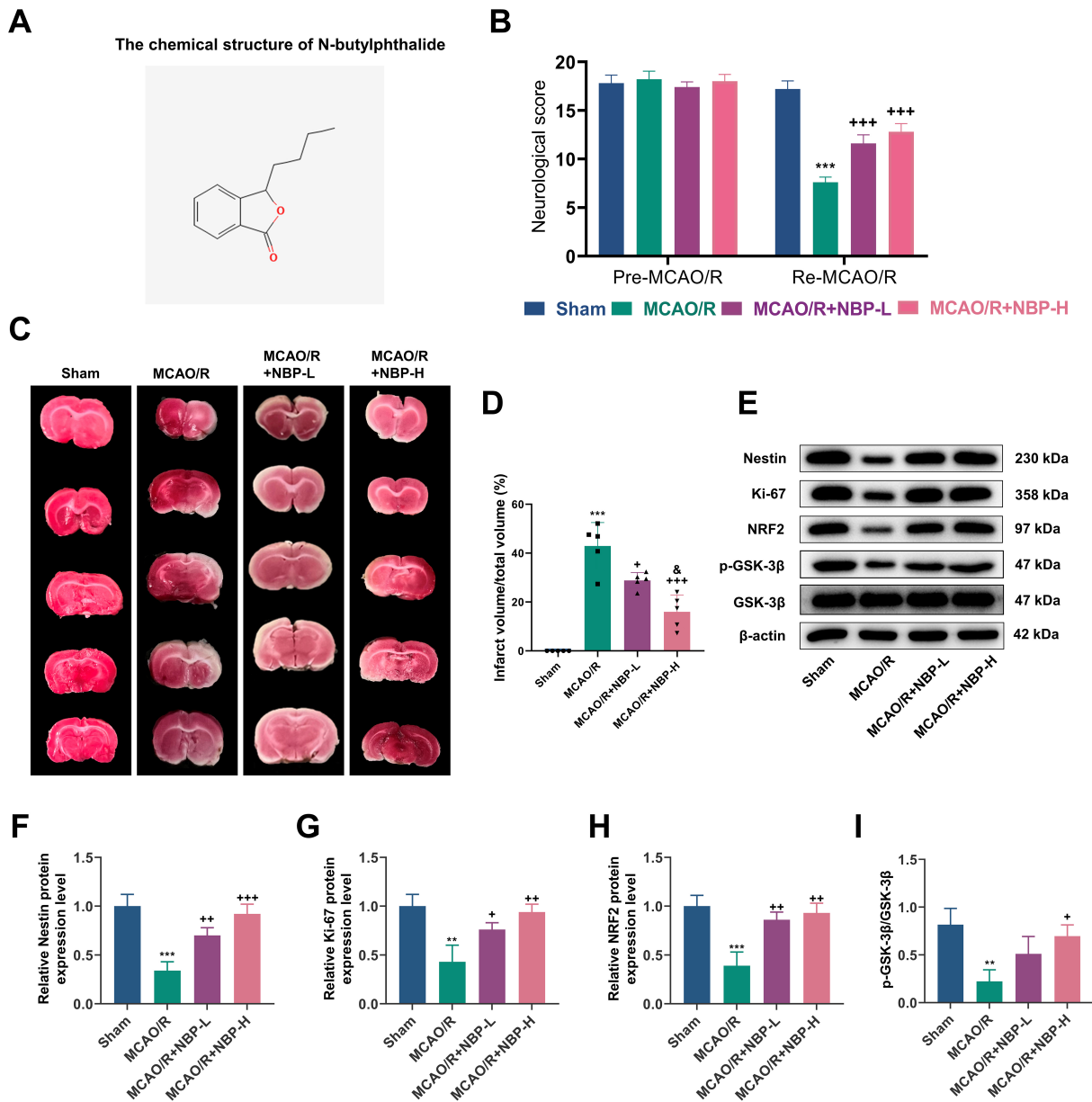


Fig. 1. N-butylphthalide (NBP) improves neurobehavioral performance and reduces infarct volume in middle cerebral artery occlusion/reperfusion (MCAO/R) mice while increasing nuclear factor erythroid 2-related factor 2 (NRF2) and phosphorylation-glycogen synthase kinase-3 β (p-GSK-3 β)/GSK-3 β expression. (A) Chemical structure of NBP. (B) Neurobehavioral test results for Sham, MCAO/R, MCAO/R+NBP-Low (NBP-L), and MCAO/R+NBP-High (NBP-H) mice before and after MCAO/R. (C,D) Brain infarct volume analysis in Sham, MCAO/R, MCAO/R+NBP-L, and MCAO/R+NBP-H mice. The sections (2 mm) shown in the figure are representative sections used for quantitative analysis of each group. (E–I) Western blot analysis of protein levels of Nestin, Ki-67, NRF2, p-GSK-3 β , and GSK-3 β in brain tissue from Sham, MCAO/R, MCAO/R+NBP-L, and MCAO/R+NBP-H mice. β -actin was used as the internal control. Sham (n = 5), MCAO/R (n = 5), MCAO/R+NBP-L (n = 5), and MCAO/R+NBP-H (n = 5). ** p < 0.01, *** p < 0.001 vs Sham; + p < 0.05, ++ p < 0.01, +++ p < 0.001 vs MCAO/R; & p < 0.05 vs MCAO/R+NBP-L.

D9542, Sigma-Aldrich, USA) was applied for 10 min in order to conduct nuclear staining. The expression of Ki-67 (red fluorescence), Nestin (green fluorescence), and nuclei (blue fluorescence) was observed with a confocal microscope (LEXT OLS5100, Olympus, Tokyo, Japan; 200 \times or 400 \times magnification), and the ratio of marker-positive cells to DAPI-positive cells was calculated.

Cell Viability Assay

NSCs (5 \times 10³ cells/well) were treated with 0, 0.1, 1, and 10 μ M NBP for 24 and 72 h after being seeded onto 96-well plates (FCP962, Beyotime, China). Following the addition of 10 μ L of the cell counting kit-8 (CCK-8) reagent (C0037, Beyotime, China) to each well, the plates were in-

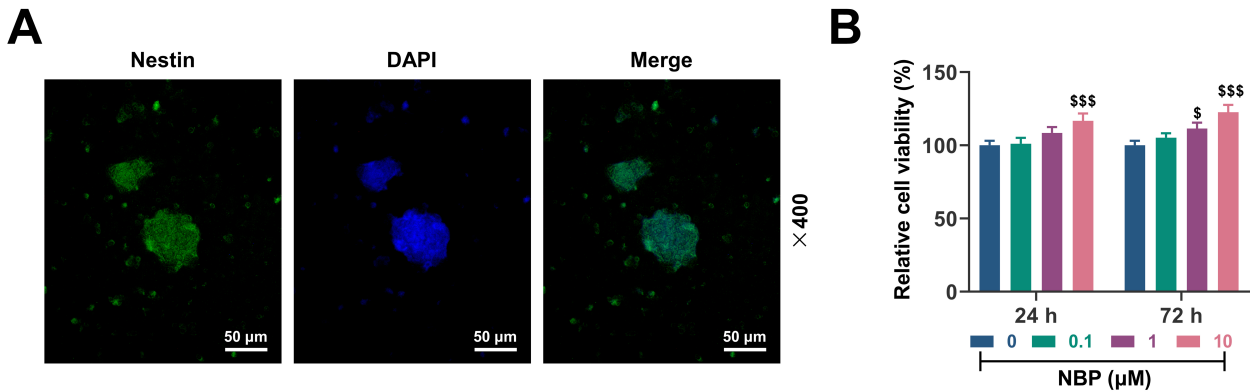


Fig. 2. NBP promotes viability of neural stem cells (NSCs). (A) Immunofluorescence analysis of Nestin levels in NSC-derived neurospheres. Magnification: $\times 400$, scale: 50 μm . 10 mice. (B) Cell viability was measured by the cell counting kit-8 (CCK-8) assay after 24 h or 72 h of treatment with 0, 0.1, 1, and 10 μM NBP in NSCs. Each experiment was performed independently in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs 0 μM .

cubated for 2 h (37 °C). At 450 nm, absorbance was determined with a microplate reader (Glomax, Promega, Madison, WI, USA). Relative cell viability (%) = $(\text{OD}_{\text{experiment}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%$.

Oxygen-Glucose Deprivation/Reoxygenation (OGD/R) Model and Treatment

There were five groups of NSCs: Control, OGD/R, NBP-1+OGD/R, NBP-10+OGD/R, and NBP-10+OGD/R+AR-A014418. The Control group's cells received no treatment, whereas the other groups had *in vitro* ischemia damage as previously reported [22]. Except for the Control group, cells in the other four groups were incubated in Earle's Balanced Salt Solution (CC0045, Reckitt Benckiser, China) devoid of glucose and serum and immediately transferred to a hypoxic chamber (94% N₂, 1% O₂, and 5% CO₂) for 4 h. During the replacement of the OGD medium, the following drugs were added accordingly: 1 μM NBP in the NBP-1+OGD/R group, 10 μM NBP in the NBP-10+OGD/R group, and 10 μM NBP combined with 5 μM GSK-3 β inhibitor (AR-A014418; S7435, Selleck, Houston, TX, USA) [23] in the NBP-10+OGD/R+AR-A014418 group. The OGD/R group received no drug. After the 4 h OGD, all cells were returned to normal complete medium and reoxygenated under normoxia for 72 h.

Statistical Analysis

GraphPad Prism 8.0 (CA, USA) was used to analyze the data, which are presented as the mean \pm standard deviation. Data were analyzed by using one-way ANOVA, and followed by Tukey's post hoc test, and *p*-values less than 0.05 were considered statistically significant.

Results

NBP Alleviates Neurofunctional Damage Induced by MCAO/R

Fig. 1A displays the chemical structure of NBP. We established a mouse model of ischemic stroke and administered NBP to assess its impact on ischemic brain damage. The neurological function scores dramatically declined after MCAO/R therapy, but NBP treatment significantly ameliorated this decline (Fig. 1B, $p < 0.05$). Both NBP-L and NBP-H treatments reduced the MCAO/R-induced infarct volume (white regions), with the effect being more pronounced in the NBP-H group (Fig. 1C,D, $p < 0.05$). Western blot examination showed that the MCAO/R group had substantially lower levels of Nestin, Ki-67, NRF2, and p-GSK-3 β /GSK-3 β in the brain tissue when compared to the sham group (Fig. 1E-I, $p < 0.05$). In contrast to the MCAO/R group, both NBP-L and NBP-H treatments significantly increased the levels of Nestin, Ki-67, and NRF2 in brain tissue, with NBP-H further enhancing the p-GSK-3 β /GSK-3 β (Fig. 1E-I, $p < 0.05$).

NBP Enhances Viability of NSCs

After being separated from C57BL/6 mice, NSCs were cultivated to create floating neurospheres. The neurospheres tested positive for the NSCs marker Nestin, as determined by immunofluorescence analysis (Fig. 2A). Next, we evaluated the effects of varying NBP concentrations on viability of NSCs. The findings demonstrated that viability of NSCs was considerably boosted by treatment with 10 μM NBP for 24 h, as well as 1 μM and 10 μM NBP for 72 h (Fig. 2B, $p < 0.05$).

NBP Ameliorates OGD/R-Induced Cell Damage by promoting GSK-3 β /NRF2 Pathway

Viability of NSCs was suppressed by OGD/R treatment, while the reduction in cell viability caused by OGD/R

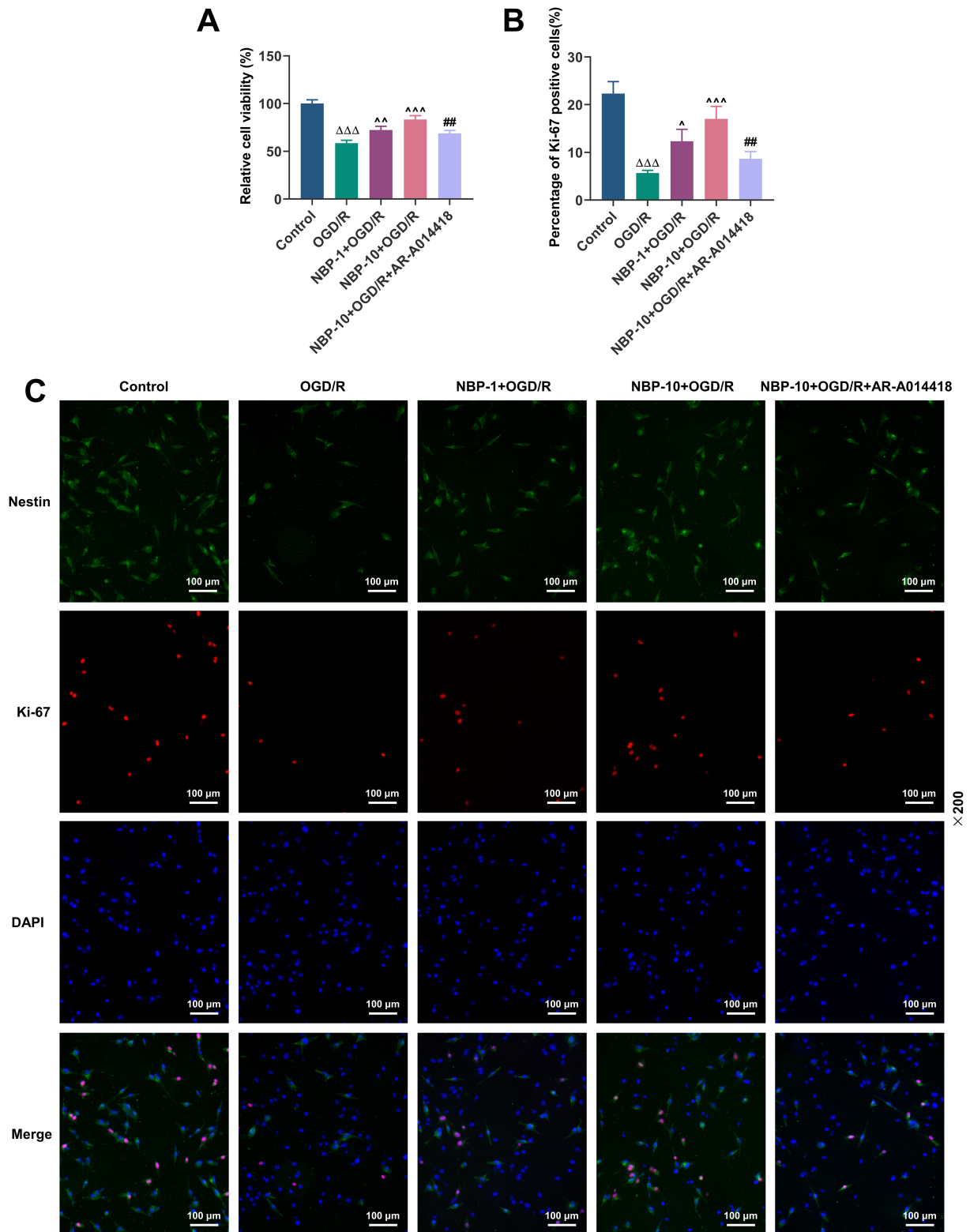


Fig. 3. NBP restores viability of NSCs reduced by oxygen-glucose deprivation/reoxygenation (OGD/R) through GSK-3 β activation. (A) CCK-8 assay showing cell viability in Control, OGD/R, NBP-1+OGD/R, NBP-10+OGD/R, and NBP-10+OGD/R+AR-A014418-treated NSCs. (B,C) Immunofluorescence analysis of Nestin and Ki-67 levels in Control, OGD/R, NBP-1+OGD/R, NBP-10+OGD/R, and NBP-10+OGD/R+AR-A014418-treated NSCs. Magnification: $\times 200$, scale: 100 μm . Nestin (+) green fluorescence, Ki-67 (+) red fluorescence, and blue fluorescence for cell nuclei. Each experiment was performed independently in triplicate. $\Delta\Delta\Delta p < 0.001$ vs Control. $^{\wedge} p < 0.05$, $^{\wedge\wedge} p < 0.01$, $^{\wedge\wedge\wedge} p < 0.001$ vs OGD/R. $^{\#\#} p < 0.01$ vs NBP-10+OGD/R.

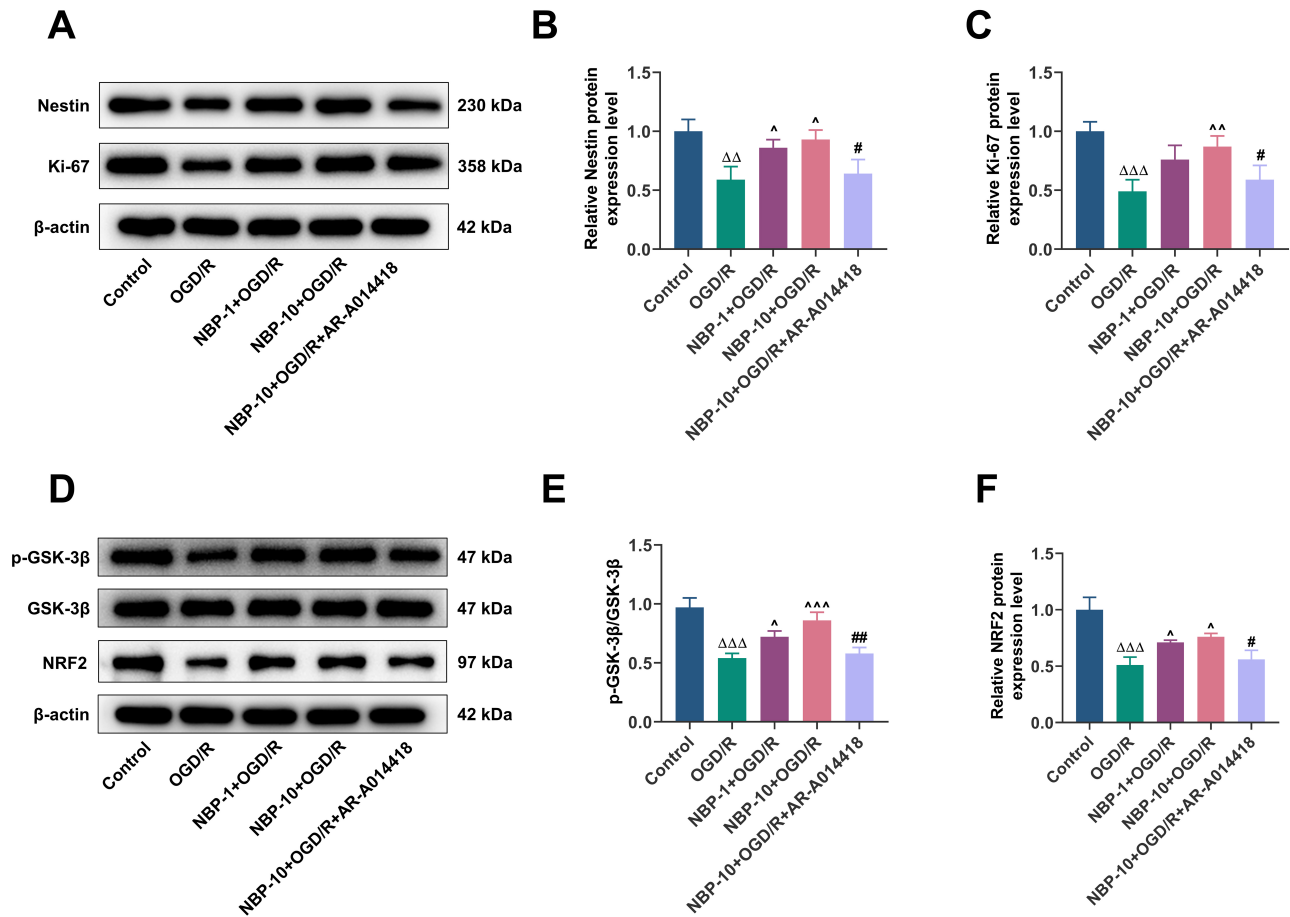


Fig. 4. NBP regulates the expression of proliferation-related proteins in NSCs under OGD/R-induced conditions via the GSK-3 β /NRF2 pathway. (A–F) Western blot analysis of protein levels of Nestin (B), Ki-67 (C), p-GSK-3 β /GSK-3 β (E), and NRF2 (F) in NSCs treated with Control, OGD/R, NBP-1+OGD/R, NBP-10+OGD/R, and NBP-10+OGD/R+AR-A014418. β -actin was used as the internal control. Each experiment was performed independently in triplicate. $\Delta\Delta\Delta p < 0.01$, $\Delta\Delta\Delta\Delta p < 0.001$ vs Control. $^{\wedge} p < 0.05$, $^{\wedge\wedge} p < 0.01$, $^{\wedge\wedge\wedge} p < 0.001$ vs OGD/R. $^{\#} p < 0.05$, $^{\#\#} p < 0.01$ vs NBP-10+OGD/R.

was mitigated by 1 μ M and 10 μ M NBP treatment (Fig. 3A, $p < 0.05$). The NBP-10+OGD/R+AR-A014418 group showed lower cell viability than the NBP-10+OGD/R group (Fig. 3A, $p < 0.05$). OGD/R-induced decreases in Nestin (green) and proliferation marker Ki-67 (red) were mitigated by 1 μ M and 10 μ M NBP treatment, according to immunofluorescence analysis, whereas AR-A014418 therapy partially reversed the impact of NBP (Fig. 3B,C). OGD/R treatment suppressed Nestin, Ki-67, p-GSK-3 β /GSK-3 β , and NRF2 levels in NSCs, according to Western blot examination (Fig. 4A–F, $p < 0.05$). NSCs in the NBP-1+OGD/R and NBP-10+OGD/R groups had higher levels of Nestin, Ki-67, p-GSK-3 β /GSK-3 β , and NRF2 than those in the OGD/R group (Fig. 4A–F, $p < 0.05$). Additionally, compared to the NBP-10+OGD/R group, NSCs in the NBP-10+OGD/R+AR-A014418 group had lower levels of these markers (Fig. 4A–F, $p < 0.05$).

Discussion

In this study, we found that NBP significantly improved the neurological function scores and reduced the infarct volume caused by ischemic stroke. Further investigation revealed that NBP increased the expression of Nestin and Ki-67 in the brain tissue following ischemic stroke. Nestin, an intermediate filament protein, is a well-known marker of NSCs and is primarily associated with the cytoskeleton [24]. Ki-67, a cell cycle-associated protein, is a classical marker for cell proliferation [25]. By upregulating these molecular markers, NBP likely plays a pivotal role in promoting NSC proliferation. A study has also indicated that overexpression of Ku70 can inhibit apoptosis of NSCs, and transplantation of Ku70-overexpressing NSCs significantly improves motor function and neurodeficits in the MCAO/R model of ischemic stroke, demonstrating its neuroprotective effects [26]. Similarly, miRNA-148b inhibitors have been shown to promote NSC proliferation, and administration of this inhibitor reduces brain ischemic

lesion volume and improves neurological outcomes [27]. Taken together, these findings suggest that NBP may promote proliferation of NSCs and alleviate the pathological changes of ischemic stroke, providing a solid foundation for exploring its therapeutic potential.

GSK-3 β is a serine/threonine protein kinase that plays a central role in cellular signal transduction. Its biological functions are broad, encompassing regulation of cell proliferation, differentiation, metabolism, and apoptosis, and it is particularly important for maintaining cellular homeostasis and function in the nervous system. The GSK-3 β pathway is closely related to proliferation of NSCs and ischemic stroke. Feng *et al.* [28] demonstrated that tetramethylpyrazine preserves viability and proliferation of NSCs through the Akt/GSK-3 β pathway, protecting NSCs from sevoflurane-induced toxicity. Yang *et al.* [29] showed that miR-199a-5p promotes proliferation of NSCs through the GSK-3 β / β -catenin signaling pathway at both cellular and *in vivo* levels. Zhang *et al.* [30] found that salvianolic acid A promotes proliferation of NSCs via Wnt3a-mediated regulation of GSK-3 β , inhibiting neuronal apoptosis and improving neurological function in rats with ischemic stroke. In this study, we found that NBP significantly enhanced the GSK-3 β signaling pathway, consistent with previous research [15]. NRF2 is a key transcription factor that regulates the antioxidant stress response and intracellular homeostasis. NRF2 plays an equally important role in proliferation of NSCs and ischemic stroke. Studies have shown that methylmercury regulates apoptosis of NSCs by inducing NRF2 expression [31]. Hu *et al.* [32] demonstrated that ginsenoside Rg1 alleviates D-galactose-induced senescence of NSCs through the NRF2 pathway. Fan *et al.* [33] showed that activation of NRF2 ameliorates blood-brain barrier injury following cerebral ischemic stroke by regulating ferroptosis and inflammation. Our study showed that NBP significantly upregulated NRF2 expression. Previous research has suggested that the GSK-3 β /NRF2 signaling pathway exerts a neuroprotection function in ischemic stroke [16]. These findings suggest that NBP may promote proliferation of NSCs through the GSK-3 β /NRF2 pathway, thereby improving neurological function after ischemic stroke and providing a new perspective for its treatment.

This study has several limitations that should be addressed in future research. First, although we assessed the short-term effects of NBP on neurological function and cell viability, the long-term outcomes, including the sustainability of functional recovery and proliferation of NSCs, remain unexplored. Second, the primary focus of this study was to investigate the regulatory role of NBP in the GSK-3 β /NRF2 pathway; however, the precise molecular mechanisms by which NBP modulates this pathway, as well as its downstream effects on neuroprotection and cellular processes, remain to be elucidated. Further investigations using advanced techniques, such as transcriptomics or proteomics, are needed to explore the intricate details of NBP's mode

of action. Moreover, the use of specific pharmacological tools (e.g., GSK-3 β inhibitors or activators) in rescue experiments *in vivo* will be essential to definitively establish GSK-3 β as the key mediator of NBP's neuroprotective effects.

Conclusion

In conclusion, this study suggests that NBP can effectively improve neurological function and promote proliferation of NSCs in a mouse model of ischemic stroke by modulating the GSK-3 β /NRF2 signaling pathway. NBP treatment significantly improves the neurological function scores, reduces infarct volume, and upregulates the expression of NSCs marker Nestin and proliferation marker Ki-67, while enhancing the expression of p-GSK-3 β /GSK-3 β and NRF2. Additionally, NBP demonstrates protective effects on viability of NSCs in the OGD/R model, primarily through the GSK-3 β /NRF2 pathway. These results highlight the potential of NBP as a novel therapeutic agent for ischemic stroke, offering promising prospects for promoting neurorepair and proliferation of NSCs.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request

Author Contributions

YC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing-original draft, Writing-review&editing. YZ: Data curation, Formal analysis, Methodology, Writing-original draft, Writing-review&editing. JW: Data curation, Validation, Methodology, Writing-original draft, Writing-review&editing. LZ: Validation, Visualization, Formal analysis, Writing-original draft, Writing-review&editing. YH: Software, Methodology, Data curation, Writing-original draft, Writing-review&editing. HL: Validation, Methodology, Supervision, Writing-original draft, Writing-review&editing. SJ: Data curation, Visualization, Investigation, Writing-original draft, Writing-review&editing. JY: Supervision, Formal analysis, Investigation, Writing-original draft, Writing-review&editing. SL: Visualization, Project administration, Resources, Writing-original draft, Writing-review&editing. ZL: Methodology, Validation, Software, Writing-original draft, Writing-review&editing. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The Animal Welfare Ethics Committee of Hebei North University gave its approval for all animal studies, which were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Approval No. HBNU20240223053).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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