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Gelsemine Exerts Neuroprotective Effects on Neonatal Mice with Hypoxic-Ischemic Brain Injury by Suppressing Inflammation and Oxidative Stress via Nrf2/HO-1 Pathway

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Abstract

Given that the role of Gelsemine in neuroinflammation has been demonstrated, this research aimed to investigate the effect of Gelsemine on neonatal hypoxic-ischemic (HI) brain injury. An in vivo HI brain injury neonatal mouse model and an in vitro oxygen–glucose deprivation (OGD) cell model were established and pretreated with Gelsemine. The brain infarct volume, neuronal loss and apoptosis, as well as spatial learning and memory were examined by TTC staining, Nissl's staining, TUNEL staining and Morris water maze test. Immunohistochemical staining was applied to detect the microglia cells and astrocytes in the mouse brain tissue. The cell viability was analyzed by CCK-8 assay. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), TNF-α, IL-1β, and IL-6 were determined via ELISA. The lactate dehydrogenase (LDH) release and reactive oxygen species (ROS) level in OGD-treated cells were detected by colorimetry and DCFH-DA staining. Nrf2, HO-1, and inflammation-related factors were analyzed by immunofluorescence, qRT-PCR, or western blot. Gelsemine reduced the infarct volume and neuronal loss and apoptosis, yet improved spatial learning and memory impairment of HI-injured mice. Gelsemine inhibited the elevated MDA, TNF-α, IL-1β, IL-6, LDH and ROS levels, promoted the reduced SOD level and viability, and strengthened the up-regulation of HO-1 and Nrf2 in brain tissues and OGD-treated cells. However, Nrf2 silencing reversed the effects of Gelsemine on the Nrf2/HO-1 pathway, inflammation, and oxidative stress in OGD-treated cells. Gelsemine produces neuroprotective effects on neonatal mice with HI brain injury by suppressing inflammation and oxidative stress via Nrf2/HO-1 pathway.

Keywords Gelsemine · Hypoxic-ischemic injury · Neonatal mice · Inflammation · Oxidative stress · Nrf2/HO-1 pathway

Introduction

Perinatal hypoxic-ischemic (HI) brain injury caused by decreased cerebral perfusion at birth is one of the main causes of acute neonatal death and chronic nerve injury [1]. Chronic nerve injury caused by HI brain injury incorporates cerebral palsy, epilepsy, as well as visual and cognitive impairments, which affects the quality of life and imposes a heavy economic burden on the family and society [2]. In view of the above, it is of great significance to explore the pathological mechanism of neonatal HI brain injury and to find promising drugs and targets for preventing and treating neonatal HI brain injury.

Gelsemine is an alkaloid extracted from *Gelsemium elegans*, which belongs to the Loganiaceae family [3]. Numerous biological activities of Gelsemine have been demonstrated in current pharmacology including anti-hyperlipidemia, anti-anxiety, analgesia, kidney protection and anti-oxidative stress. Among them, the anti-hyperlipidemic effect can be exerted on rabbits fed with high-fat diet [4], and the renoprotective and anti-oxidative stress effects can counteract cisplatin-induced toxicity [5]. Besides, Gelsemine is reported to alleviate neuropathic pain in partial sciatic nerve ligation mice [6]. Recently, Chen et al. have verified that Gelsemine can mitigate neuroinflammation and cognitive impairments in Aβ oligomer-treated mice [7]. The above evidence indicates the possible therapeutic effect of Gelsemine

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on the nervous system. Therefore, we eager to fathom out whether Gelsemine plays a role in neonatal HI brain injury.

Nrf2, a member of the basic leucine zipper family, mediates the function of assorted antioxidant and cytoprotective factors [8]. Under normal circumstances, Nrf2 is located in the cytoplasm and binds to Keap1 which promotes the degradation of Nrf2 [9]. When the cell is stimulated by different abnormalities such as hypoxia, Nrf2 can be activated as evidenced in the phenomenon that Nrf2 is released from Keap1 and translocates from cytoplasm to nucleus [9, 10]. After entering into the nucleus, Nrf2 generates antioxidative effects by promoting the transcription of downstream antioxidative enzymes such as HO-1 [11, 12]. The role of the Nrf2/HO-1 pathway in protecting the brain from HI-induced injury has been proven [13, 14]. What's more, the conduction of Nrf2/HO-1 signaling in HI brain injury, can be regulated by active ingredients from plants such as resveratrol and apigenin [15, 16]. However, whether the role of the Nrf2/HO-1 signaling in HI brain injury could be regulated by Gelsemine awaits to be expounded.

Correspondingly, in the present research, we established both the HI brain injury model in neonatal mice and oxygen–glucose deprivation (OGD) model in BV2 microglial cells, and the roles of Gelsemine in neonatal HI brain injury and the Nrf2/HO-1 signaling were firstly evaluated. Our research was devoted to unveiling novel drugs and targets to prevent and treat neonatal HI brain injury.

Materials and Methods

Animals and Ethics Statement

Animals, purchased from ALF Biotechnology (Jiangsu, Nanjing, China), were reared under 12 h of light/dark cycle in a specific pathogen-free environment with free access to food. All experiment procedures were approved by the Committee of Experimental Animals of Nanfang Hospital (Z2020120635X) and performed at Nanfang Hospital.

Neonatal HI Brain Injury Model Establishment and Grouping

Total 72 postnatal 7-day-old C57BL/6 mouse pups were used in this research and randomly divided into three groups: the sham group (n = 24), the HI group (n = 24), and the HI + Gelsemine group (n = 24). Mice in the HI group received HI brain injury model establishment based on Rice-Vannucci methods [17]. In brief, after the mouse pups were comatose by inhaling isoflurane (R510-22, RWD, Shenzhen, Guangdong), the right common carotid artery (CCA) of all mice was subjected to electrocoagulation by an electric knife, and then all pups were allowed to stay with their mothers for 1 h

(h). After that, the pups were placed in an oxygen-deficient box (gas mixture of 8% O_2 and 92% N_2 at a flow rate of 2 l/min) for 2 h and then returned to normoxic conditions. Mice in the sham group were anesthetized by inhaling isoflurane, and the CCA was exposed without ligation and hypoxia. Mice in the HI + Gelsemine group were treated with 10 μg/kg Gelsemine (N2313, APExBIO, Houston, Texas, USA) via intraperitoneal injection 20 min before the establishment of HI brain injury model [7].

Seventy-two hours after surgery, 16 mouse pups in each group were euthanized by isoflurane. The brain tissues were harvested and evenly divided into 4 parts to participate in 4 groups of assays, namely, 2,3,5-Triphenyltetrazolium chloride (TTC) staining; Nissl's staining; terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining; as well as enzyme-linked immunosorbent assay (ELISA), immunofluorescence staining and western blot. 4 weeks after surgery, the remaining 8 mice in each group received Morris water maze (MWM) tests and then euthanized.

TTC Staining

Seventy-two hours after surgery, 4 mouse pups in each group were euthanized by isoflurane and the brain tissues were collected for TTC staining [17]. In short, the brain tissues were collected and sectioned into 2 mm coronal slices, followed by being soaked in 1% TTC staining buffer (G3005, Solarbio, Beijing, China) for about 2 min (min). The normal brain tissues were stained red with TTC, and the infarct tissues were stained white with TTC. The staining images of brain tissues were observed under the P30 mobile phone (Shenzhen, Guangdong, China) and the infarct volume of brain tissues was analyzed using version 1.8.0 Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Nissl's Staining

Seventy-two hours after surgery, 4 mouse pups in each group were euthanized by isoflurane and the brain tissues were removed for assessing neuron loss through Nissl's staining according to the previous research with some modifications [18]. Concretely, the brain tissues were firstly fixed with 4% paraformaldehyde fixative buffer (P1110, Solarbio) for 24 h, followed by being embedded into paraffin (YA0012, Solarbio). Then, the tissues were sectioned into 5 μm slices, incubated with xylene (B50009, Meryer, Shanghai, China), 100% ethanol (E111991, Aladdin, Shanghai, China), 90% ethanol, 80% ethanol and 70% ethanol, and washed by distilled water for 2 min successively. Subsequently, the tissues were stained with 1% toluidine blue solution (G3668, Solarbio) for 25 min and washed with distilled water for 3 min. After further being cultivated with 70% ethanol, 80%

ethanol, 90% ethanol, 100% ethanol and xylene, and sealed with neutral gum (G8590, Solarbio), the image was observed under a microscopic imaging system (THUNDER, Leica, Weztlar, Germany). Results are expressed as the number of surviving neuron within each group relative to that of the Sham group.

TUNEL Staining

Seventy-two hours after surgery, 4 mouse pups in each group were euthanized by isoflurane and the brain tissues were taken out for analyzing cell apoptosis in the hippocampus through TUNEL staining based on the previous research with some modifications [19]. The TUNEL staining kit was bought from Beyotime (C1091, Shanghai, China). In a nutshell, the paraffin-embedded brain tissue slices were digested by 2% proteinase K (P9460, Solarbio) for 30 min and washed with phosphate buffered saline (PBS, P1010, Solarbio) three times. Thereafter, the tissues were incubated with endogenous peroxidase strong blocking solution (P0100B, Beyotime) for 20 min and washed with PBS three times. Next, the tissues were cultured with 50 μl biotin labeling solution at 37 °C for 1 h and washed with PBS three times, before 300 μl stopping buffer was used to stop the reaction. Then, the tissues were cultivated with 50 μl Streptavidin-HRP working buffer for 30 min and washed with PBS three times. 200 μl 3,3'-Diaminobenzidine (DAB) chromogenic solution was incubated with the tissues for 15 min, followed by the washing with PBS three times. The image was observed under a microscopic imaging system. And the cell apoptosis rate was calculated using the following formula: apoptosis rate = the number of positive cells (yellowish brown cells)/ the number of total cells × 100%.

Immunohistochemical (IHC) Staining

IHC staining was applied to detect the microglia cells and astrocytes in the brain tissue sections with reference to the previous study [20]. As mentioned before, brain tissue sections were dewaxed and hydrated with xylene and gradient ethanol. The antigen retrieval process was performed twice in Tris/EDTA buffer solution (pH 9.0, for Iba-1 antibody) or citrate buffer (pH 6.0, for GFAP antibody) for 5 min, followed by maintenance in a microwave (37 °C) for 30 min. Next, sections were allowed to cool to room temperature for 20 min and washed twice in distilled water for 5 min. And then sections were blocked with 5% goat serum (C0265, Beyotime, China) for 1 h and incubated with anti-Iba-1 primary antibody (ab178846, 1: 2000, abcam, UK) and anti-GFAP primary antibody (1: 1000) at 4 °C overnight. After washing three times in PBS, the slices were incubated with the goat anti-rabbit secondary antibody (ab6721, 1:1000, abcam, UK) at 37 °C for 2 h. After the washing with PBS,

diaminobenzidine (DAB) staining kit (P0202, Beyotime, China) was applied to stain the sections for 10 min. Two minutes post the counterstaining with hematoxylin (C0107, Beyotime, China), the tissue sections were differentiated in hydrochloric acid and alcohol. Finally, the tissue sections were dehydrated, transparentized, and mounted, followed by the observation on the color development under a microscope.

MWM Test

The MWM test was performed 4 weeks after surgery to evaluate the spatial learning and memory of the experimental mice in line with the previous research [21]. The MWM device was a circular pool with 120 cm in diameter and 70 cm in height, and the water depth of the pool was 25–35 cm with water temperature of 21–25 °C. The pool contained four quadrants, and a circular escape platform with a diameter of 10 cm was located 1.5 cm below the water surface in the center of the fourth quadrant. The formal test referred to location navigation and space exploration tests. In the location navigation test, the mice received training 4 times a day, with an interval of 15–20 min, for 5 consecutive days. In each test, the mice were admitted to find the platform within 60 s (s) and received any support from experimenter if it was applicable. The time for mice to find platform was termed as escape latency. On the sixth day, the platform was removed for a space exploration test which evaluated the ability of mice to remember the location of platform. The time of mice spending on the first visit to the platform and the number of platform crossing were documented.

Cell Culture and Gelsemine Treatment

BV2 microglial cells were bought from Procell (CL-0493, Hubei, Wuhan, China) and grown in a specific medium (CM-0493, Procell). BV2 cells were cultured in a normoxic environment at 37 °C with saturated humidity. In order to single out the appropriate concentration of Gelsemine, BV2 cells were treated with different doses of Gelsemine (0, 1, 3, 10, 30, 100 nM) for 12 h, and then collected for cell viability detection.

Cell OGD Treatment

For the cell model construction, cells were treated with OGD as previously described [21, 22], and then classified into three groups: the control group, the OGD group, and the OGD + Gelsemine group. Cells in the control group were normally cultured. Cells in the OGD group were placed into Dulbecco's Modified Eagle's Medium (DMEM, 11966025, Thermo, Waltham, Massachusetts, USA) without glucose

and FBS, followed by the culture in a hypoxia environment with 1% O_2, 5% CO_2 and 94% N2 at 37 °C for 3 h. Then, the cells were immediately placed into a medium specific for BV2 cells and cultured under the normoxic condition for 12 h. Cells in the OGD + Gelsemine group were treated with 100 nM Gelsemine for 3 h before OGD treatment.

Cell Viability Detection

The viability of cells after Gelsemine or OGD treatment was evaluated through CCK-8 assay. Briefly speaking, the cells were seeded into a 96-well plate (5000 cells/well) and treated with Gelsemine or OGD. Then 10 μl CCK-8 solution (C0037, Beyotime) was added into the cell medium and incubated for 2 h. After that, the 96-well plate was placed under a microplate reader (Varioskan LUX, Thermo) and the optical density of each well was read at a wavelength of 450 nm.

Nrf2 Immunofluorescence Staining

The Nrf2 immunofluorescence staining was carried out using the brain tissues of mice 72 h after surgery and BV2 cells after Gelsemine and OGD treatments as per previous research [23, 24]. In short, the paraffin-embedded brain tissue slices and the fixed cell slices were blocked with 10% goat serum (C0265, Beyotime) for 1 h and then incubated with Nrf2 antibody (#12721, Cell Signaling Technology (CST), Boston, Massachusetts, USA) at 4 °C overnight. After washing with PBS three times, the slices were incubated with Alexa Fluor 647-labeled goat anti-rabbit IgG (ab150083, Abcam, Cambridge, UK) for 2 h followed by being stained with 4',6-diamidino-2-phenylindole (DAPI, C0065, Solarbio) for 5 min. Finally, the slices were covered with neutral gum and the image was observed under a fluorescence microscope (DM2500, Leica).

Cell Transfection

Small interfering RNA (siRNA) against Nrf2 (siNrf2; siG150114100151-1-5) and siRNA negative control (siNC; siN0000001-1-5) were obtained from RIBOBIO (Shenzhen, Guangzhou, China). For cell transfection, the cells were cultured in a 6-well plate until the cell confluence reached about 80%, and then transfected with siNrf2 and siNC by Lip2000 (L7800, Solarbio). 48 h post transfection, the cells were collected for later processes such as Gelsemine and OGD treatment.

Western Blot Assay

Total protein in BV2 cells and brain tissue which was collected from mice after 72 h of surgery was firstly extracted through NP-40 lysis buffer (N8032, Solarbio) blended with protease inhibitor mixture (P6730, Solarbio) and phenyl-methylsulfonyl fluoride (PMSF, P0100, Solarbio). For the detection of nuclear Nrf2 protein level, NE-PER Nuclear and cytoplasmic extraction reagent (78833, Thermo Scientific, USA) was utilized to extract the nuclear proteins. Then, the protein concentration was evaluated using a BCA Protein Concentration Detection Kit (PC0020, Solarbio) under a microscope reader (562 nm wavelength). After the protein was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (P1040, Solarbio) and incubated at 100 °C for 5 min, 20 μg protein was subjected to electrophoresis in SDS-PAGE gel (P1200, Solarbio) and transferred onto PVDF membrane (ISEQ00010, Solarbio) which was pre-incubated with Activation Buffer (P0021S, Beyotime). Then, the membrane was sealed with 5% nonfat milk for 2 h, incubated with primary antibodies against Nrf2 (ab92946, 80 kDa, Abcam), HO-1 (#43966, 28 kDa, CST), H3 (17 kDa, #9728, CST) and β-actin (45 kDa, #4970, CST) at 4 °C overnight, and cultured with a secondary antibody goat-anti rabbit IgG (ab6721, Abcam) for 2 h the next day. The membrane was further cultured with ECL Western Blotting Substrate (PE0010, Solarbio) for 1 min. Finally, the protein signaling on the membrane was determined under the Image Lab 3.0 detector (Bio-Rad, Hercules, California, USA).

Quantitative RT-PCR (qRT-PCR) Assay

Total mRNA in BV2 cells after treatment was firstly extracted through Total RNA Extraction Kit (R1200, Solarbio). Then, the mRNA was reverse-transcribed into cDNA using QuantiNova Reverse Transcription Kit (205413, Qiagen, Dusseldorf, Germany) after the concentration was determined. Subsequently, the cDNA was mixed with SYBR Green buffer (A25742, Thermo) and relative gene primers for amplification reaction under Real-Time PCR System (QuantStudio 6 Flex, Thermo). Gene primer information is as follows: TNF-α forward: 5′-CCCTCACACTCAGATCATCTTCT-3′, TNF-α reverse: 5′-GCTACGACGTGGGCTACAG-3′; IL-1β forward: 5′-GCAACTGTTCCTGAACTCAACT-3′, IL-1β reverse: 5′-ATCTTTTGGGGTCCGTCAACT-3′; IL-6 forward: 5′-TAGTCCTTCCTACCCCAATTTCC-3′, IL-6 reverse: 5′-TTGGTCCTTAGCCACTCCTTC-3′, β-actin forward: 5′-GGCTGTATTCCCCTCCATCG-3′, β-actin reverse: 5′-CCAGTTGGTAACAATGCCATGT-3′.

Inflammatory Factor and Oxidative Stress Factor Detection

Mouse TNF-α ELISA kit (DY410), mouse IL-1β ELISA kit (DY401), and mouse IL-6 ELISA kit (DY406) were acquired from R&D System (Minneapolis, Minnesota,
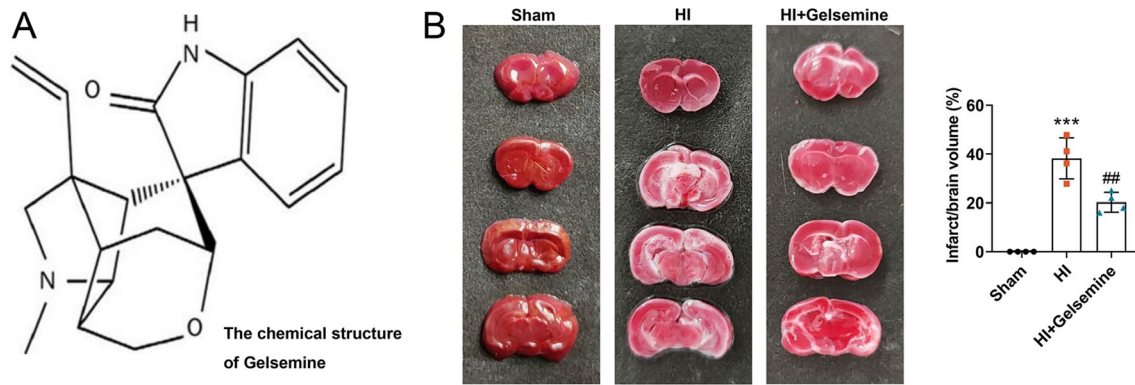
Fig. 1 Gelsemine pretreatment reduced brain infarct volume of neonatal mice with HI brain injury. **A** The chemical structure of Gelsemine was presented. **B** Seventy-two hours after HI injury, the brain infarct of neonatal mice was evaluated through TTC staining. (***$P < 0.001$, vs. sham; ##$P < 0.01$, vs. HI). (*HI* hypoxic-ischemic, *TTC* 2,3,5-Triphenyltetrazolium chloride)

USA). Specifically, 100 µl supernatant of BV2 cells and homogenate of brain tissue were added into a 96-well plate, incubated for 2 h, and rinsed off using wash buffer. Then, 100 µl Detection Antibody was added to each well, incubated for 2 h and again washed with wash buffer. 100 µl working dilution of Streptacidin-HRP was introduced into each well for 2-h incubation in the dark, followed by washing with wash buffer. 100 µl substrate solution was added into each well for 20-min incubation in the dark followed by the addition of 50 µl Stop Solution. Finally, the optical density of each well was immediately determined using a microplate reader at a wavelength of 450 nm. In addition, the content of oxidative stress factors including malondialdehyde (MDA) and superoxide dismutase (SOD) in brain tissues and BV2 cells was detected using MDA ELISA detection kit (F01963, Xitang biotechnology, Shanghai, China) and SOD ELISA detection kit (F11502, Xitang biotechnology) in the light of the manufacturer's instructions. The levels of TNF-α, IL-1β, IL-6, MDA, and SOD were calculated based on the optical density in accordance with the manufacturer's protocol.

Lactate Dehydrogenase (LDH) Release Detection

The release level of LDH in BV2 cells after treatment was evaluated using an LDH detection kit (C0017, Beyotime). In detail, BV2 cells were placed into a 96-well plate and treated with Gelsemine and OGD. Subsequently, the supernatant of BV2 cells was collected and treated on the basis of the procedure proposed by the manufacturer of the LDH detection kit. Finally, the optical density of each well was immediately determined using a microplate reader at a wavelength of 490 nm. The release of LDH was lastly calculated according to a formula provided by the manufacturer.

Reactive Oxygen Species (ROS) Detection

The intracellular ROS level of BV2 cells was determined using DCFH-DA regent (S0033S, Beyotime). In brief, after treatment, the culture medium was discarded and 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) buffer diluted with DMEM without FBS (1:1000) was cultivated with the cells for 30 min, followed by washing with PBS three times. Ultimately, the DCFH-DA fluorescence in cells was observed under a fluorescence microscope.

Statistical Analysis

All data were analyzed in Graphpad prism 8.0 using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. Statistical data were presented as Mean ± Standard Deviation (SD). $P < 0.05$ meant a statistically difference.

Results

Gelsemine Pretreatment Reduced Brain Infarct Volume of Neonatal Mice with HI Brain Injury

To investigate the role of Gelsemine in neonatal HI brain injury, the models were constructed in neonatal mice with HI brain injury which were pretreated with Gelsemine (whose chemical structure was presented in Fig. 1A). Seventy-two hours after HI injury, the brain infarct was evaluated through TTC staining, as depicted in Fig. 1B, no infarction was observed in mouse brain tissue of the sham group, while the brain infarct volume surged in the HI group ($P < 0.001$), but Gelsemine pretreatment reduced such evident increment ($P < 0.01$). These findings indicated that Gelsemine exerted improving effects on brain infarct of neonatal mice with HI brain injury.
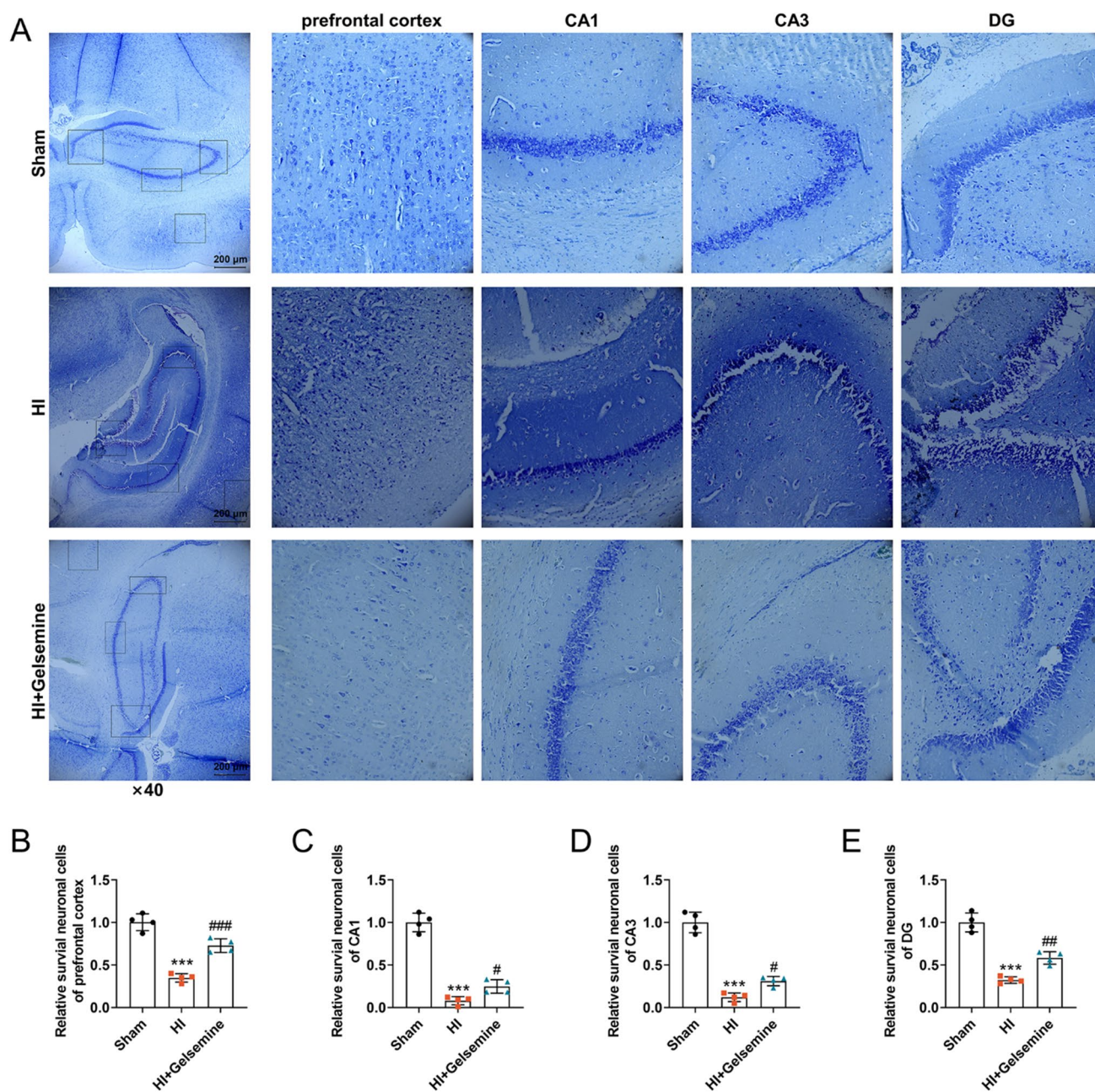
Fig. 2 Gelsemine pretreatment attenuated HI-induced neuronal loss and apoptosis of neonatal mice with HI brain injury. **A** Seventy-two hours after HI injury, the neuronal loss of mouse brain tissues was determined using Nissl's staining, with magnification ×40 and scale bar = 200 μm (left), and the relative enlarged image of the left was shown in the right. **B**, **E** Relative survival neuronal cells of prefrontal cortex (**B**), CA1 (**C**), CA3 (**D**), and DG (**E**). (***$P < 0.001$, vs. sham; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, vs. HI). (*HI* hypoxic-ischemic, *CA* hippocampus, DG: dentate gyrus)

Gelsemine Pretreatment Attenuated HI-Induced Neuronal Loss and Apoptosis of Neonatal Mice with HI Brain Injury

The neuronal loss and apoptosis of mouse brain tissues were determined using Nissl's staining (Fig. 2A-E) and TUNEL staining (Fig. 3A, B). As exhibited in Fig. 2, heaps of neuronal losses were discovered in the prefrontal cortex (Fig. 2A and B, $P < 0.001$), CA1 (Fig. 2A and C, $P < 0.001$), CA3 (Fig. 2A and D, $P < 0.001$), and DG regions (Fig. 2A and E, $P < 0.001$) in neonatal mice with HI brain injury, whereas the neuronal loss in each region was then alleviated by Gelsemine pretreatment (Fig. 2A–E, $P < 0.05$). Similarly, loads of neuronal apoptosis was observed in
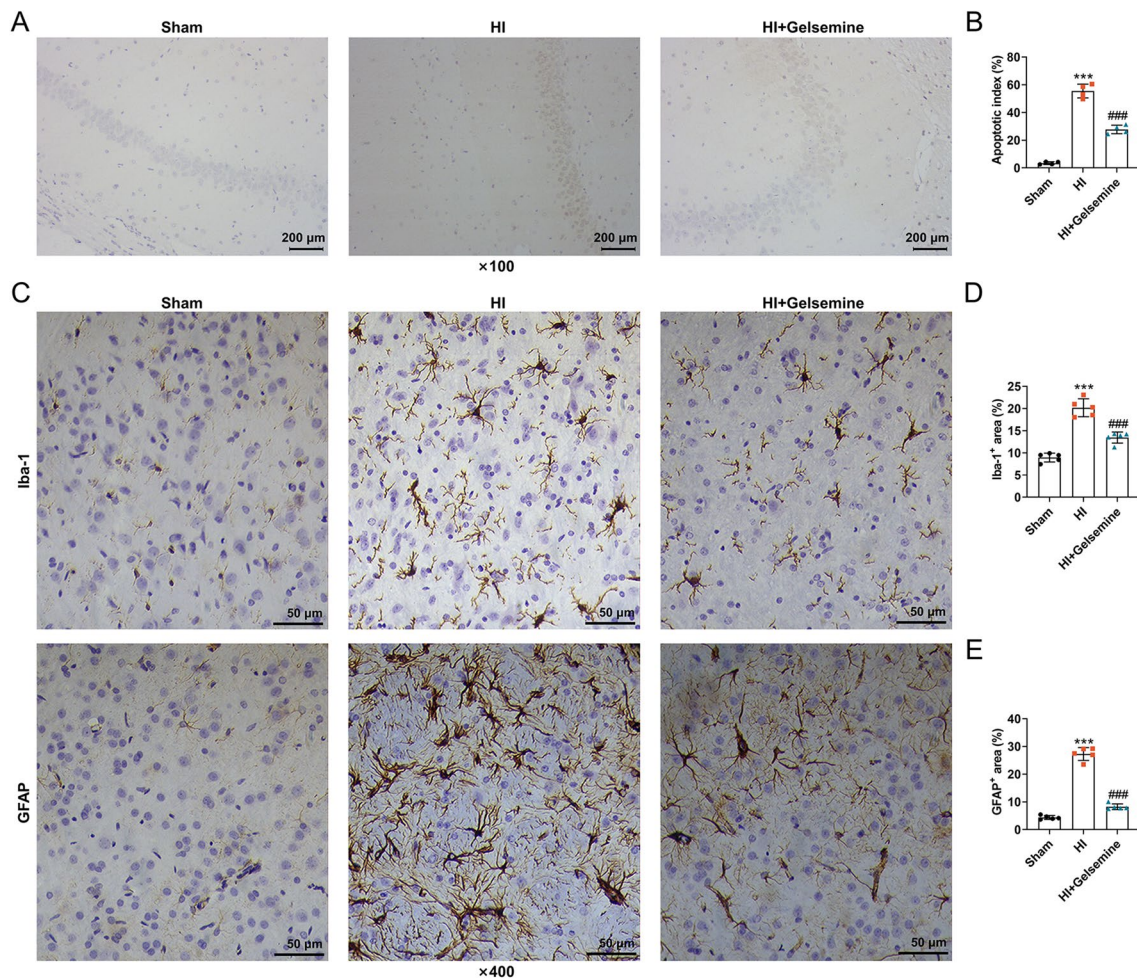
Fig. 3 Gelsemine pretreatment attenuated HI-induced neuronal apoptosis and activation of microglia cells and astrocytes in neonatal mice with HI brain injury. **A**, **B** Seventy-two hours after HI injury, the neuronal apoptosis of mouse brain tissues was determined using TUNEL staining, with magnification ×100 and scale bar = 200 μm. **C–E** Immunohistochemical staining was applied to detect the microglia cells and astrocytes in the mouse brain tissue, with magnification ×400 and scale bar = 50 μm. (***$P < 0.001$, vs. sham; ###$P < 0.001$, vs. HI). (*HI* hypoxic-ischemic)

hippocampus tissues of mice with HI brain injury (Fig. 3A and B, $P < 0.001$), which was also mitigated by Gelsemine pretreatment (Fig. 3A and B, $P < 0.001$). In addition, IHC staining showed that microglia cells and astrocytes were activated in neonatal mice with HI brain injury (Fig. 3C–E, $P < 0.001$), while the treatment of Gelsemine could reduce the activation of microglia cells and astrocytes in model mice (Fig. 3C–E, $P < 0.001$).

Gelsemine Pretreatment Mitigated the Oxidative Stress and Inflammation and promoted the Nrf2 Nuclear Translocation of Neonatal Mice with HI Brain Injury

The factors related to oxidative stress and inflammation in mouse brain tissues were examined. In accordance with

Fig. 4A-B, the content of MDA (Fig. 4A) was increased and the activity of SOD (Fig. 4B) was dampened in HI group ($P < 0.001$), but the two trends were reversed by Gelsemine pretreatment ($P < 0.001$). Meanwhile, the levels of TNF-α (Fig. 4C), IL-1β (Fig. 4D), and IL-6 (Fig. 4E) were all up-regulated in HI mice ($P < 0.001$), while Gelsemine pretreatment down-regulated the above three levels ($P < 0.001$). Owing to that Nrf2 is a widely recognized factor in restoring oxidative stress in the brain, we detected the nuclear translocation of Nrf2 through immunofluorescence. It can be noted from Fig. 4F that Nrf2 fluorescence was enhanced in nucleus of HI mice, and further strengthened by Gelsemine pretreatment.

Fig. 4 Gelsemine pretreatment attenuated the oxidative stress and inflammation and promoted the translocation of Nrf2 from the cytoplasm to nucleus after HI injury. **A–E** Seventy-two hours after HI injury, the levels of factors related to oxidative stress (MDA, SOD) and inflammation (TNF-α, IL-1β, and IL-6) in brain tissues were examined using ELISA. **F** Seventy-two hours after HI injury, the nuclear translocation of Nrf2 in brain tissue was determined using immunofluorescence, with magnification ×200 and scale bar = 100 μm. (***$P < 0.001$, vs. sham; ###$P < 0.001$, vs. HI). (*HI* hypoxic-ischemic, *MDA* malondialdehyde, *SOD* superoxide dismutase)
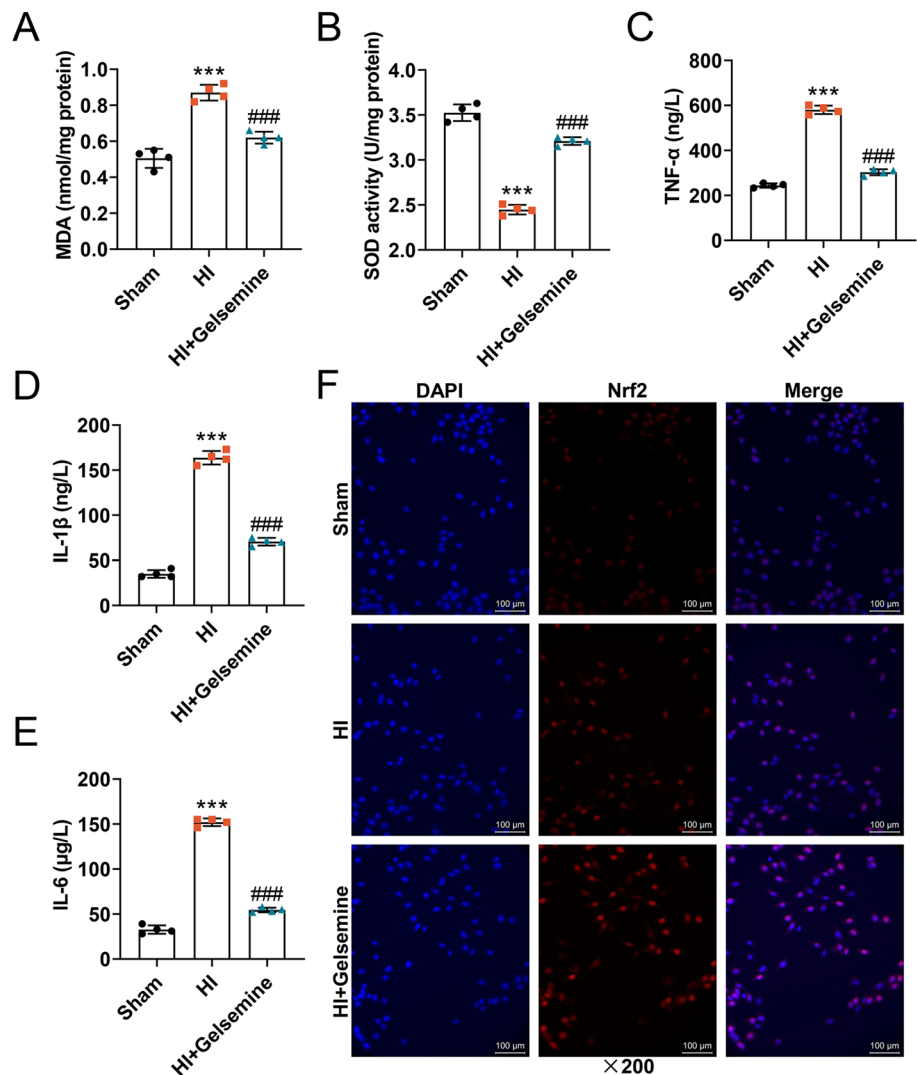
Gelsemine Pretreatment Promoted Nrf2/HO-1 Pathway and Improved the Spatial Learning and Memory of Neonatal Mice with HI Brain Injury

Considering that down-regulation of Nrf2 in oxidative stress was mediated by regulating down-stream antioxidant factors such as HO-1 [11], Nrf2 and HO-1 protein expressions were detected through western blot. The data revealed that Nrf2 level in the nucleus and HO-1 level were up-regulated in the brain tissue of HI mice (Fig. 5A, B, $P < 0.001$), and further intensified by Gelsemine pretreatment (Fig. 5A, B, $P < 0.001$). Four weeks after HI injury, the spatial learning and memory of the remaining mice were evaluated using the MWM test. As illustrated in Fig. 5C–E, the escape latency (Fig. 5C) and the time spending on the first visit to the platform (Fig. 5D) were increased, and the number of platform crossing (Fig. 5E) was lessened ($P < 0.001$), but Gelsemine pretreatment offset such alterations of the three indexes ($P < 0.001$), manifesting that Gelsemine pretreatment improved the spatial learning and memory of neonatal mice with HI brain injury.

Gelsemine Ameliorated the Injury, Inflammation, and Oxidative Stress in OGD-Treated BV2 Cells

In vitro experiments were implemented hereby. The role of Gelsemine in the viability of BV2 cells was evaluated, uncovering that the cell viability exhibited no statistical change after being treated with 1, 3, 10, 30, and 100 nM Gelsemine for 12 h (Fig. 6A). Then 100 nM Gelsemine was chosen to pretreat the OGD-induced BV2 cells, revealing that Gelsemine boosted the decreased viability of BV2 cells induced by OGD ($P < 0.001$, Fig. 6B). Meanwhile, the release and mRNA expressions of inflammation-related factors were examined, as mirrored in Fig. 6C-H that the mRNA expressions and release of TNF-α (Fig. 6C and F), IL-1β (Fig. 6D and G), and IL-6 (Fig. 6E and H) were promoted by OGD ($P < 0.001$), while Gelsemine pretreatment neutralized the promoting effect of OGD ($P < 0.001$).
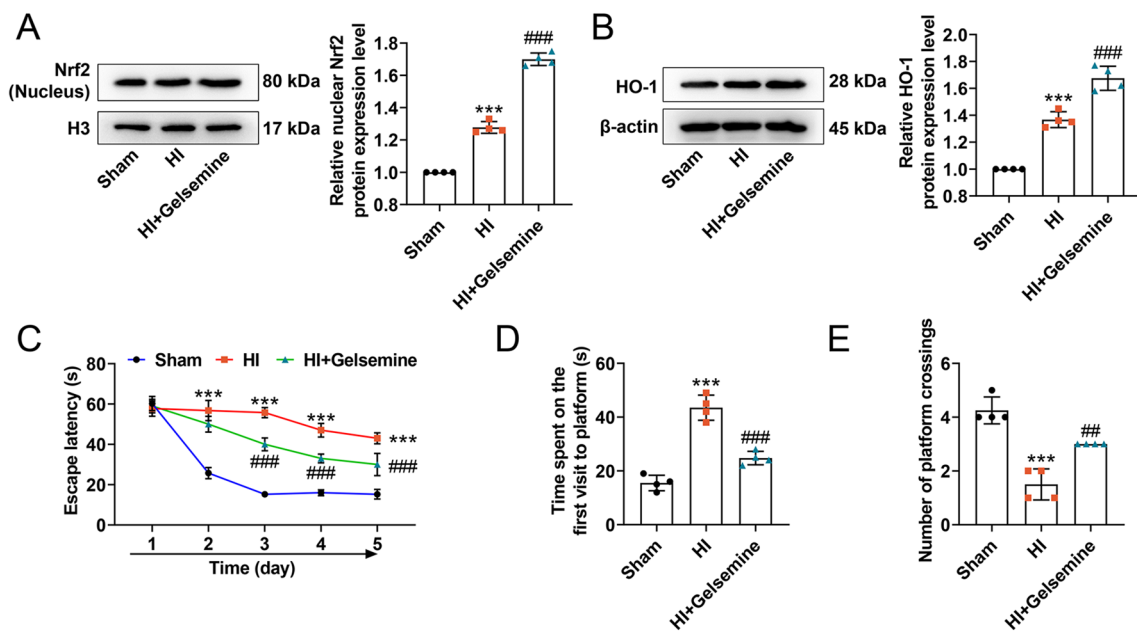
Fig. 5 Gelsemine pretreatment promoted Nrf2/HO-1 pathway and improved the spatial learning and memory of mice with HI brain injury. **A**, **B** Seventy-two hours after HI injury, the expressions of nucleus Nrf2 (**A**) and total HO-1 (**B**) in brain tissues were examined using western blot. **C–E** Four weeks after HI injury, the spatial learning and memory of the remaining mice were evaluated using the MWM test. (***$P < 0.001$, vs. sham; ##$P < 0.01$, ###$P < 0.001$, vs. HI). (*HI* hypoxic-ischemic, *MWM* Morris water maze)

To check the injury of BV2 cells after OGD, the release of LDH in BV2 cells was determined to be increased ($P < 0.001$, Fig. 6I), but was then dwindled by Gelsemine pretreatment ($P < 0.001$, Fig. 6I). Furthermore, oxidative stress was evaluated. The ROS level in cells was firstly evaluated through DCFH-DA staining. Figure 6J reflected that the fluorescence intensity was stronger in the OGD group than in the control group, but was recovered to be nearly normal in the OGD+Gelsemine group, implying that Gelsemine pretreatment offset the up-regulating effect of OGD on ROS level in BV2 cells. Therefore, the content of MDA and the activity of SOD in cells were evaluated soon after, identifying that the content of MDA was increased and the activity of SOD was suppressed in OGD-induced BV2 cells ($P < 0.001$, Fig. 7A, B), while Gelsemine pretreatment weakened such regulatory role of OGD ($P < 0.001$).

Gelsemine Pretreatment Promoted the Nrf2/HO-1 Pathway of OGD-Treated BV2 Cells

The Nrf2/HO-1 pathway of BV2 cells after OGD treatment was subsequently detected (Fig. 7C-D). Nrf2 expression in nucleus and total HO-1 of BV2 cells were up-regulated by OGD ($P < 0.001$), which was then further enhanced by Gelsemine pretreatment ($P < 0.001$). Consistent with the above results of western blot, the nuclear translocation of Nrf2 was then visualized by immunofluorescence (Fig. 7E), confirming that the Nrf2 fluorescence in nucleus

was increased in OGD-treated cells, which was also further intensified by Gelsemine.

Nrf2 Down-Regulation Reversed the Role of Gelsemine in the Nrf2/HO-1 Pathway, Inflammation, and Oxidative Stress in OGD-Induced BV2 Cells

To further confirm whether the role of Gelsemine in OGD-induced BV2 cells was realized through modulating the Nrf2/HO-1 pathway, siNrf2 was transfected into the BV2 cells. The results verified that the expressions of nuclear Nrf2 and total Nrf2 as well as the protein expression of the downstream HO-1 were dwindled by siNrf2 ($P < 0.001$, Fig. 8A-C). Then, the inflammation-associated factors and cell injury-related factors were evaluated. The levels of TNF-α (Fig. 8D and G), IL-1β (Fig. 8E and H), IL-6 (Fig. 8F and I), and LDH (Fig. 8J) in OGD-induced BV2 cells were discovered to be down-regulated by Gelsemine ($P < 0.001$), while siNrf2 starkly neutralized the effects of Gelsemine on these factors ($P < 0.001$). Meanwhile, the evaluation of oxidative stress was depicted in Fig. 9A. The fluorescence intensity of OGD-induced BV2 cells was diminished by Gelsemine, but the inhibiting effect of Gelsemine was offset by siNrf2. Gelsemine reduced the content of MDA and increased the activity of SOD in OGD-induced BV2 cells ($P < 0.001$, Fig. 9B, C), while
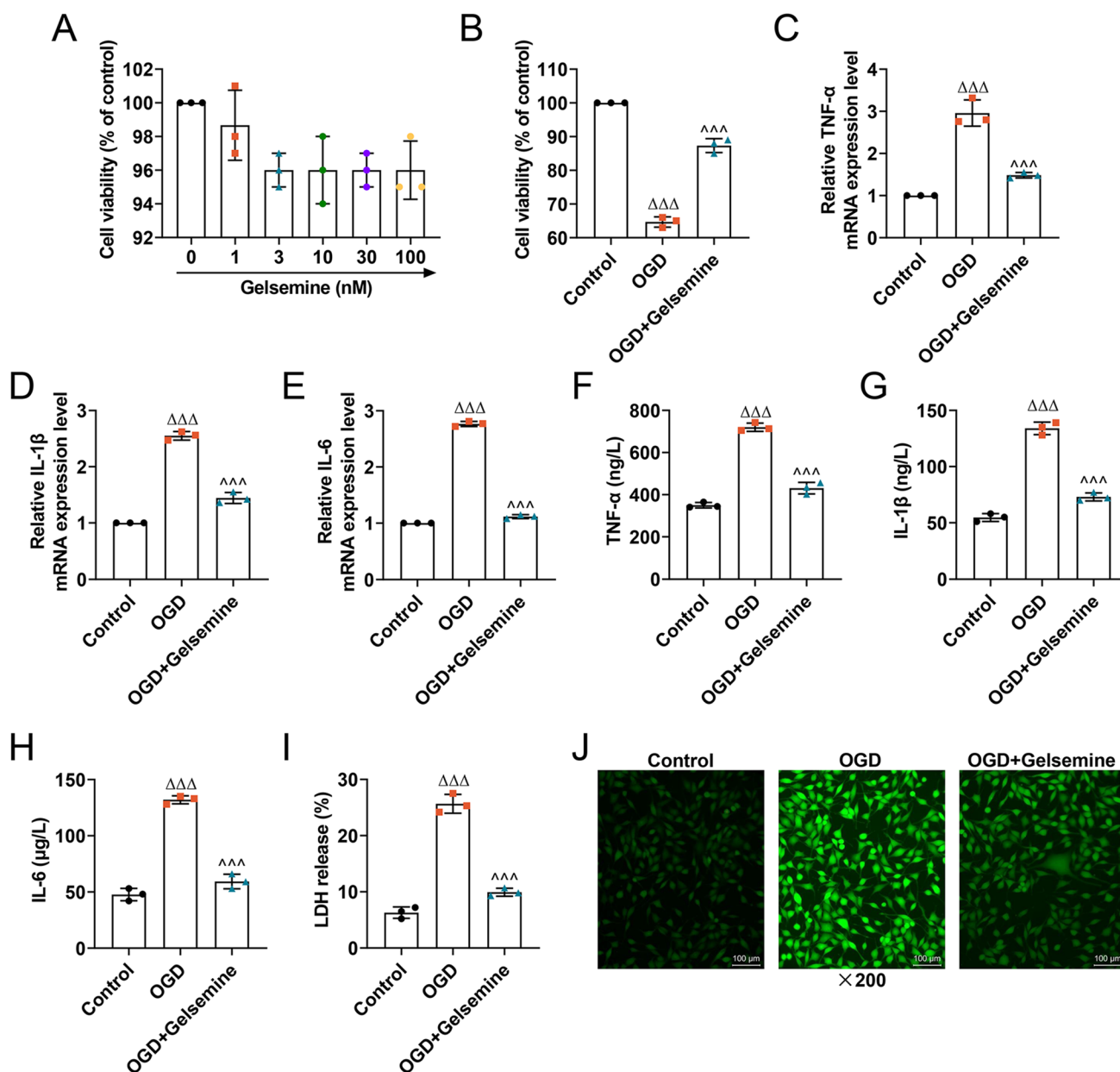
Fig. 6 Gelsemine mitigated the injury, inflammation, and oxidative stress in BV2 cells after OGD treatment. **A** The viability of BV2 cells after being treated with different doses of Gelsemine for 12 h was detected using CCK-8 assay. **B** The viability of BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment was detected using CCK-8 assay. **C–E** The mRNA expressions of TNF-α (**C**), IL-1β (**D**), and IL-6 (**E**) in BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment were quantitated through qRT-PCR. **F–H** The levels of TNF-α (**F**), IL-1β (**G**), and IL-6 (**H**) in BV2 cells after OGD treat-

ment and 100 nM Gelsemine pretreatment were determined using ELISA. **I** The release of LDH in BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment was analyzed by colorimetry. **J** The ROS level of BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment was visualized by DCFH-DA staining, with magnification ×200 and scale bar = 100 μm. ($^{\triangle\triangle\triangle}P < 0.001$, vs. Control; $^{\wedge\wedge\wedge}P < 0.001$, vs. OGD). (*OGD* oxygen–glucose deprivation, *qRT-PCR* quantitative RT-PCR, *LDH* lactate dehydrogenase, *ROS* reactive oxygen species)

Nrf2 silencing reversed the role of Gelsemine in regulating MDA and SOD levels ($P < 0.01$, Fig. 9B, C).

Discussion

As an alkaloid extracted from *Gelsemium elegans*, Gelsemine possesses multiple biological activities such as anti-hyperlipidemia, anti-anxiety, analgesia, and kidney
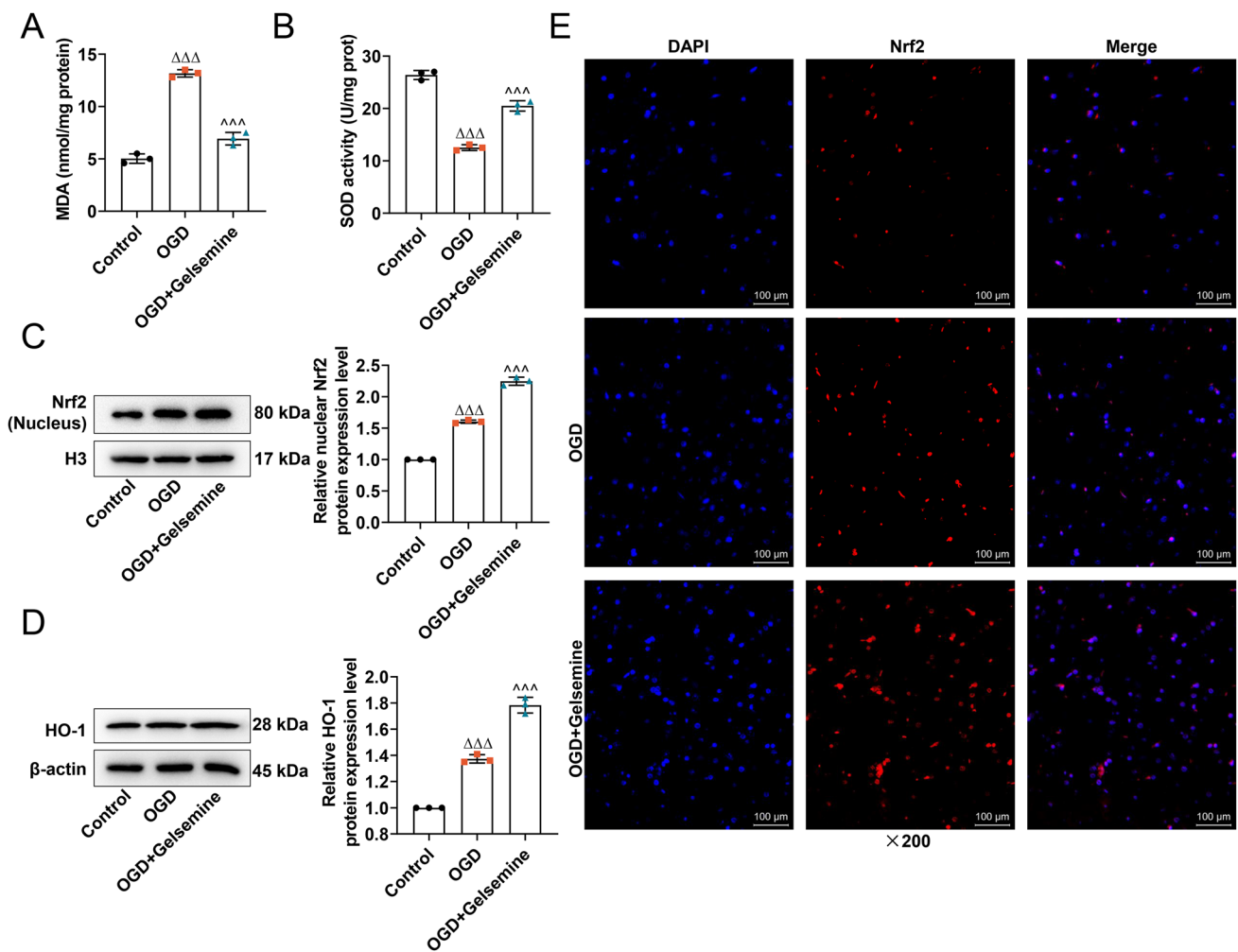
Fig. 7 Gelsemine pretreatment promoted the Nrf2/HO-1 pathway of BV2 cells after OGD treatment. **A-B** The contents of MDA (**A**) and SOD (**B**) in BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment were evaluated through ELISA. **C-D** The expressions of nuclear Nrf2 (**C**) and total HO-1 (**D**) in BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment were determined using western blot. **E** The nuclear translocation of Nrf2 in BV2 cells after OGD treatment and 100 nM Gelsemine pretreatment was determined using immunofluorescence, with magnification ×200 and scale bar = 100 μm. ($^{\triangle\triangle\triangle}P < 0.001$, vs. Control; $^{\wedge\wedge\wedge}P < 0.001$, vs. OGD). (*OGD* oxygen–glucose deprivation, *MDA* malondialdehyde, *SOD* superoxide dismutase)

protection in different types of diseases such as hyperlipidemia, chronic pain, and tumor [4, 25]. Recently, Gelsemine is reported to alleviate neuropathic pain in partial sciatic nerve ligation mice [6]. Besides, Chen et al. demonstrated that Gelsemine can mitigate neuroinflammation and cognitive impairments in Aβ oligomer-treated mice [7]. In the present research, the mitigative effect of Gelsemine on brain infarct volume as well as spatial learning and memory impairments of neonatal mice with HI brain injury was first discovered, signifying that Gelsemine might have a therapeutic effect on neonatal HI brain injury, the regulated mechanism of which acquires more exploration.

The brain infarct and spatial learning and memory impairments may result from neuronal apoptosis and loss [26, 27]. In this study, the improving effect of Gelsemine on neuronal

apoptosis and loss was also verified in neonatal mice with HI brain injury. In addition, biological genetic failure such as cognitive impairment and cell damage containing apoptosis of neurons and microglia are caused by OGD which is induced by HI injury. Moreover, the exacerbated cascade of oxidative stress and inflammatory response further leads to cell death and ultimately dysfunction [21]. Hence, blocking oxidative stress and inflammation in the early stage is instrumental in reducing injury. SOD is an important antioxidant and MDA is the final oxidation product. A previous research has reported that during HI brain injury, SOD level is down-regulated while MDA level is up-regulated [14]. In the meantime, the levels of inflammation mediators including TNF-α, IL-1β and IL-6 are elevated to stimulate various molecular signaling pathways and to take part in neuron
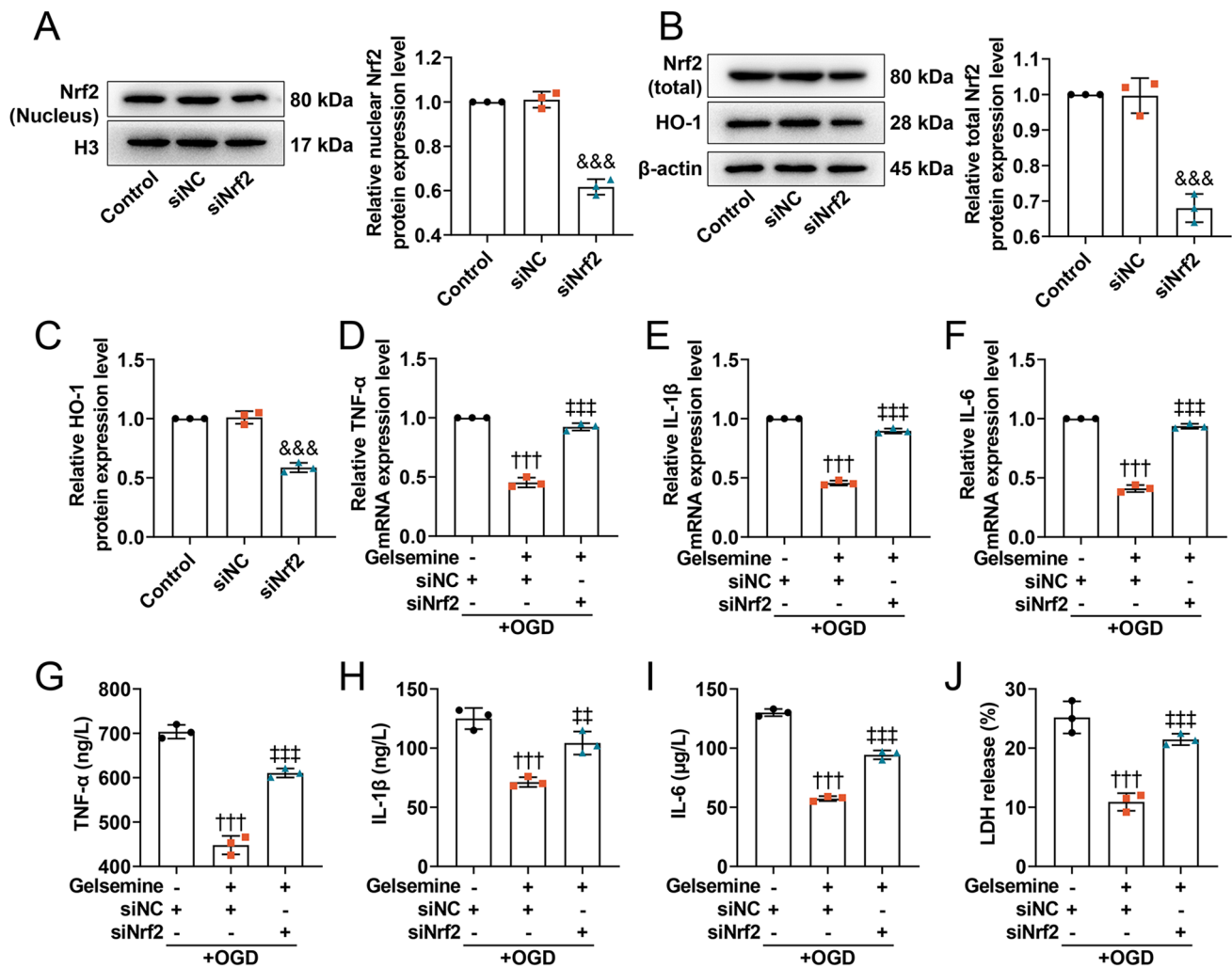
Fig. 8 Nrf2 down-regulation reversed the role of Gelsemine in the Nrf2/HO-1 pathway and inflammation in OGD-induced BV2 cells. **A–C** After cells were transfected with siNrf2, the expressions of nuclear Nrf2 (**A**), total Nrf2 and HO-1 (**B**, **C**) in BV2 cells were quantitated by western blot. **D–F** After siNrf2 transfection, Gelsemine pretreatment and OGD treatment, the mRNA expressions of TNF-α (**D**), IL-1β (**E**), and IL-6 (**F**) in BV2 cells were evaluated through qRT-PCR. **G-I** After siNrf2 transfection, Gelsemine pretreat-ment and OGD treatment, the release of TNF-α (**G**), IL-1β (**H**), and IL-6 (**I**) in BV2 cells was evaluated by ELISA. (**J**) After siNrf2 transfection and Gelsemine pretreatment, the release of LDH in BV2 cells was evaluated by colorimetry. ($^{\&\&\&}P < 0.001$, vs. siNC; $^{\dagger\dagger\dagger}P < 0.001$, vs. OGD + siNC; $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$, vs. OGD + Gelsemine + siNC). (*OGD* oxygen–glucose deprivation, *qRT-PCR* quantitative RT-PCR, *LDH* lactate dehydrogenase)

loss and brain repair [28]. Similar to previous research, the present study evidenced that Gelsemine down-regulated the MDA, TNF-α, IL-1β, and IL-6 levels and up-regulated SOD level, corroborating the suppressive effects of Gelsemine on the oxidative stress and inflammation in HI brain injury.

Microglia, which is important for developing brain tissue, is regarded to play a crucial role in the maturation of white matter and axon intersections, thereby forming a highly integrated network of neuronal fibers [29]. Furthermore, the activation of microglia, considered to be the marker of neuroinflammation in the brain after HI injury, takes the charge of stimulating the early and palpable inflammation in the immature brain [30]. Additionally, microglia-produced

ROS after OGD induction damages the microglia itself, which further leads to nerve damage [31]. Therefore, in this research, we established OGD-induced microglia model using BV2 cells and demonstrated the alleviating impacts of Gelsemine on oxidative stress and inflammation in OGD-induced BV2 cells through regulating the expressions of MDA, SOD, TNF-α, IL-1β and IL-6, which further proved the in vivo discoveries.

Nrf2/HO-1 signaling pathway modulates oxidative stress response in different types of diseases, for instance, type 2 diabetic osteoporosis, osteoarthritis, ulcerative colitis, and cerebral ischemia/reperfusion injury [32–35]. Nrf2, a member of the basic leucine zipper family, is normally located in

Fig. 9 Nrf2 down-regulation reversed the role of Gelsemine in the oxidative stress of OGD-induced BV2 cells. **A** After siNrf2 transfection, Gelsemine pretreatment and OGD treatment, the ROS level of BV2 cells was visualized by DCFH-DA staining, with magnification ×200 and scale bar = 100 μm. **B**, **C** After siNrf2 transfection, Gelsemine pretreatment and OGD treatment, the content of MDA (**B**) and the activity of SOD (**C**) in BV2 cells were determined using ELISA. (&&&$P < 0.001$, vs. siNC; †††$P < 0.001$, vs. OGD + siNC; ‡‡$P < 0.01$, ‡‡‡$P < 0.001$, vs. OGD + Gelsemine + siNC). (*OGD* oxygen–glucose deprivation, *ROS* reactive oxygen species, *MDA* malondialdehyde, *SOD* superoxide dismutase)
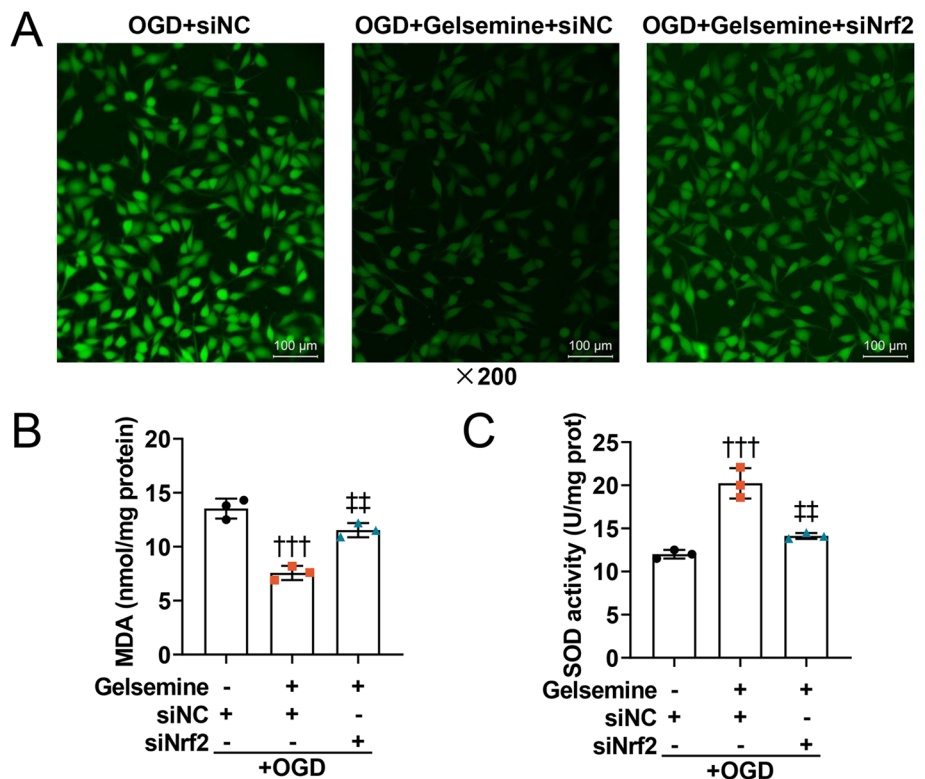
the cytoplasm and translocated from cytoplasm to nucleus under a stress environment such as hypoxia [10, 36]. After Nrf2 enters the nucleus, the levels of downstream factors such as HO-1 can be up-regulated by Nrf2 which exerts an antioxidative effect [11]. Hence, Nrf2 is regarded as a neuroprotective factor in brain HI injury [14]. For example, the protective effect of sevoflurane against HI-induced brain injury is mediated by up-regulating Nrf2 level [13], and the inhibiting effect of resveratrol on oxidative stress in neonatal rats with HI brain injury is realized through promoting Nrf2/HO-1 signaling pathway [15]. Besides, the conduction of Nrf2/HO-1 signaling in diverse diseases including HI brain injury can be regulated by active ingredients from plants such as resveratrol and apigenin [15, 16]. In this research, we evidenced that the expressions of nuclear Nrf2 and total HO-1 were up-regulated in mice with HI brain injury and in OGD-treated cells, which were further enhanced by Gelsemine, reflecting that the role of Gelsemine in neonatal HI brain injury was achieved by promoting Nrf2/HO-1 signaling. Moreover, this indication was further verified through data from experiments involving Nrf2 silencing.

In conclusion, our research authenticated that Gelsemine alleviates the inflammation and oxidative stress, and improves the spatial learning and memory impairments of neonatal mice with HI brain injury through promoting the Nrf2/HO-1 pathway. The discoveries in this research provide novel drugs and targets for treating neonatal HI brain injury.

Data Availability The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Declarations

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