Genistein mitigates oxidative stress and inflammation by regulating Nrf2/HO-1 and NF-κB signaling pathways in hypoxic-ischemic brain damage in neonatal mice

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Background: Oxidative stress and neuroinflammation play crucial roles in the progression of neonatal hypoxic-ischemic brain damage (HIBD). Genistein, a natural phytoestrogen, has been found to protect against ischemic brain injury. However, its effects and potential mechanisms in HIBD have not yet been explored.

Methods: A neonatal mouse model of hypoxia-ischemia (HI) and a cell model of oxygen-glucose deprivation/reperfusion (OGD/R) were employed. In the *in vivo* study, genistein (10 mg/kg; ip) was administered in mice once daily for 3 consecutive days before the operation and once immediately after HI. The effects of genistein treatment on acute brain damage and long-term responses were evaluated. Neuronal injury and apoptosis were estimated using hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, respectively. The expression of apoptosis-related proteins were also measured by Western blot analysis. Dihydroethidium (DHE) staining and glutathione (GSH) and malondialdehyde (MDA) production were determined to assess the extent of oxidative stress. The messenger RNA (mRNA) levels of proinflammatory cytokines were detected using real-time quantitative polymerase chain reaction (RT-qPCR) to evaluate the extent of neuroinflammation. In the *in vitro* study, cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assays, as well as propidium iodide (PI) staining, were performed to analyse the neuroprotective effects of genistein on primary cortical neurons. Western blot assays were used to detect the levels of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), phosphorylated inhibitor kappa B-α (p-IκB-α) and phosphorylated nuclear factor-kappa B (p-NF-κB) both *in vivo* and *in vitro*.

Results: Our results showed that genistein treatment effectively reduced cerebral infarction, attenuated neuronal injury and apoptosis, and contributed to the long-term recovery of neurological outcomes and brain atrophy in neonatal HIBD mice. Moreover, genistein ameliorated HIBD-induced oxidative stress and neuroinflammation. Meanwhile, genistein significantly increased cell viability, reversed neuronal injury and decreased cell apoptosis after OGD/R injury. Finally, the activation of the Nrf2/HO-1 pathway and inhibition of the NF-κB pathway by genistein were verified in the brain tissues of neonatal mice subjected to HIBD and in primary cortical neurons exposed to OGD/R.

Conclusions: Genistein exerted neuroprotective effects on HIBD by attenuating oxidative stress and neuroinflammation through the Nrf2/HO-1 and NF-κB signalling pathways.

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Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE), which is induced by the interruption of oxygenated blood supply to the brain, is a worrying personal health and socioeconomic problem in perinatal foetuses or newborns (1). Over the past several decades, although pre-emptive and neonatal care have improved, the incidence of HIE is 1–8 per 1,000 live births in developed countries and approximately 26 cases per 1,000 live births in low-resource settings countries (2,3). Currently, the clinical treatment of HIE focuses mainly on maintenance therapy and special neuroprotective measures, including stem cell transplantation, anticonvulsant (anti-epileptic) drugs, and mild hypothermia. However, the abovementioned treatments are only partially effective (4,5). Therefore, new treatment methods for neonatal HIE must be developed.

Genistein, 4′,5,7-trihydroxyisoflavone, is a bioactive isoflavone phytoestrogen found in soybean that has received the most attention because it exerts a wide variety of pharmacological effects, including tyrosine kinase inhibition and anxiolytic, weak oestrogenic, neuroprotective, cardioprotective, anticarcinogenic, anti-inflammatory, and antioxidant properties (6). In rodents, a high-soy diet has been reported to be neuroprotective in an experimental stroke model (7-9). Interestingly, high isoflavone intake reduces cerebral infarction in Japanese women (10). Similarly, a previous study found that the neuroprotective effects of genistein involve interactions with antioxidants in the nuclear factor erythroid 2-related factor 2 (Nrf2) signalling cascades and suppressing the nuclear factor-kappa B (NF-κB) signalling pathway, which help to mitigate oxidative stress and neuroinflammation in *in vivo* and *in vitro* models of cerebral ischemia (11). However, the role and mechanism of genistein in neonatal hypoxic-ischemic-induced brain injury remain unexplored.

Excessive generation of reactive oxygen species/reactive nitrogen species (ROS/RNS) occurs in response to mitochondrial dysfunction, Ca^{2+} overload, and inflammatory processes that contribute to oxidative stress, leading to ischemic cell death (12,13). When cells are exposed to oxidative stress, Nrf2 dissociates from Kelch-like ECH-associated protein 1 (Keap1), translocates into the nucleus to bind antioxidant response elements (AREs) and upregulates the expression of many antioxidant-encoding genes, particularly *heme oxygenase-1* (*HO-1*). Then, these proteins will constitute chief cellular defence mechanisms that remove ROS/RNS and confer generalized endogenous protection against oxidative stress (14,15). NF-κB activation plays a vital role in the development of neonatal HI brain injury by regulating inflammatory mediators (16). Under basal conditions, NF-κB is inactive in the cytoplasm and forms a complex with inhibitor kappa B (IκB) proteins that mask their nuclear localization signal. When cells are exposed to hypoxia (17), NF-κB becomes activated and translocates into the nucleus, where it upregulates the expression of inflammatory factors such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), all of which are considered to play crucial roles in ischemic brain damage (18-21). Thus, strategies targeting multiple molecular pathways are promising for HI treatment and prevention.

Accordingly, the current study examined the neuroprotective activity of genistein against HIE in neonatal mice and investigated whether the Nrf2/HO-1 and NF-κB signalling pathways were involved in this process.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-4958/rc).

Methods

Animals

For this study, C57BL/6 mice were purchased from the Hubei Research Center of Laboratory Animals (Wuhan, China). The mice were reared at the appropriate density in an environment with suitable temperature and humidity, a 12-h light/dark cycle, and freely available food and water. All animal operations were approved by the Animal Research Ethics Committee of South-Central University for Nationalities (No. SYXK2016-0089) and complied

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with the NIH guiding principles for the care and use of animals. A protocol was prepared before the study without registration.

Hypoxic-ischemic brain damage (HIBD) model and treatments

The mouse HIBD model was established based on the Rice-Vannucci model and our previous work (22,23). Briefly, at postnatal Day 10, male C57BL/6 pups (mean body weight of 5 g) were anaesthetized with isoflurane (RWD, Shenzhen, China) and the right common carotid artery (CCA) was double-ligated and cut between the ligatures. The operating time for each pup was maintained within 5 min. The body temperature of the mice was maintained at 37±0.5 ℃ throughout the operation using a heating pad. Afterwards, the pups were returned to their dams and allowed to recover for 2 h. Then, mice were placed in a 37 ℃ chamber containing 10% O_2 and 90% N_2. When 1 h of hypoxia was complete, the pups were returned to their mothers and observed until they were sacrificed. The sham group was only anaesthetized, and the right CCA was exposed. Operators were blinded to the study procedures, and the mice were randomly divided into 3 groups: the sham group (n=40), HI group (n=38), and HI + genistein group (n=39). Genistein (10 mg/kg, Sigma, St. Louis, MO, USA) (11) was dissolved in dimethylsulfoxide (DMSO) and injected intraperitoneally once daily for 3 consecutive days before the operation and once immediately after hypoxia (*Figure 1A*).

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

The mice were sacrificed 24 h after HIBD, and brain tissues were removed and frozen at −20 ℃ for 12 min. Then, tissues were cut into four 1–2 mm slices. The sections were incubated with a 2% TTC (Sigma-Aldrich, Hamburg, Germany) solution at 37 ℃ for 10–15 min and fixed with 4% paraformaldehyde at 4 ℃ overnight. Images of stained tissues were collected using a scanner (EPSON, Nagano, Japan), and the infarct area was measured using Image-Pro Plus 6.0 software (24).

Haematoxylin and eosin (H&E) staining

The mice were sacrificed 24 h after HIBD, the brain tissues were harvested, fixed with 4% paraformaldehyde for 24 h, and embedded in paraffin. Afterwards, the brain tissues were cut into 4 μm-thick sections. Subsequently, H&E staining was performed using a previously described protocol (25). Images were obtained in parietal cortex using a Nikon DS-U3 camera (Tokyo, Japan) with a ×40 objective.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The mice were sacrificed 24 h after HIBD, neuronal apoptosis in the ipsilateral cortex was examined with a TUNEL assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions (26). Two sections in the same plane of the cerebral parietal cortex for each animal were selected to detect cells that exhibited positive TUNEL staining. Apoptotic neurons in 6 randomly selected fields in parietal cortex on each section were photographed with a Nikon DS-U3 camera and a ×40 objective. Apoptotic cells were counted using Image-Pro Plus 6.0 software, and the rate of apoptosis was calculated with the following formula: number of apoptotic neurons/number of total neurons ×100%.

Neurobehavioral assessments

The behavioural tests were conducted 3 weeks after HIBD as described previously (23). For the grip test, the YLS-13A Grip Strength System (Jinan Yiyan Technology Development Co., Ltd., Jinan, China) was used to measure the grip strength of the contralateral forelimb in mice. The mean peak force of 3 trials was analysed. For the corner test, the mice were placed in a 30° corner, which was created with 2 boards (30 cm × 20 cm × 1 cm). The number of right turns during 20 trials was recorded.

Brain atrophy quantification

The mice were sacrificed, and brains were collected 3 weeks after HIBD. The ipsilateral hemispheres and contralateral hemispheres were weighed separately. The percent of brain tissue loss was reported as ipsilateral hemisphere weight/contralateral hemisphere weight ×100% (23).

Nissl staining

The mice were anaesthetized with 4% chloral hydrate (10 mL/kg), and the brain tissues were harvested 3 weeks after HIBD, fixed with 4% paraformaldehyde for 24 h, and embedded in paraffin. Next, the brain tissues were cut into

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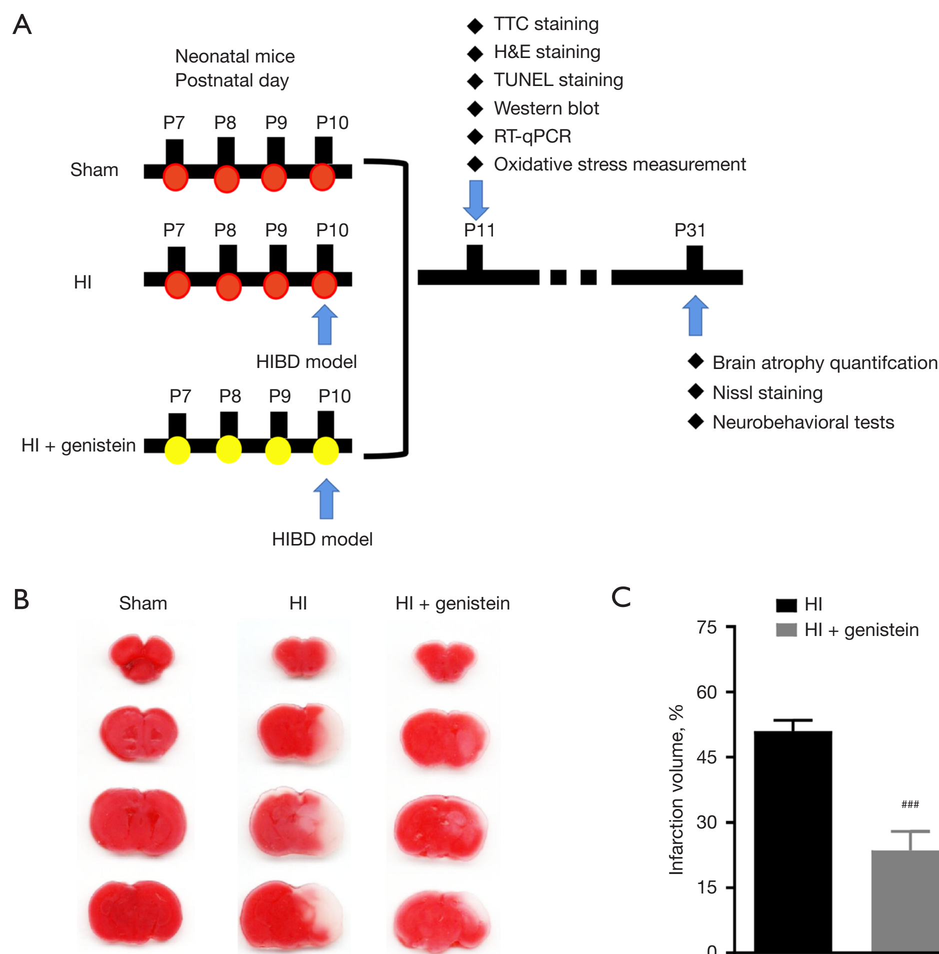

Figure 1 Genistein attenuated HIBD-induced cerebral infarction. (A) Diagram of the experimental design for the *in vivo* study. Vehicle (red circle) and genistein (yellow circle) were administered intraperitoneally once daily for three consecutive days before the induction of HIBD and once immediately after HIBD. (B) Representative images of TTC-stained coronal brain slices. (C) Evaluation of the brain infarct volume after HIBD. Data are shown as the means ± SEM, n=10 animals per group. ###, P<0.001 *vs.* the HI group. HI, hypoxia-ischemia; HIBD, hypoxic-ischemic brain damage; TTC, 2, 3, 5-triphenyltetrazolium chloride; H&E, haematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard errors of the means.

4 μm-thick slices. Subsequently, Nissl staining was performed as described previously, and the images were obtained using a scanner (EPSON) (23).

Dihydroethidium (DHE) staining

The mice were anaesthetized with 4% chloral hydrate (10 mL/kg), and the brain tissues were harvested 24 h after HIBD. DHE staining was performed as described previously. Two sections in the same plane of the cerebral parietal cortex for each animal were selected to detect the intensity of DHE fluorescence. The images were obtained in 6 randomly selected fields in the ipsilateral parietal cortex on each section using a Nikon DS-U3 camera with a ×40 objective (27).

Malondialdehyde (MDA), and glutathione (GSH) measurement

MDA was detected using a Lipid Peroxidation MDA Assay Kit (Beyotime), and GSH levels were determined using a Glutathione Assay Kit (Beyotime) according to the

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Table 1 Primers for RT-qPCR detection

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	CACTGCAAACGGGGAAATGG	TGAGATGGACTGTCGGATGG
TNF-α	GTGCCTATGTCTCAGCCTCT	CTGATGAGAGGGAGGCCATT
IL-1β	TGACGGACCCCAAAAGATGA	TCTCCACAGCCACAATGAGT
IL-6	CTCCCAACAGACCTGTCTATAC	CCATTGCACAACTCTTTTCTCA

RT-qPCR, real-time quantitative polymerase chain reaction.

manufacturer's instructions (28).

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Ipsilateral cortex was collected 24 h after HIBD, and total RNA was extracted using TRIzol reagent (Ambion, Austin, TX, USA) and reverse transcribed to complement DNAs (cDNAs) with a Goldenstar RT6 cDNA Synthesis Kit (TSINGKE, Beijing, China). RT-qPCR was performed using 2× TSINGKE Master qPCR Mix (SYBR Green I) (TSINGKE). Fold changes in messenger RNA (mRNA) expression were determined based on β-actin expression. The primer sequence information is shown in *Table 1*.

Primary cortical neuron cultures

Primary cortical neurons were obtained from embryonic C57BL/6 mouse brains (e16–18 d) as described previously (29). The cells were cultured in polylysine-coated 96-, 24- or 6-well plates in neuronal culture medium containing neurobasal medium, 2% B27 supplement, 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) at 37 ℃ in a 5% CO_2 incubator.

Oxygen-glucose deprivation/reperfusion (OGD/R) model and treatments

The neuronal culture medium was replaced with glucose- and oxygen-free Dulbecco's modified Eagle's medium (DMEM) in a mixture of 95% N_2 and 5% CO_2 in a hypoxic chamber at 37 ℃ for 4 h to establish an OGD/R model. Afterwards, the cells were reoxygenated under normoxic conditions (5% CO_2 and 95% air, 37℃) and cultured in neuronal culture medium for 24 h (27). Cells in the control group were incubated with neuronal culture medium under normoxic conditions. Cells in the OGD/R group were exposed to OGD/R. Cells in the OGD/R + genistein group were pretreated with genistein for 24 h prior to OGD/R and during OGD/R.

Lactate dehydrogenase (LDH) determination

The LDH level was determined using commercial kits (Nanjingjiancheng, Nanjing, China) according to the instructions (30).

Cell viability assay

Cell viability was detected using the cell counting kit-8 (CCK-8) (MedChemExpress, Monmouth Junction, NJ, USA) according to a standard protocol (31).

Propidium iodide (PI) staining

Cells were stained with PI (Yeason, Shanghai, China), incubated overnight with an anti-NeuN antibody (Abcam, Cambridge, MA, USA) and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (Biosharp, Hefei, China). Images were captured using a fluorescence Nikon C2 confocal microscope with a ×40 objective. The percentage of PI-positive cells (%) = (PI-positive cell number/total cell number) ×100% (32).

Western blotting

Western blotting experiments were performed as described previously (23). Briefly, nuclear and cytoplasmic proteins were extracted with kits (BioVision, Milpitas, CA, USA) according to the instructions. Proteins (30–50 μg) were electrophoretically separated on 10% SDS-PAGE gels (Boster, Wuhan, China) and transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA).

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Then, the membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 ℃ with the following primary antibodies: anti-Bax (1:1,000), anti-Bcl-2 (1:1,000), anti-cleaved caspase-3 (1:500), anti-p-NF-κB (1:1,000), anti-NF-κB (1:1,000), anti-p-IκBα (1:1,000), anti-IκBα (1:1,000), anti-β-actin (1:1,000) (Cell Signaling Technology, Danvers, MA, USA), anti-Nrf-2 (1:1,000) (Proteintech, Rosemont, IL, USA), anti-HO-1 (1:500) (Abclonal, Woburn, MA, USA), and anti-histone H3 (1:1,000) (Abcam). The membranes were subsequently incubated with a 1:5,000 dilution of the HRP-labelled secondary antibody (Biosharp) at room temperature for 1 h. The signals were detected using chemiluminescence (ECL) (Beyotime) and a bioanalytical imaging system (Azure Biosystems, Inc., Dublin, CA, USA; c300). The data were analysed using Quantity One 4.6.1 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Each group was analysed in at least 3 independent experiments. The primary outcome for HIBD experiments was infarct volume, which was used to determine the group size for each experiment based on our preliminary experiments. Statistical analyses were conducted using GraphPad Prism v7.0 (GraphPad Software, Inc., La Jolla, CA, USA), and experimental data are presented as the means ± standard errors of the means (SEM). For statistical analyses, *t*-test was used for comparisons between two groups, and one-way ANOVA followed by Tukey's post hoc test was used for comparisons between more than 2 groups. A P value <0.05 was considered to indicate a statistically significant difference.

Results

Genistein attenuated HIBD-induced cerebral infarction

The cerebral infarct area in the pup brain was assessed using TTC staining at 24 h after HIBD. As shown in *Figure 1B,1C*, no obvious cerebral infarct areas were observed in the sham group. In HI neonatal mice, a severe lesion was observed. However, the total infarct volume was significantly decreased (P<0.001) in the HI + genistein group compared with that in the HI group. This finding clearly indicates that genistein effectively decreases HIBD-induced cerebral infarction.

Genistein ameliorated HIBD-induced neuron damage and apoptosis

Based on H&E staining, neurons were morphologically intact, arranged regularly and stained uniformly in the ipsilateral parietal cortex of the sham group. In the HI group, neurons exhibited atrophic structures and a disordered distribution, and the nuclei of neurons were stained and pyknotic. However, following genistein treatment, the pathological changes were significantly improved. Similarly, TUNEL staining showed a large number of TUNEL-positive neurons in the ipsilateral parietal cortex of the HI group compared with the sham group (P<0.001). However, compared with the HI group, neuronal apoptosis was decreased significantly by treatment with genistein (*Figure 2A*; P<0.001). Furthermore, Western blot data showed that the levels of the apoptosis-related proteins Bax and cleaved caspase-3 were significantly increased in the HI group compared with the sham group. Meanwhile, the levels of Bax and cleaved caspase-3 were markedly deceased in the HI + genistein group. Genistein treatment also significantly increased the level of the antiapoptotic protein Bcl-2 compared to that in the HI group (*Figure 2B-2E*). These results suggest that genistein alleviates HIBD-induced neuronal damage and apoptosis in the brain.

Genistein contributed to the long-term recovery of neurological outcomes and brain atrophy after HIBD

We then explored whether genistein improved long-term neurological performance 3 weeks after HI. In the grip strength test, a significant reduction in the grip strength of the contralateral forelimb was observed in the HI group compared with that obtained in the sham group (*Figure 3A*; P<0.001). However, genistein administration significantly ameliorated this change. In addition, compared with the HI group, the percentage of right turns was decreased in the HI + genistein group (*Figure 3B*; P<0.01). Furthermore, *Figure 3C* shows that no brain tissue loss occurred in the sham group, while different levels of brain tissue loss were observed in the other 2 groups. Nissl staining revealed extensive cerebral atrophy and damage to the ipsilateral hemisphere in the HI group, which was attenuated in the genistein-treated group. Moreover, genistein also increased the mass ratio of the ipsilateral/contralateral hemispheres compared with the HI group (*Figure 3D*; P<0.01). Based on

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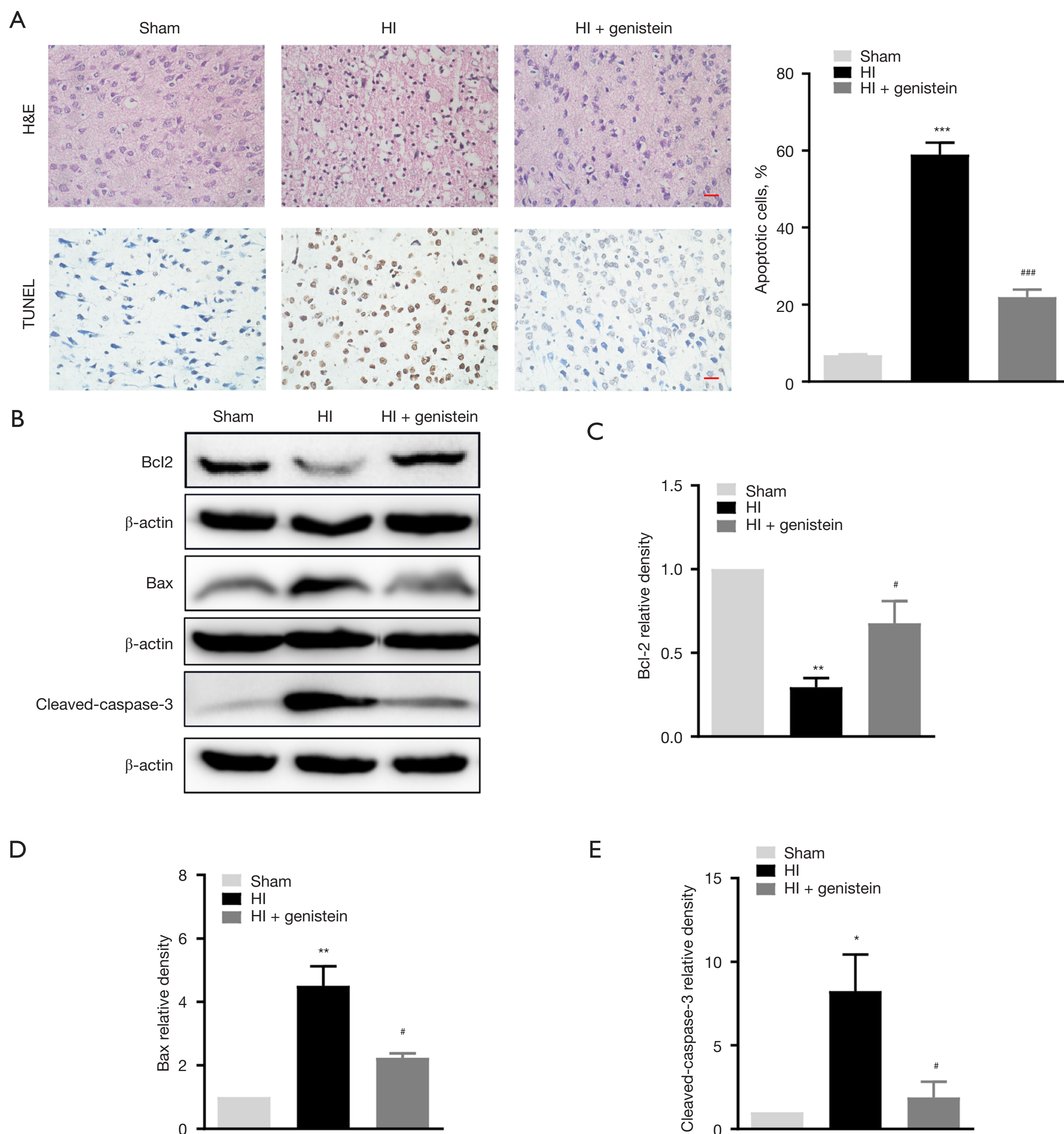

Figure 2 Genistein ameliorated HIBD-induced neuronal damage and apoptosis. (A) Representative photomicrographs of H&E staining and TUNEL staining (scale bar: 20 μm). The bar graph represents the percentage of apoptotic cells in the ipsilateral parietal cortex. (B) Representative western blots showing Bcl-2, Bax and cleaved caspase-3 levels. (C-E) Quantitative analysis of Bcl-2, Bax and cleaved caspase-3 levels. Data are presented as the means ± SEM, n=3 animals per group. *, P<0.05; **, P<0.01; ***, P<0.001 *vs.* the sham group; $^{\#}$, P<0.05; $^{\#\#\#}$, P<0.001 *vs.* the HI group. HI, hypoxia-ischemia; H&E, haematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; HIBD, hypoxic-ischemic brain damage; SEM, standard errors of the means.

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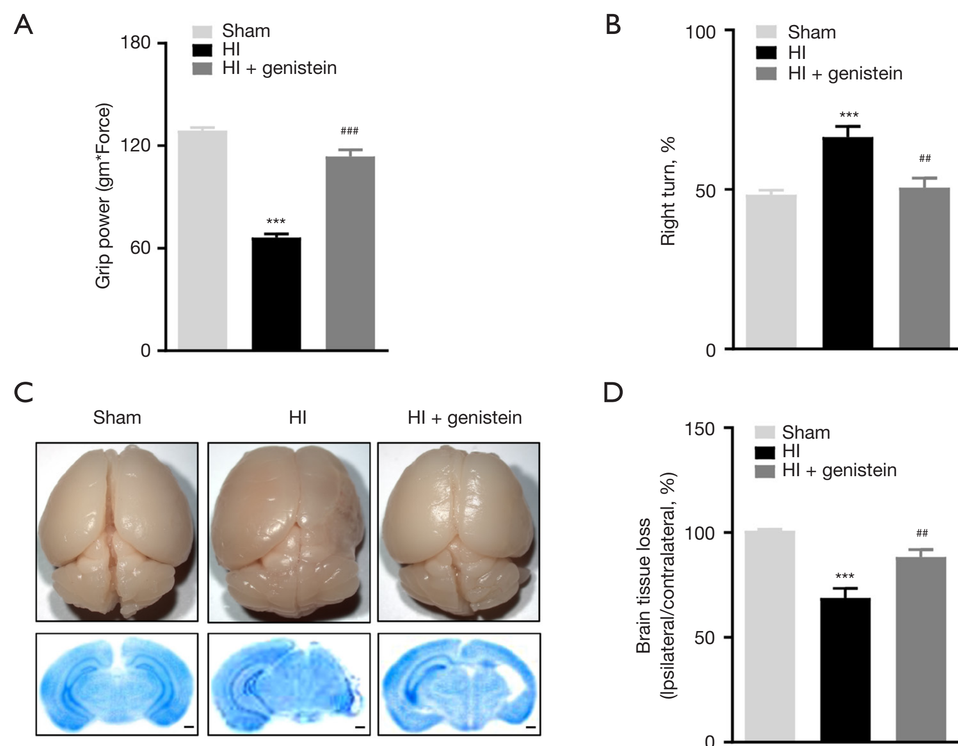

Figure 3 Genistein contributed to the long-term recovery of neurological outcomes and brain atrophy after HIBD. (A,B) Neurological outcomes were evaluated using the grip strength test and corner test, n=9 animals in the sham group; n=7 mice in the HI group; n=8 mice in the HI + genistein group. (C) Representative images of whole brains and Nissl-stained brain slices (scale bar: 0.5 mm). (D) Tissue loss is presented as the mass ratio of the ipsilateral/contralateral hemispheres. Data are shown as the means ± SEM, n=8 mice in the sham group; n=6 animals in the HI group; n=7 animals in the HI + genistein group. ***, P<0.001 *vs.* the sham group; ##, P<0.01; ###, P<0.001 *vs.* the HI group. HI, hypoxia-ischemia; HIBD, hypoxic-ischemic brain damage; SEM, standard errors of the means.

these findings, genistein also provides long-term protection in neonatal mice following HIBD.

Genistein reduced HIBD-induced oxidative stress and acute inflammation

As shown in *Figure 4A*, the superoxide level was measured using DHE staining, and a few DHE-positive cells were observed in the ipsilateral parietal cortex of the sham group, probably due to the physiological level of superoxide. A marked increase in red fluorescence was detected in the ipsilateral parietal cortex of the HI group (P<0.001). However, genistein treatment significantly suppressed superoxide production (P<0.001). In addition, the levels of the antioxidant GSH and lipid peroxidation product MDA were determined. HI significantly decreased the GSH content while increasing the MDA concentration. However, these changes were reversed by the genistein intervention (*Figure 4B,4C*). We also explored the effect of genistein on the inflammatory response following neonatal HIBD. RT-qPCR analysis revealed that the levels of proinflammatory markers (TNF-α, IL-1β, and IL-6) were significantly increased in the HI group compared to the sham group. However, genistein treatment reduced the levels of these factors (*Figure 4D-4F*). Overall, genistein exerts an inhibitory effect on HIBD-induced oxidative damage and acute inflammation.

Genistein activated the Nrf2/HO-1 pathway and inhibited the NF-κB pathway following HIBD

Western blot analysis was used to clarify the mechanisms underlying the neuroprotective effects of genistein on neonatal HIBD mice. As shown in *Figure 5A,5B*, the

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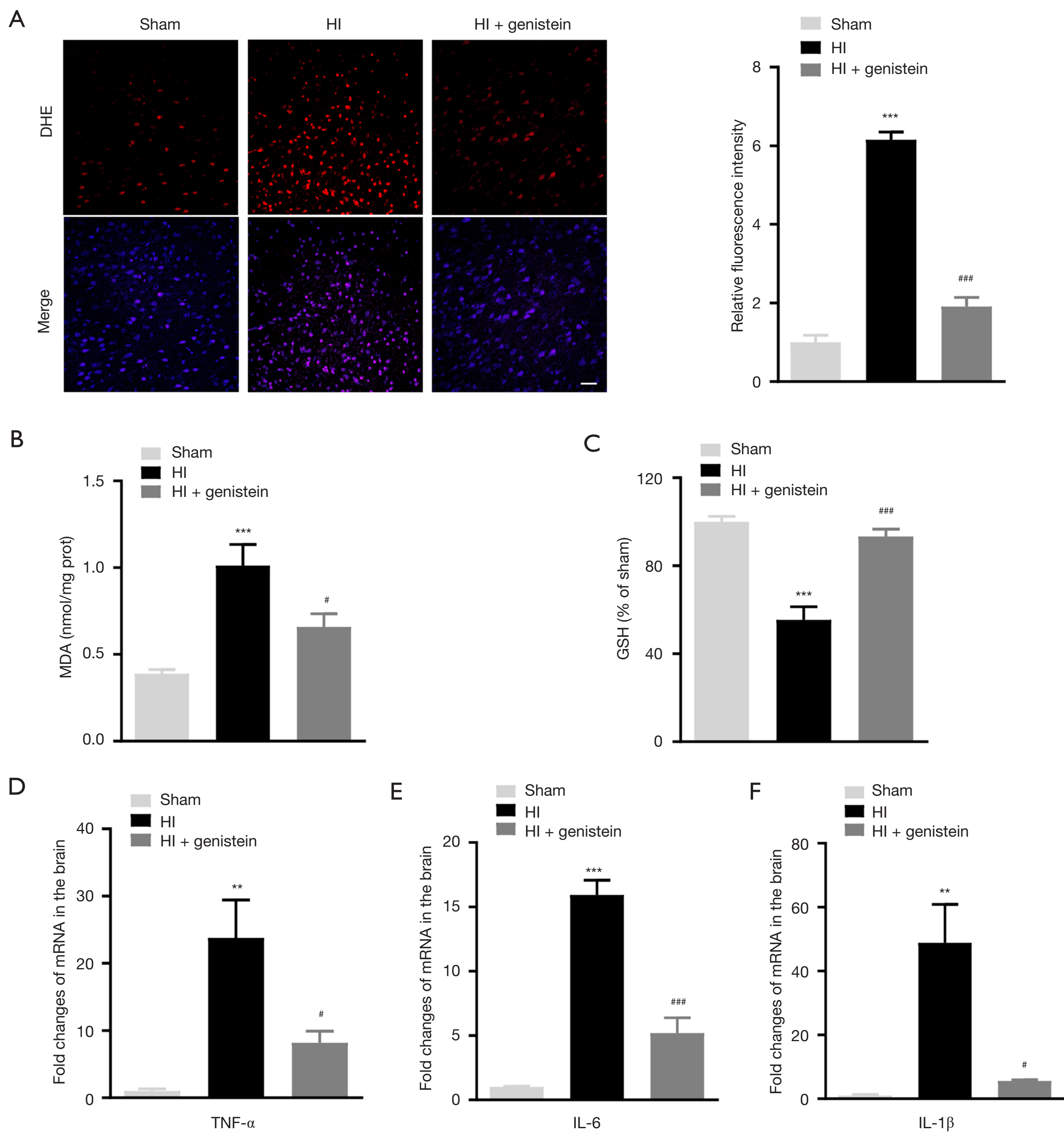

Figure 4 Genistein reduced HIBD-induced oxidative stress and acute inflammation. (A) Representative images of DHE staining and semiquantitative analysis of the DHE fluorescence intensity in the ipsilateral parietal cortex, n=3 animals per group (scale bar: 20 μm). (B,C) Quantification of MDA and GSH levels, n=6 animals per group. (D-F) Expression of the TNF-α, IL-6, and IL-1β mRNAs in the brain. Data are presented as the means ± SEM, n=3 per group. **, $P<0.01$; ***, $P<0.001$ *vs.* the sham group; #, $P<0.05$; ###, $P<0.001$ *vs.* the HI group. HI, hypoxia-ischemia; DHE, dihydroethidium; MDA, malondialdehyde; GSH, glutathione; HIBD, hypoxic-ischemic brain damage; SEM, standard errors of the means.

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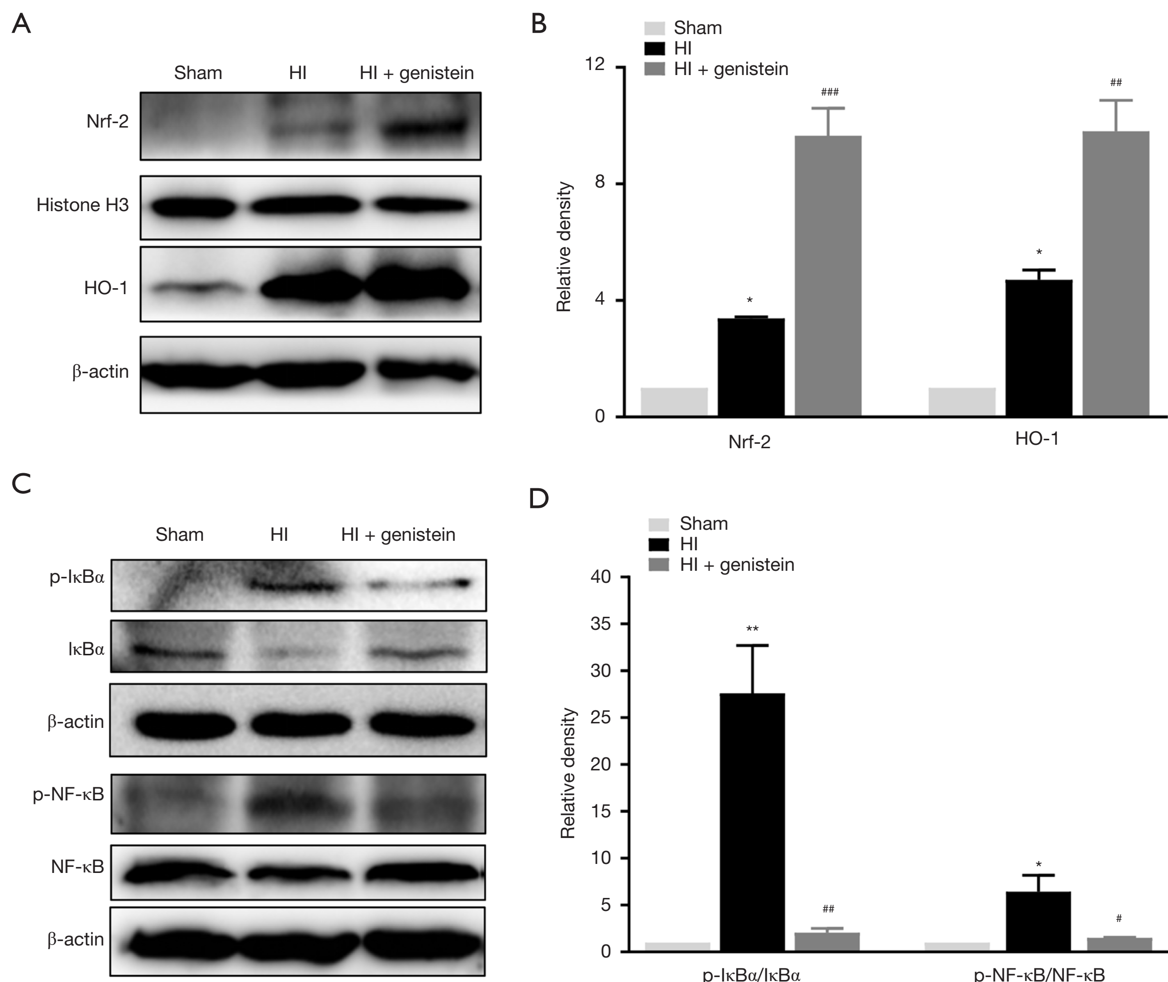

Figure 5 Genistein activated the Nrf2/HO-1 pathway and inhibited the NF-κB pathway following HIBD. (A) Levels of the nuclear Nrf2 and cytosolic HO-1 proteins. (B) Analyses of nuclear Nrf2 and cytosolic HO-1 levels. (C) Levels of the IκB-α, p-IκB-α, NF-κB, and p-NF-κB proteins. (D) Analyses of IκB-α, p-IκB-α, NF-κB, p-NF-κB levels. Data are presented as the means ± SEM, n=3 animals per group. *, P<0.05; **, P<0.01 *vs.* the sham group; #, P<0.05; ##, P<0.01; ###, P<0.001 *vs.* the HI group. HI, hypoxia-ischemia; HIBD, hypoxic-ischemic brain damage; SEM, standard errors of the means.

expression levels of Nrf2 (nuclear fraction) and HO-1 (cytosolic fraction) in the ipsilateral cortex were substantially increased in the HI group compared with the sham group (P<0.05). Interestingly, genistein treatment significantly increased Nrf2 and HO-1 levels compared with those in the HI group. Furthermore, the ratios of p-NF-κB/NF-κB and p-IκB/IκB were markedly increased in the HI group, and these changes were reversed by genistein treatment (*Figure 5C,5D*). All these data illustrate that genistein protects mice from brain injury after HIBD by activating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway.

Genistein protected primary cortical neurons against OGD/R-induced neuronal injury

Primary cortical neurons were treated with 5, 7.5, 10, 12.5, or 15 μM genistein for 24 h, and a CCK-8 assay was performed to assess the effect of genistein on the viability of primary cortical neurons and its potential cytotoxicity. As shown in *Figure 6A*, genistein (5–15 μM) treatment did not alter the viability of primary cortical neurons. Next, to study the role of genistein in HIBD *in vitro*, primary cortical neurons were subjected to OGD/R to induce injury

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(*Figure 6B*). OGD/R remarkably reduced the viability of primary cortical neurons compared with those in the control group (P<0.001). However, genistein (5–15 µM) treatment increased cell viability compared to the OGD/R group (*Figure 6C*). Because the greatest neuroprotective effect of genistein was observed at 12.5 µM, this concentration was used in subsequent *in vitro* studies. Indeed, genistein reduced OGD/R-induced neuronal injury, as also reflected by decreased LDH release (*Figure 6D*). Genistein protected against OGD/R-induced neuronal injury, which was further confirmed by PI staining. As shown in *Figure 6E,6F*, the percentage of PI-positive cells in the OGD/R group was significantly increased compared with that in the control group (P<0.001), but was significantly decreased following treatment with genistein (P<0.001). All these data indicate the neuroprotective effect of genistein on OGD/R-induced neuronal injury.

Genistein attenuates OGD/R-induced injury in primary cortical neurons via activating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway

Western blot assays were also performed to explore whether genistein protected against OGD/R-induced neuronal cell injury by regulating the Nrf2/HO-1 and NF-κB pathways. As indicated in *Figure 7A-7D*, consistent with our *in vivo* data, the levels of Nrf2 (nuclear fraction) and HO-1 (cytosolic fraction) and the ratios of p-NF-κB/NF-κB and p-IκB/IκB were noticeably increased in OGD/R-treated primary cortical neurons. However, these OGD/R-induced changes were significantly reversed by genistein treatment. Thus, genistein attenuates OGD/R-induced injury in treated primary cortical neurons by modulating the Nrf2/HO-1 and NF-κB pathways.

Discussion

HIE is a brain lesion caused by decreased cerebral blood flow and hypoxia in the perinatal period in newborns, and no acknowledged clinical treatments are available for neonatal HIE other than therapeutic hypothermia (33). Therefore, the development of new synergistic therapies for the prevention and treatment of neonatal HIE is urgently needed.

Genistein crosses the blood-brain barrier and its neuroprotective action without appreciable toxic effects on animal models has been documented, which highlights the importance of further characterizing its mechanisms of action in the brain (34). Indeed, many previous studies have shown that genistein protects against focal and global cerebral ischemia (35-37). However, the neuroprotective effects of genistein on neonatal HIE have not yet been reported. Thus, in this study, we explored the effect of genistein treatment on HIBD in neonatal mice. First, we revealed that genistein treatment decreased the brain infarct volume, attenuated neuronal damage and apoptosis, as well as contributed to the long-term recovery of neurological outcomes and brain atrophy in the neonatal mice induced by HIBD. Second, we found that genistein reduced HIBD-induced oxidative stress and neuroinflammation. Third, genistein significantly increased cell viability, rescued neuronal injury and decreased cell apoptosis after OGD/R injury. Finally, we discovered that genistein significantly alleviated neuronal injury and apoptosis by upregulating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway *in vivo* and *in vitro*. Taken together, genistein may exert a neuroprotective effect on neonatal HIE.

Based on accumulating evidence, oxidative stress and subsequent inflammatory reactions after the HI insult mainly contribute to the pathophysiology of HIE (38,39). Oxidative stress leads to nerve injury and neuronal apoptosis following HIBD (12,40). Genistein rescues neurons from death following transient global cerebral and focal cerebral ischemia injury in rats by attenuating oxidative stress (37,41). Consistent with previous findings, in the present study, H&E staining showed that genistein treatment attenuated the prominent HIBD-mediated morphological injuries in neurons in the ipsilateral parietal cortex. Genistein also reduced the number of TUNEL-positive cells in the ipsilateral parietal cortex after HIBD. Simultaneously, HIBD induced an increase in the levels of the apoptosis-related proteins Bax and cleaved caspase-3. However, genistein significantly decreased the levels of Bax and cleaved caspase-3 and upregulated the expression of the antiapoptotic protein Bcl-2. *In vitro* data also indicated that genistein inhibited OGD/R-induced neuronal death, neuronal injury and neuronal apoptosis, consistent with a previous study (42). Furthermore, a recent study showed that genistein treatment prevents hypoxia-induced long-term cognitive dysfunction in mice (43). Our results also showed that genistein exerted long-term neuroprotective effects on the immature brain in terms of both morphology and neurological function following HI. Therefore, genistein restored damaged neurons, inhibited neuronal apoptosis and exerted a long-term neuroprotective effect on neonatal mice after HIBD.

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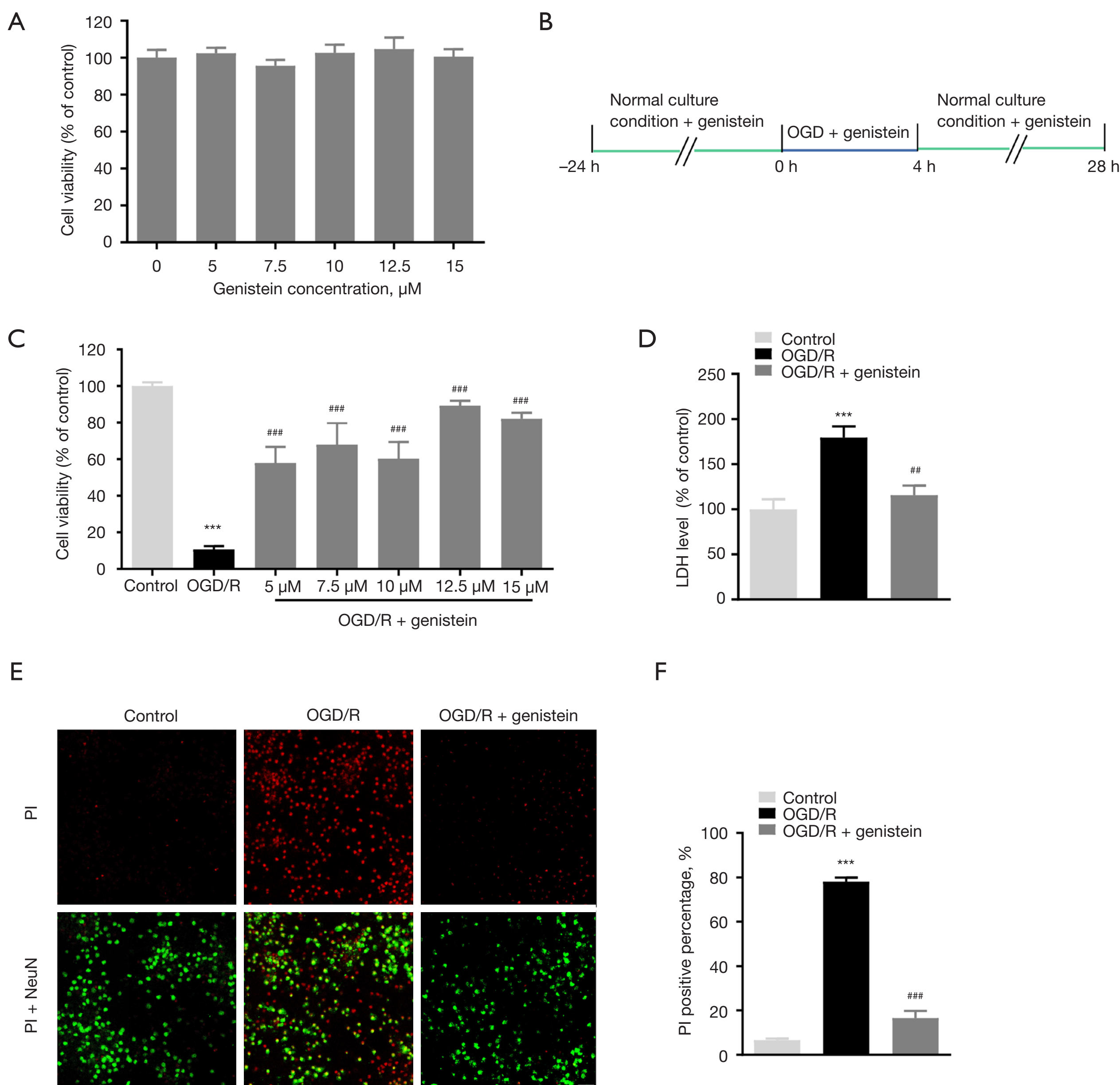

Figure 6 Genistein protected primary cortical neurons from OGD/R-induced neuronal injury. (A) Viability of primary cortical neurons after treatment with 0, 5, 7.5, 10, 12.5, or 15 μM genistein for 24 h. (B) Diagram of the experimental design for the OGD/R and genistein treatments. (C) Viability of primary cortical neurons exposed to OGD/R with or without genistein treatment (5, 7.5, 10, 12.5, and 15 μM). (D) LDH release assay, n=8 samples per group. (E) Representative images of PI staining (scale bar: 20 μm). (F) Quantitative analysis of PI-positive cells. Data are presented as the means ± SEM, n=4 samples per group. ***, P<0.001 *vs.* the control group; ##, P<0.01; ###, P<0.001 *vs.* the OGD/R group. OGD/R, oxygen-glucose deprivation/reperfusion; PI, propidium iodide; LDH, lactate dehydrogenase; SEM, standard errors of the means.

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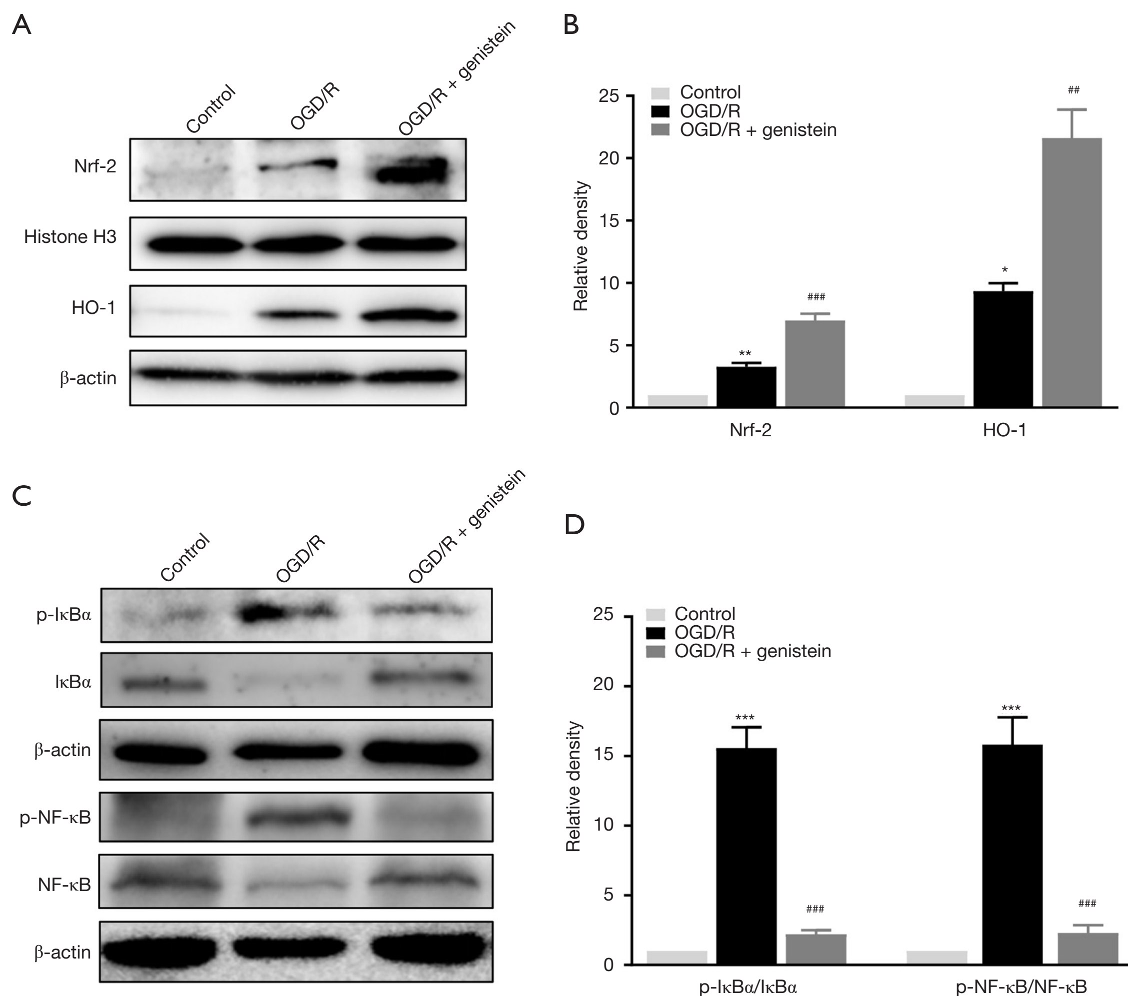

Figure 7 Genistein attenuates OGD/R-induced injury in primary cortical neurons by activating the Nrf2/HO-1 pathway and inhibiting the NF-κB pathway. (A) Levels of the nuclear Nrf2 and cytosolic HO-1 proteins. (B) Analyses of nuclear Nrf2 and cytosolic HO-1 levels. (C) Levels of the IκB-α, p-IκB-α, NF-κB, and p-NF-κB proteins. (D) Quantification of IκB-α, p-IκB-α, NF-κB, p-NF-κB levels. Data are presented as the means ± SEM, n=3 samples per group. *, P<0.05; **, P<0.01; ***, P<0.001 *vs.* the control group; ##, P<0.01; ###, P<0.001 *vs.* the OGD/R group. OGD/R, oxygen-glucose deprivation/reperfusion; SEM, standard errors of the means.

Excessive ROS production caused by oxidative stress has been widely reported to play a key role in the pathological process of ischemic brain injury (44,45). ROS destroy cellular macromolecules, leading to lipid peroxidation, protein breakdown, and DNA damage, which also result in brain damage after HI (46,47). In the present study, DHE staining was performed to assess the production of superoxide anion, a ROS, in the peri-infarct region (48). Genistein treatment significantly reduced the DHE fluorescence density. Moreover, MDA is a convenient biomarker for lipid peroxidation, and GSH is a key antioxidant equivalent to antioxidant enzymes that scavenge excessive oxygen free radicals (43), which was also measured in the current study. Genistein treatment decreased the levels of MDA in pups following HIBD and increased the levels of GSH following HIBD. In recent years, accumulating evidence has indicated that the Nrf2/HO-1 pathway activates the antioxidant system, which plays a pivotal role in mediating oxidative stress responses and neuronal cell death in response to ischemic stroke (49-51). Genistein has been reported to protect against global cerebral ischemia in rats by upregulating the Nrf2/HO-1 antioxidant signaling pathway (36). Consistently, our present data showed that HIBD or OGD/R significantly increased

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the levels of Nrf2 (nuclear) and HO-1 (cytoplasmic), which were related to the body's own ability to resist oxidative stress. Additionally, the expression of Nrf2 and HO-1 was significantly upregulated after treatment with genistein. Taken together, these results indicate that genistein alleviates HIBD-induced oxidative stress in neonatal mice, perhaps by increasing antioxidant levels and decreasing lipid peroxide levels through the modulation of the Nrf2/HO-1 pathway.

Neuroinflammation has been recognized as one of the important contributors to neonatal ischemic brain injury (52). NF-κB is an essential transcription factor that promotes the production of proinflammatory cytokines that further aggravate tissue damage (53). Under regular physiological conditions, NF-κB is localized in the cytoplasm and maintained an inactive state by binding to the inhibitory protein IκB. Upon stimulation, IκB is phosphorylated, which leads to the degradation of IκB and translocation of NF-κB p65 to the nucleus, where it binds to specific DNA sequences, resulting in alterations in the expression of target genes and release of more inflammatory factors, including *TNF-α*, *IL-1β*, and *IL-6* (54-56). Furthermore, we cannot ignore the correlation between oxidative stress in the brain and neuroinflammation during ischemic brain injury (57). ROS activate NF-κB, and the activation of NF-κB may result in increased ROS production, potentially contributing to sustained oxidant production during cerebral ischemia (58). A previous study found that genistein exerts neuroprotective effects on ischemic brain injury by inhibiting ROS-induced NF-κB activation (59). Moreover, genistein has been shown to protect cultured cortical neurons from oxidative stress at least partially by inactivating the NF-κB signalling pathway (60). The present study also showed that genistein treatment reversed the increases in the mRNA levels of proinflammatory cytokines, including IL-6, IL-1β, and TNF-α, in mice following HIBD. Western blot data indicated that the genistein intervention attenuated the activation of the NF-κB pathway *in vivo* and *in vitro*. These findings suggest that genistein exerts its neuroprotective effects on HIBD by inhibiting inflammatory responses and oxidative stress through the inhibition of the NF-κB pathway.

Some limitations exist in the current study. First, specific inhibitors of each pathway or transgenic mice will be utilized to further elucidate whether genistein directly or indirectly regulates the Nrf2/HO-1 and NF-κB pathways in neonatal HIBD mice in future studies. Second, the mRNA expression of endogenous antioxidants related to Nrf2 and the protein levels of inflammatory markers related to NF-κB were not assessed, which would have strengthened the results. Third, it was indicated that genistein provided the neuroprotective effects on HIBD in the neonatal mice when we combined pre-treatment with post-treatment. However, only post-treatment of genistein would be of more important clinical value in neonatal HIE. Finally, we only focused on apoptosis, oxidative stress and neuroinflammation. Glutamate excitotoxicity and mitochondrial dysfunction are also major contributors to HIE, and these processes will be examined in future studies.

Conclusions

In summary, genistein treatment ameliorates HIBD in neonatal mice by alleviating oxidative stress and inflammation through the activation of the Nrf2/HO-1 pathway and inhibition of the NF-κB pathway. These findings suggest that genistein should be explored further as treatment for neonatal HIE.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-4958/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-4958/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

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to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal operations were approved by the Animal Research Ethics Committee of South-Central University for Nationalities (No. SYXK2016-0089) and complied with the NIH guiding principles for the care and use of animals.

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