European Review for Medical and Pharmacological Sciences 2019; 23: 5923-5933

Nodal mitigates cerebral ischemia-reperfusion injury via inhibiting oxidative stress and inflammation

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Abstract. – OBJECTIVE: Nodal is a member of the transforming growth factor β (TGF-β) family, which induces the activation of the cytoplasmic Smad2 and Smad3, both of which play a neuroprotective role against cerebral ischemia-reperfusion (I/R) injury. However, the role of Nodal in cerebral I/R is unclear. Thus, the aim of the present study was to shed light on the function of Nodal in cerebral I/R injury.

MATERIALS AND METHODS: Cerebral I/R injury was induced in the Sprague Dawley (SD) rats by middle cerebral artery occlusion (MCAO) and reperfusion and in murine hippocampal neuronal cells (HT22) by oxygen-glucose deprivation/reperfusion (OGD/R) stimulation. The lentivirus vectors (Nodal overexpressing lentivirus vector [OE-Nodal] and the short hair RNA of Nodal [sh-Nodal]) were used to upregulate and downregulate Nodal in SD rats or cells.

RESULTS: Nodal expression increased in the cerebral I/R models and reached a peak after 12 h of reperfusion. OE-Nodal administration to the cerebral I/R rats significantly reduced the cerebral infarction volume and inhibited the brain cell apoptosis. It also increased the level of superoxide dismutase (SOD), an antioxidant enzyme, and decreased the levels of the lipid peroxides (malondialdehyde [MDA] and lactate dehydrogenase [LDH]), in addition to those of the proinflammatory factors. Consistently, the upregulation of Nodal in HT22 by OGD/R significantly increased the SOD level and decreased the levels of MDA, LDH, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α).

CONCLUSIONS: This study revealed that Nodal exerted a protective role during cerebral I/R by inhibiting excessive oxidative stress and inflammation.

Key Words:
Nodal, Cerebral I/R injury, HT22 cells, SD rats, Oxidative stress, Inflammation.

Introduction

Ischemic stroke is one of the three leading causes of deaths worldwide, after malignant tumors and heart diseases[1]. Ischemic strokes place a heavy burden on society, as they are associated with high morbidity, mortality, and disability, as well as with high recurrence rates[2-3]. Currently, the clinical therapeutic approach to ischemic strokes is mainly focused on reperfusion in the ischemic area via drugs or early thrombolysis, with the aim of restoring the oxygen and glucose supply[4-5]. A range of factors, including inflammatory mediators, excitatory amino-acid toxicity, and associated oxidative stress, can lead to ischemia-reperfusion (I/R) injury[6-8]. Therefore, an effective means to protect against cerebellar I/R injury is urgently needed.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine belonging to the TGF superfamily, which consists of more than 30 proteins, such as bone morphogenetic protein, activin, and nodal[9]. This superfamily plays a crucial role in tissue homeostasis via regulation of cell proliferation and matrix formation[10-11]. By binding to different receptors of the activin receptor-like kinase family (ALK1–7), TGF-β induces intracellular phosphorylation of receptor-regulated Smad (R-Smad) proteins, including Smad1/2/3/5/8. The phosphorylated R-Smad proteins combine with the common-mediator Smads, such as Smad4, and translocate to the nucleus to regulate the gene transcription[12,13]. Increasing evidence points to a neuroprotective role for the TGF-β/Smad2/3 signaling pathway[14]. Previous studies[14,15] reported increased expression levels of TGFβ1 and phosphorylated Smad2/3 in brain tissues with-

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in 72 h after reperfusion. The inhibition of the TGF-β/Smad3 signaling pathway with a specific blocker of the pathway, LY2157299, significantly increased the neurological deficit scores and infarct volumes and induced cell apoptosis in hippocampal tissues in a murine model of I/R[15].

In humans, the Nodal gene, which is located on chromosome 10q22, propagates signaling via a heterodimeric complex comprising type I (ALK4/7) and type II (ActRIIB) ALK receptors. This interaction induces ALK4/7 phosphorylation and cytoplasmic Smad2 and/or Smad3 activation, resulting in the formation of the transcriptional complexes, including cyclin D1[16], c-Myc[17], and c-jun[18] via interactions with Smad4. However, the role of Nodal in the pathogenesis of cerebral I/R injury remains unclear.

In the present study, we investigated the function of Nodal in the development of the cerebral I/R injury. Cerebral I/R injury was induced in the Sprague Dawley (SD) rats by middle cerebral artery occlusion (MCAO) and reperfusion (*in vivo* model) and in murine hippocampal neuronal cells (HT22) by the oxygen-glucose deprivation/reperfusion (OGD/R) stimulation (*in vitro* model). The results revealed that Nodal played a neuroprotective role during cerebral I/R by repressing oxidative stress and inflammatory reactions.

Materials and Methods

Ethics Statement

The animal study was performed in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of the Laboratory Animals and was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University.

Animals and Groups

Fifty-four SD male rats were purchased from Better Biotechnology Co., Ltd. (Nanjing, Jiangsu, China) and raised in specific-pathogen-free conditions. They were housed under conditions of 21 ± 2°C and 30-35% humidity, under a 12 h day/night cycle, with access to food and water ad libitum.

The animals (N = 54) were randomly divided into five groups: Sham group, Model group, Model + OE-Nodal group, and Model + sh-Nodal group. The Model group was divided into six subgroups according to different sampling times after reperfusion (2, 6, 12, 24, 48, and 72 h after reperfusion). There were six rats in each group.

MCAO Procedure and Lentivirus Administration

The MCAO procedure was performed after the rats had been allowed to acclimate for 1 week in accordance with a previously reported protocol[19]. The rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g) via intraperitoneal injection and fixed on the operation table. An incision was then made in the cervical region. The right common carotid artery (CCA) was isolated, and a thread was ligated near the bifurcation. Subsequently, the external carotid artery and the internal carotid artery (ICA) were isolated, followed by separation of the palatine artery along the ICA, with the external carotid artery and palatine artery ligated in the bifurcation. The thread was prepared near the bifurcation of the ICA. A V-shaped incision was made 3 mm from the bifurcation of the CCA, through which the disinfected thread was inserted, with the end of the thread left upside. The thread was carefully inserted first for about 10 mm and then to 20 mm. The prepared thread at the distal end of ICA and CCA was then tightly ligated, followed by the closure of the neck incision with intermittent sutures. The end of the thread outside of the skin was cut off, and reperfusion was performed.

In the Sham group, the CCA was separated without ligation and suture insertion. Then, 2 h after the occlusion, the occlusion sutures were pulled to restore the blood flow in the middle cerebral artery.

The rats in Model + OE-Nodal group and Model + sh-Nodal group were given 100 µl of a Nodal overexpressing lentivirus vector (OE-Nodal; GenePharm, Shanghai, China) or short hair RNA of Nodal (sh-Nodal) 24 h prior to MCAO via an intravenous tail injection. The rats in other groups were given equal amounts of a control lentivirus vector. After 12 h of reperfusion, the rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g) and sacrificed via spinal dislocation.

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

After sacrifice, the rat brains were perfused with saline, dissected, and stored at -20°C for 20 min. Brain samples were obtained from the midpoint of the line between the forebrain and optic chiasm, suprachiasmatic site, infundibular stem, and between the infundibular stem and the posterior lobe. The slices 2-mm thick were cut at these points and stained with 2% TTC solution (Solarbio, Beijing, China) for 30 min at 37°C without light. The sec-

tions were then fixed with 4% paraformaldehyde for 10 min. Normal tissues showed a pink or red stain, whereas the ischemic tissues were white. The infarction area was evaluated using ImageJ software (NIH, Bethesda, MD, USA), and the total infarction volume was calculated by multiplying the infarction area in each section by the slice thickness[20].

Hematoxylin-Eosin (HE) Staining

After fixing in 4% paraformaldehyde overnight, rinsing with double-distilled water, and dehydration and embedding in paraffin, the brain tissues were cut into serial sections 3-4 μm thick and stained with HE to assess the pathological lesions on the brain tissues.

Terminal Deoxyribonucleotide Transferase (TDT)-Mediated dUTP-Digoxigenin Nick End Labeling (TUNEL) Assay

Cell apoptosis in rat brain tissues was measured using a TUNEL assay kit (Beyotime, Jiangsu, China) in accordance with the manufacturer's instructions. In brief, the brain tissues were paraffin embedded and cut into 5-μm sections, followed by deparaffinization for 3 h and incubation with protease K for 30 min at 37°C. After washing with Phosphate-Buffered Saline (PBS) three times, the sections were treated with 50 μl of TUNEL detection buffer, which consisted of 2 μl of TDT and 48 μl of FITC-marked solution, for 60 min at room temperature in the dark. The specimens were then washed in PBS again three times. The apoptotic cells were counted under a fluorescence microscope.

Cell Line and OGD/R Stimulation

The murine hippocampal neuronal cell line HT22 (No. BNCC337709) was obtained from the BeNa Culture Collection (Beijing, China) and grown in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and maintained at 37°C in 5% carbon dioxide.

For OGD/R stimulation[21], the HT22 cells were first cultured in glucose-free Earle's balanced salt solution and placed in an oxygen-deprived incubator (95% nitrogen, 3% carbon dioxide, and 2% oxygen) at 37°C for 2 h. The cells were then returned to normoxic conditions.

Cell Transfection

Twenty-four hours before OGD/R stimulation, the HT22 cells were transiently transfected with an overexpressing vector of Nodal (OE-Nodal; No. MC209046, Origene, Beijing, China), small interfering RNA (si-Nodal), or a negative control vector (NC) using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Western Blotting Analysis

Hippocampal tissues and HT22 cells were lysed with RIPA buffer (Beyotime, Shanghai, China) for 30 min on ice and centrifuged for 20 min at 4°C to obtain the total protein. After quantitation using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, the same amount of proteins from each sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride (Thermo Fisher Scientific, Waltham, MA, USA) membranes. The membranes were then blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with anti-Nodal antibody (1:1000 dilution; No. ab55676, Abcam, MA, USA) or anti-GAPDH antibody (1:6000 dilution; No. 5174, Cell Signaling Technology, MA, USA) overnight at 4°C. Next, the membranes were probed with secondary antibody (1:10000 dilution; No. 7074 and 7056, Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature. The bands were visualized using enhanced chemiluminescence reagent and were quantified using ImageJ software (NIH).

Real Time-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from brain tissues or cells from the various treatment groups using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Subsequently, the reverse transcription was carried out using EasyScript Reverse Transcriptase (TransGen Biotech, Beijing, China). The RT-PCR was then performed on a DA7600 Real-Time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad, Hercules, CA, USA) in a 25 μl reaction system using TransStart Green qPCR SuperMix (TransGen Biotech Co., Beijing, China). The primers were obtained from Shanghai Sangon Biotech (Shanghai, China) and are listed in Table I.

Evaluation of Biochemical Parameters

After 12 h of rat reperfusion or OGD/R stimulation, the contents of lactate dehydrogenase (LDH), superoxide dismutase (SOD), and

Table I. Primer sequences used in RT-PCR assay.

Gene	Sense (5′-3′)	Antisense (5′-3′)
Rat Nodal	GCGTGTTTGGATGGAGAGGA	CCACCTGGAACTTGACCCTC
Mouse Nodal	TCTCAGGTCACGTTTGCCTC	TTTCTGCTCGACTGGACACC
Rat GAPDH	AGTGCCAGCCTCGTCTCATA	GATGGTGATGGGTTTCCCGT
Mouse GAPDH	AGCTACTCGCGGCTTTACG	ATGAAGGGGTCGTTGATGGC

malondialdehyde (MDA) in rat brain or the supernatant of HT22 cells were assessed. The commercially available kits, including a rat/mouse LDH kit (Reckon diagnostics P. Ltd., Vadodara, Gujarat, India), SOD kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and MDA kit (Nanjing Jiancheng Bioengineering Institute), were used according to the manufacturers' instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Inflammatory factors, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in rat brain or the supernatant of the HT22 cells, were assessed by an ELISA assay using rat/mouse IL-1β and TNF-α ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were carried out using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The data were presented as the mean ± SD and analyzed using the Student's *t*-test or a One-way analysis of variance, followed by the Dunnett's test. A value of $p<0.05$ was considered statistically significant in between-group comparisons.

Results

Nodal Expression Increased in the Hippocampus of I/R Mice

To explore the role of Nodal in the progression of I/R, we first assessed its expression pattern in the hippocampus of I/R mice through RT-PCR and Western blotting assays. The Nodal expression significantly increased in the hippocampus of I/R mice as compared with that in the Sham mice, with Nodal expression increasing in a time-dependent manner, and a peak after 12 h of reperfusion (Figures 1A, B). Thus, 12 h after the reperfusion was selected as the measurement time in subsequent analyses.

The HE staining results demonstrated a normal arrangement of nerve cells and normal nuclei in the hippocampus of the Sham group. In contrast, in the hippocampus of the I/R mice, the neurons showed a disordered arrangement, and the nuclei exhibited high heterogeneity (Figure 1C). In addition, cell apoptosis significantly increased in the hippocampus of the I/R mice as compared with that in the Sham group (Figure 1D). Overall, these results suggested that Nodal was highly expressed in this murine model of I/R.

Overexpression of Nodal Reduced the Cerebral Infarction Volume Induced by I/R

Next, we explored the protective role of Nodal in cerebral I/R mice in which Nodal was overexpressed. The results showed that administration with OE-Nodal lentivirus vector significantly reduced the cerebral infarction volume in I/R mice as compared with that in the Model group (Figure 2A). The neurons in the Model + OE-Nodal group were less disordered and exhibited nuclear heterogeneity (Figure 2B). Furthermore, OE-Nodal administration reduced cell apoptosis induced by I/R (Figure 2C).

We then assessed the effects of Nodal on oxidative stress, LDH expression, and inflammatory responses in response to cerebral I/R. The results showed that the level of SOD significantly decreased, whereas that of MDA significantly increased in the hippocampus of I/R rats as compared with the levels in the Sham group. OE-Nodal administration remarkably increased the SOD expression and decreased MDA expression (Figures 3A, B). Moreover, the overexpression of Nodal decreased the contents of LDH, IL-1β, and TNF-α, all of which were induced in the hippocampi of I/R rats by MCAO (Figures 3C-E). These results suggested that Nodal protected the brain from I/R injury by inhibiting oxidative stress and the inflammatory response.

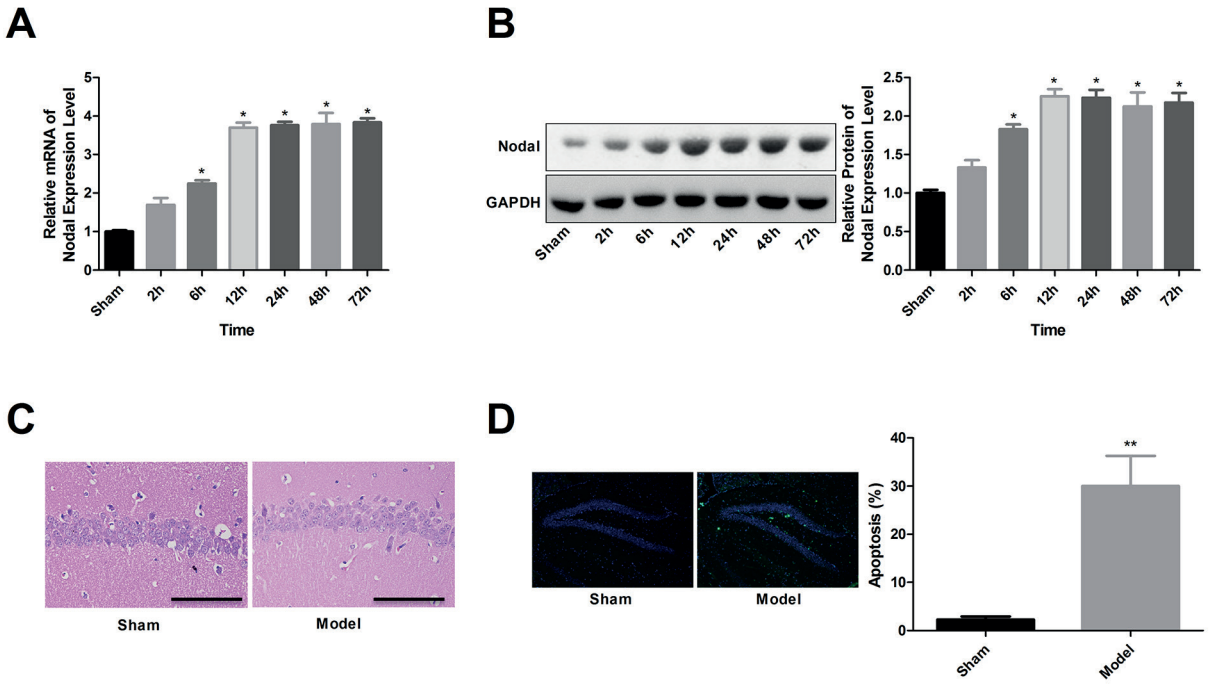

Figure 1. Nodal expression increased in I/R mice. ***A, B,*** RT-PCR and Western blotting assays were performed to detect the Nodal expression in the hippocampus of mice in the Sham and I/R groups (2, 6, 12, 24, 48, and 72 h after reperfusion). ***C,*** HE staining was performed to assess the histological morphology of the hippocampus of mice in the Sham and I/R groups (Scale bar=100 μm; Magnification: 40 × objective). ***D,*** The TUNEL assay was carried out to evaluate cell apoptosis in the hippocampus of mice in the Sham and I/R groups ($^{*}p<0.05$, $^{**}p<0.01$).

Downregulation of Nodal Aggravated Cerebral I/R Injury by Enhancing Oxidative Stress and the Inflammatory Response

To further verify the mitigating role of Nodal in the I/R-induced brain injury, we downregulated Nodal in the cerebral I/R rats. As shown by the results of the RT-PCR and Western blotting, the Nodal expression notably decreased in the Model + sh-Nodal group at both mRNA (Figure 4A) and protein levels (Figure 4B), compared with Nodal expression in the Model and Model + sh-Nodal groups. In contrast to OE-Nodal administration, the sh-Nodal administration resulted in disorganized neuronal arrangements (Figure 4C) and reduced the cerebral infarction volumes (Figure 4D). Furthermore, the sh-Nodal administration decreased the SOD level (Figure 5A) and increased MDA, LDH, IL-1β, and TNF-α contents in the hippocampus of the I/R rats (Figures 5B-E). Overall, these results demonstrated that the downregulation of Nodal significantly aggravated I/R injury by enhancing oxidative stress and inflammatory responses.

Evaluation of Nodal Function in Cerebral I/R Injury In Vitro

Next, we investigated the role of Nodal in cerebral I/R injury by building *in vitro* models. As compared with the Control group, the mRNA and protein expression levels of Nodal were significantly increased when the HT22 cells were exposed to OGD/R stimulation, with a peak value of 12 h after reperfusion. Thus, this time point was chosen for subsequent experiments (Figures 6A, B). The si-Nodal transfection significantly decreased the expression of Nodal at both mRNA and protein levels (Figures 7A, B). As compared with the Control group, the level of SOD (Figure 7C) clearly decreased in the supernatant sample of the HT22 cells with OGD/R stimulation, and the levels of MDA (Figure 7D), LDH (Figure 7E), IL-1β (Figure 7F), and TNF-α (Figure 7G) significantly increased. The upregulation of Nodal notably increased the SOD levels and decreased the levels of MDA, LDH, IL-1β, and TNF-α (Figures 7C-G). The downregulation of Nodal produced opposite results (Figures 7C-G). These results verified the beneficial effects of Nodal in cerebral I/R injury.

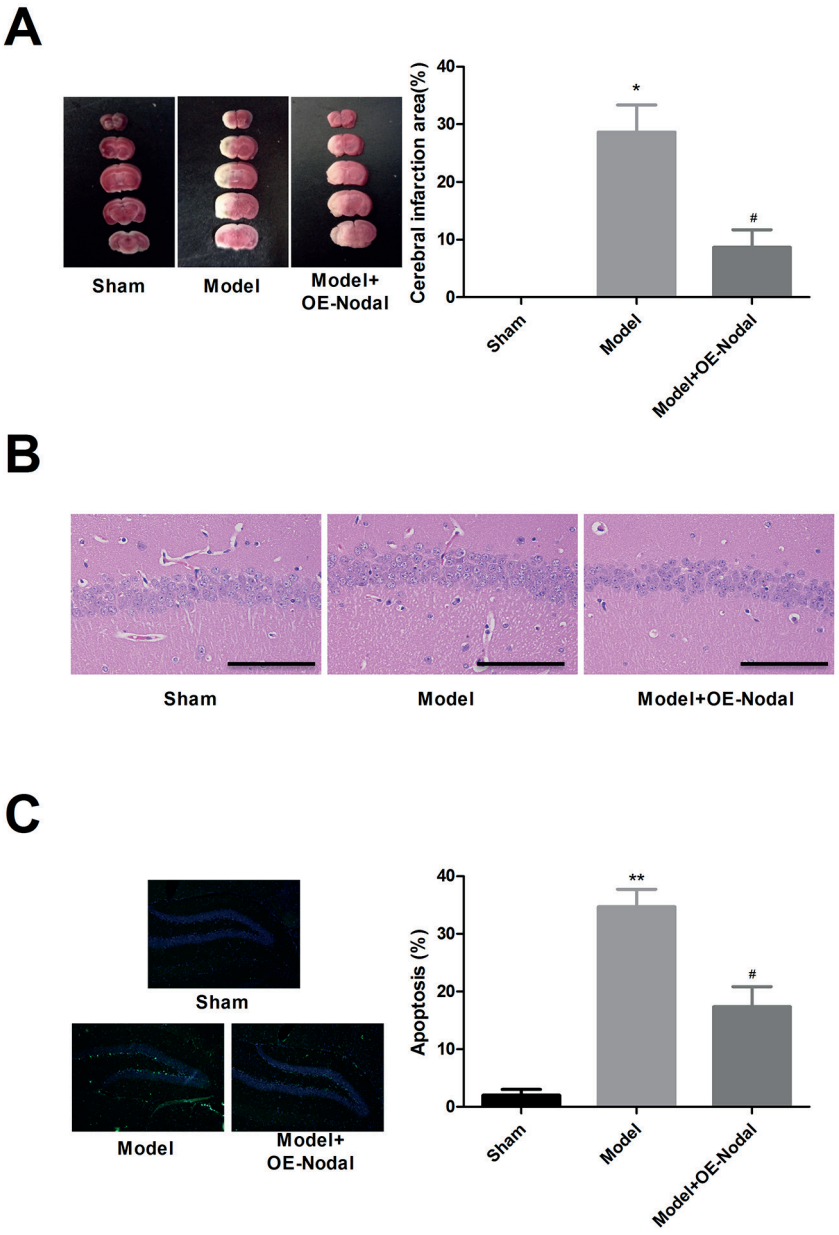

Figure 2. Nodal overexpression mitigated the injury induced by cerebral I/R injury in rats. ***A,*** TTC staining was performed to evaluate brain infarct volumes of rats in the Sham, Model, and Model + OE-Nodal groups. ***B,*** Histomorphological evaluation of the hippocampal tissue of rats was determined by HE staining (scale bar = 50 μm). Magnification: 40 × objective. ***C,*** Cell apoptosis in rat brain tissues was detected by TUNEL staining. ($^{*}p<0.05$, $^{**}p<0.01$, compared with the Sham group; $^{\#}p<0.05$, compared with the Model group).

Discussion

Many studies[22,23] showed the crucial roles of both inflammation responses and oxidative stress in secondary brain injury after cerebral ischemia. The overproduction of the reactive oxygen species accounts for much of this oxidative stress, which leads to neural dysfunction, cell death, and inflammatory response. Oxidative stress is common in the pathology of neurodegenerative disorders, such as Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and ischemic strokes[24]. Thus, the anti-inflammation agents and antioxidants are effective for ischemic stroke prevention and treatment. In the present study, through the use of *in vitro* and *in vivo* studies, we demonstrated for

the first time that Nodal played a protective role against cerebral I/R injury.

Many researches have provided evidence that the TGF-β family plays an essential role in the pathogenesis of several central nervous system disorders, such as neurodegenerative disorders and ischemic strokes[25,26]. For example, some works reported that TGFβ1 alleviated cerebral edema, reduced infarct areas, and promoted angiogenesis by inhibiting the inflammation responses in the early stages of cerebral ischemia, thereby exerting a protective role in the brain against ischemic injury[27-30]. Nodal pathway ligands are members of the TGF-β superfamily, which bind to type I and type II receptors, and then send signals downstream via Smad2/Smad3[31,32]. The co-receptors of the EGF-CFC family are required for Nodal to send signals downstream[31]. Such co-receptors are unique to the Nodal pathway. According to Gong et al[33], Nodal signaling not only plays an important role in the differentiation of the endoderm and mesoderm during embryogenesis but also plays a major role in carcinogenesis. Notably, Nodal signaling often elicits similar results to activin both at membrane receptor and intracellular signal transduction levels. Previous research reported that activin exhibited neuroprotective effects against the I/R injury. For example, activin A treatment significantly increased PC12 cell growth and protected the cells from OGD damage by increasing the expression ActRIIA, Smad3, and Smad4, reducing nitric oxide levels, and increasing SOD activity, indicating that activin A provided protection against cerebral I/R injury by inhibiting oxidative stress[34]. In the present study, we focused on the role of Nodal in cerebral I/R injury. The results showed that Nodal expression was significantly upregulated after cerebral I/R in both rats and cells, which was similar to the expression pattern of TGFβ1 reported by previous studies[14-15]. Moreover, we observed that the upregulation of Nodal in cerebral I/R rats significantly reduced the infarction volume, reduced cell apoptosis, and inhibited excessive oxidative stress and inflammation. Similarly, the upregulation of Nodal in HT22 cells reduced the injury induced by OGD/R stimulation, with decreased expression of MDA, LDH, IL-1β, and TNF-α. These results suggested that Nodal exerted a protective role against the cerebral I/R injury and that its function was similar

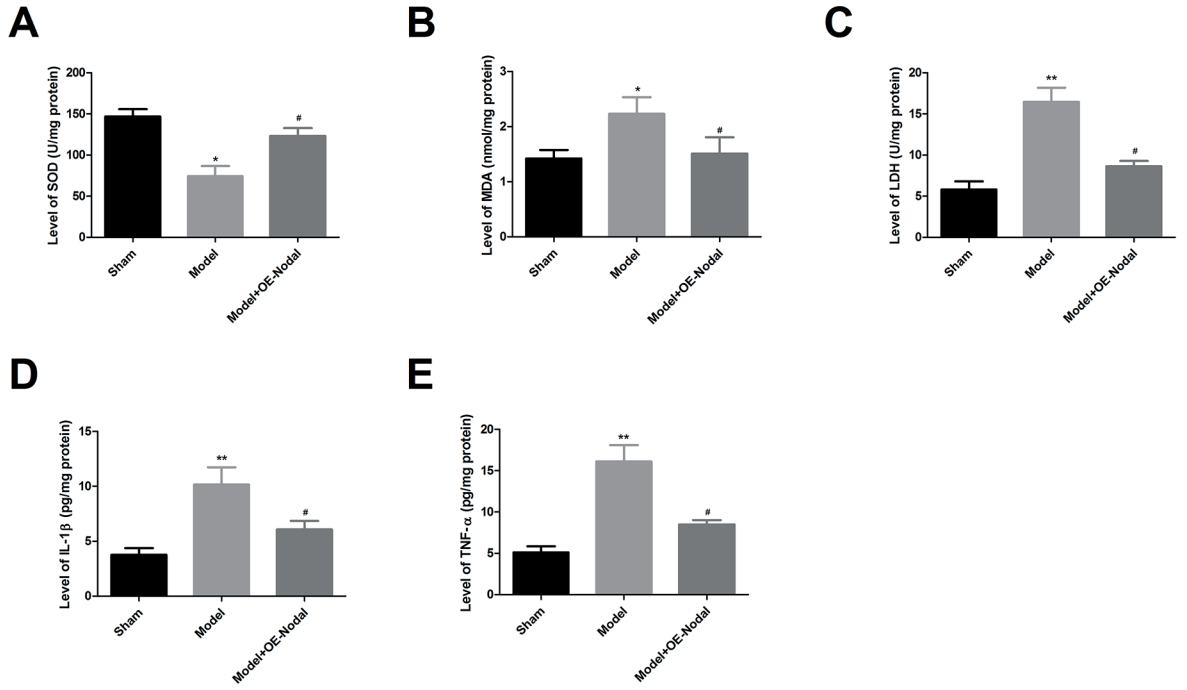

Figure 3. Evaluation of the effects of Nodal overexpression on the levels of SOD, MDA, LDH, IL-1β, and TNF-α in cerebral I/R rats. ***A, B, C,*** The levels of SOD, MDA, and LDH in rat brain tissues were assessed using commercially available kits. ***D, E,*** ELISA was carried out to determine the levels of IL-1β and TNF-α. ($^{*}p<0.05$, $^{**}p<0.01$, compared with the Sham group; $^{\#}p<0.05$, compared with the Model group).

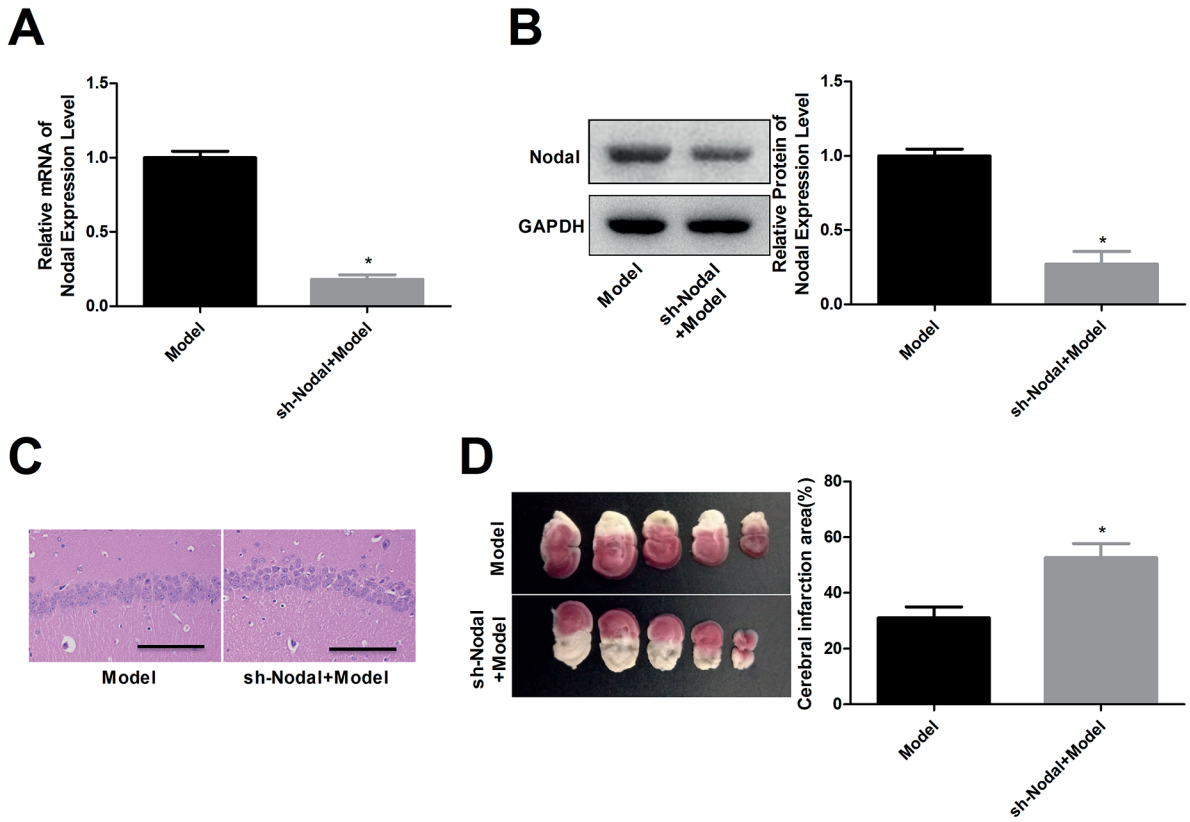

Figure 4. Effect of downregulation of Nodal on cerebral I/R injury in rats. ***A-B,*** The mRNA and the protein expression of Nodal in rat brain tissues were tested by RT-PCR and Western blotting assays. ***C,*** Histomorphological evaluation of rat hippocampal tissue was determined by HE staining (scale bar = 50 μm). Magnification: 40 × objective. ***D,*** TTC staining was performed to evaluate the brain infarct volumes of rat in the Model and Model + sh-Nodal groups ($^{*}p<0.05$).

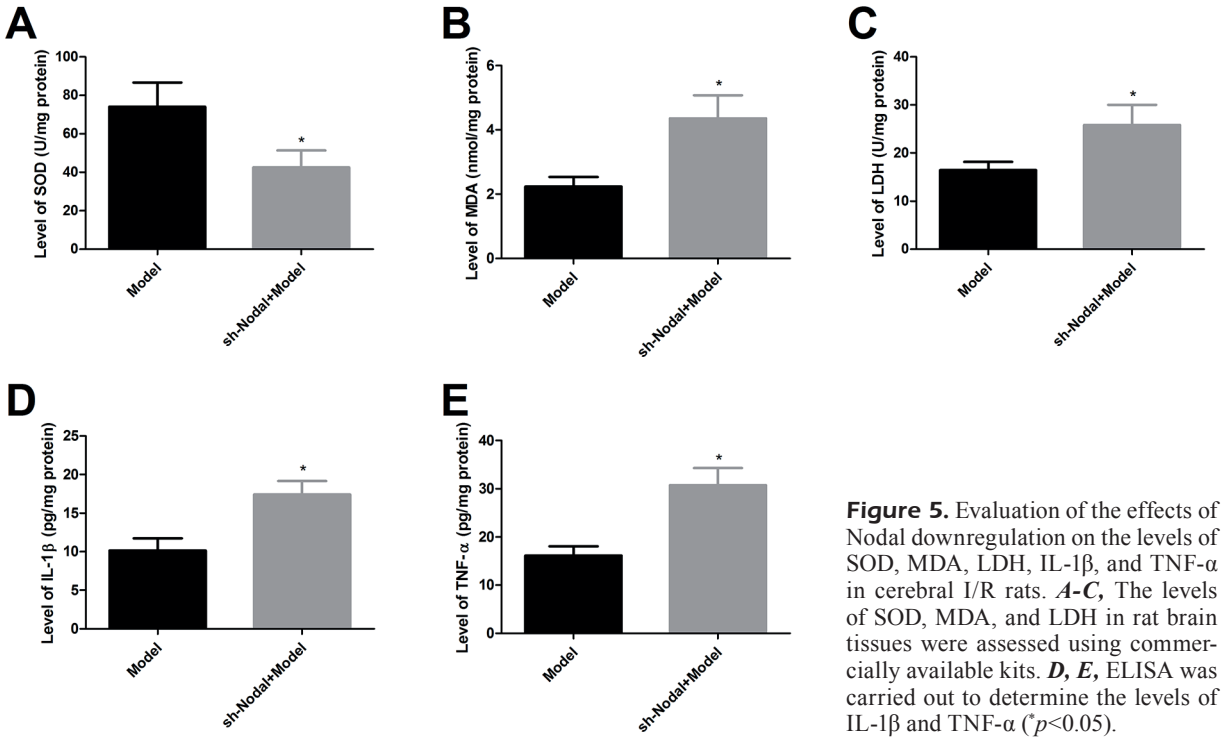

Figure 5. Evaluation of the effects of Nodal downregulation on the levels of SOD, MDA, LDH, IL-1β, and TNF-α in cerebral I/R rats. ***A-C,*** The levels of SOD, MDA, and LDH in rat brain tissues were assessed using commercially available kits. ***D, E,*** ELISA was carried out to determine the levels of IL-1β and TNF-α ($^{*}p<0.05$).

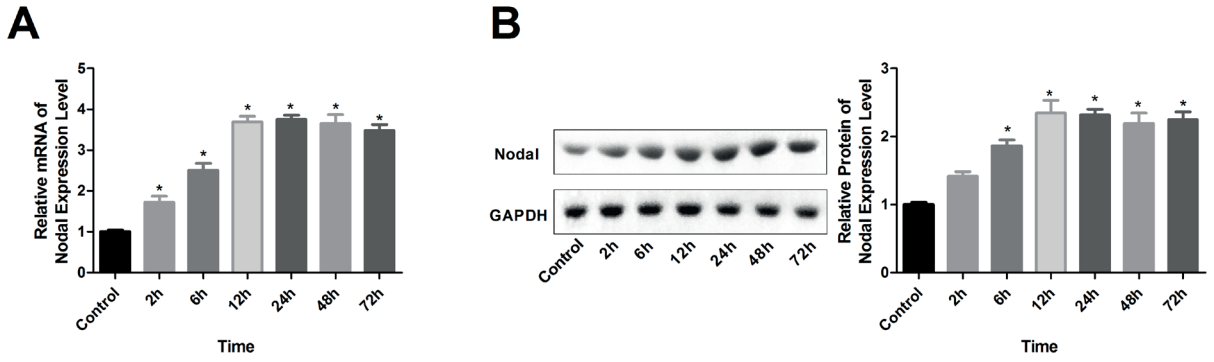

Figure 6. Impact of OGD/R stimulation on Nodal expression in HT22 cells. ***A, B,*** The mRNA and protein expression levels of Nodal in HT22 cells were detected by RT-PCR and Western blotting assays after 2, 6, 12, 24, 48, and 72 h of reperfusion ($^{*}p<0.05$).

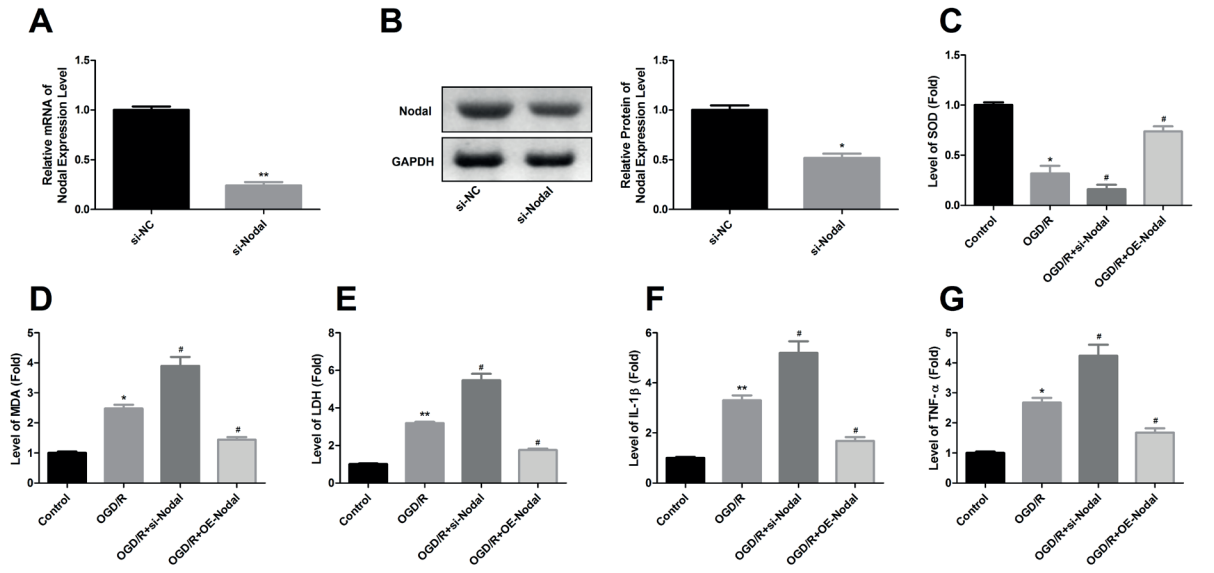

Figure 7. Assessment of the effects of Nodal up/downregulation on the levels of SOD, MDA, LDH, IL-1β, and TNF-α in HT22 cells. ***A-B,*** The expression of Nodal was detected in HT22 cells transfected with si-NC or si-Nodal in RT-PCR and Western blotting assays to determine its mRNA and protein levels, respectively ($^{*}p<0.05$, $^{**}p<0.01$). ***C-E,*** The levels of SOD, MDA, and LDH in cell supernatant samples were assessed using commercially available kits. ***F-G,*** ELISA was carried out to determine the levels of IL-1β and TNF-α ($^{*}p<0.05$, compared with the Control group; $\#p<0.05$, compared with the OGD/R group).

to that of other TGF-β superfamily members, such as activin A[34] and TGFβ1[15].

A major limitation of the present study was that we did not explore the mechanism underlying Nodal in alleviating the cerebral injury induced by I/R, such as downstream signaling such as Smad2/3/4 and signaling related to inflammation and oxidative stress. We intend to explore this topic in a subsequent study.

Conclusions

We elucidated the neuroprotective role of Nodal in hypoxic ischemic brain injury. Nodal provided protection against cerebral I/R-induced injury via anti-inflammation and antioxidant effects. These results may aid the design of the neuroprotective agents against ischemic stroke.

Conflict of interest
The authors declare no conflicts of interest.

Funding
The work was supported by the National Natural Science Foundation of China [No. 81371298], the National Natural Science Foundation of China [No. 81671159], and the National Natural Science Foundation of China [No. 31700927].

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