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RESEARCH

Low-Dose Lipopolysaccharide Alleviates Neuronal Apoptosis and Oxidative Stress in Rats with Spinal Cord Injury by Inducing Nrf2 m6A Methylation Modification via Suppressing ALKBH5

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Abstract

This work reported the neuronal protection of low-dose lipopolysaccharide (LD-LPS) after spinal cord injury (SCI). SCI rat model was constructed, after adenovirus-mediated ALKBH5 vectors and shRNA transfection and LD-LPS pre-treatment. Hematoxylin and eosin, Nissl, TUNEL staining of spinal cord tissues were adopted to monitor pathological changes, neuronal survival and apoptosis. PC12 cells transfected with ALKBH5 vectors and ALKBH5/Nrf2 siRNAs were treated by LD-LPS, followed by oxygen and glucose deprivation/reoxygenation (OGD/R). Cell viability and apoptosis were assessed by cell counting kit-8 and TUNEL assays. Neuronal oxidative stress was evaluated by appraising MDA and SOD levels. ALKBH5 and Nrf2 expression was monitored through immunohistochemistry, Western blot and qRT-PCR. Methylated RNA immunoprecipitation assay and Dot-blot experiment were for Nrf2 m6A modification detection, while RNA pull-down assay was for the binding validation between ALKBH5 and Nrf2. In rats with SCI, LD-LPS relieved spinal cord tissue damage and neuronal apoptosis; enhanced neuronal survival; decreased MDA content; elevated SOD activity; down-regulated ALKBH5; up-regulated Nrf2; and facilitated Nrf2 m6A methylation. These above influences by LD-LPS were eliminated by ALKBH5. Similar results were found in the OGD/R-induced PC12 cells after LD-LPS treatment. ALKBH5 significantly blocked Nrf2 m6A methylation, and pulled down Nrf2 protein. In the OGD/R-induced PC12 cells, the repressed oxidative stress and apoptosis by ALKBH5 silencing was abrogated by Nrf2 knockdown. LD-LPS might alleviate neuronal apoptosis and oxidative stress after SCI by facilitating Nrf2 m6A methylation via reducing ALKBH5. It was proposed to be a novel strategy for SCI treatment.

Keywords SCI · LD-LPS · Neuronal damage · Nrf2 m6A methylation · ALKBH5

Introduction

Spinal cord injury (SCI) is caused by mechanical trauma in the vast majority of cases, including primary and secondary stages of injury [1]. In addition to neuronal damage caused by primary injury, secondary pathological changes such as inflammatory response, oxidative stress, and neuronal apoptosis are also the major causes of limited axonal regeneration, permanent neurological dysfunction and disrupted spinal cord function [2]. Heretofore, despite of progress in clinical management of SCI, complete repair of spinal cord function after SCI cannot be achieved due to the limited regenerative capacity of neurons [3]. The situation of the impaired motor and sensory function and even paralysis caused by SCI underlines the urgent need to develop effective therapeutic strategies.

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Lipopolysaccharide (LPS) is a common bacterial endotoxin, and high doses of LPS is capable of inducing the release of a series of pro-inflammatory factors to result in severe inflammatory reactions and even sepsis; intriguingly, due to the cross-tolerance property of LPS, low dose of LPS (LD-LPS) can stimulate a positive immune response, which in turn promotes the production of endogenous anti-inflammatory mediators, activates anti-apoptotic and anti-oxidative stress pathways, and ultimately exerts a protective effect against injury and irritation [4]. Researchers have highlighted the effectiveness of LD-LPS against neuronal damage, as evidenced by the mitigating effect of LD-LPS on neurological dysfunction and neuronal apoptosis caused by subarachnoid hemorrhage [5]. LD-LPS preconditioning exerts neuroprotective role after traumatic brain injury without disrupting the blood-brain barrier [6]. It prevents neuronal damage by inducing the conversion of microglia to neuroprotective phenotype, which provides new insights into the treatment of neurodegenerative diseases such as Alzheimer's disease [7]. Thus, these previous studies suggested the neuroprotective role of LD-LPS. In our previous studies, we have researched that LD-LPS could alleviate nerve damage after SCI [8–11]. However, to promote the widespread use of LD-LPS in SCI treatment, molecular mechanisms by which LD-LPS exerts neuroprotective function after SCI still need to be revealed in detail.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is known as an important antioxidant and anti-inflammatory factor, which activation helps to protect neurons from damage after SCI [12]. N6-methyladenosine (m6A) is the most conserved and abundant internal cotranscriptional modification in eukaryotic RNAs; m6A modification is modified by m6A methyltransferases, but removed by demethylases such as AlkB homolog 5 (ALKBH5); m6A modification is critical for maintaining RNA stability and its translation [13]. In our previous study, one of the ways in which LD-LPS exert neuroprotective effects might be through the activation of Nrf2 [14]. Currently, there are very few studies exploring the mechanism of action of LD-LPS to alleviate SCI based on the m6A mechanism. Our preliminary experiments showed that LD-LPS exerted a regulatory effect on ALKBH5. As we know, ALKBH5 is an m6A demethylase. Moreover, it has been reported that ALKBH5 could suppress the expression of Nrf2 and cause post-transcriptional repression of NRF2 through inhibiting its m6A methylation modification [15]. Thus, focusing on Nrf2 m6A methylation modification, this paper explored the mechanisms underlying the neuroprotective function of LD-LPS after SCI. Although there have been some studies about LD-LPS relieving SCI in recent years, the mechanism involved in this process is still unclear. To the best of our knowledge, this study was the first to explore the mechanism of action of LD-LPS to relieve SCI based on the m6A mechanism. The elucidation of this mechanism will lay a reliable molecular foundation for the mechanism of LD-LPS in alleviating SCI, and will help to promote the application of LD-LPS in the treatment of SCI.

Materials and Methods

Animals and Animal Ethics Committee

Sprague-Dawley male rats ($n=72$, 8-week old, weight 220–250 g) were supplied by Kaixue Biotechnology (Shanghai, China). The rats were housed in a specific pathogen-free room kept at a temperature of 22 °C with a 12-h day/night cycle. Approval from the Animal Ethics Committee of The First People's Hospital of Yunnan province, Affiliated Hospital of Kunming University of Science and Technology was obtained before conducting the animal experiments.

Animal SCI Model and Transfection

Rats that had been deeply anaesthetized by inhalation of 2% isoflurane (Reward Life Technology, Shenzhen, China) were fixed on the operating table in the prone position. After the routine skin sterilization, the skin at the site of the T9/T10 vertebrae was cut. The exposed T9/T10 vertebrae were subjected to SCI construction by vertically dropping an Allen's weight drop apparatus (weighing 10 g) from a height of 25 mm. The injured T9/T10 vertebrae area was flushed with 0.9% normal saline, and then the skin wound was sutured and routinely sterilized. The presence of the following symptoms in rats implied the successful establishment of the SCI model: oedema and haemorrhage of spinal cord, spastic tail wagging, retraction-like flutter and flaccid paralysis of bilateral hind limbs.

Adenovirus-mediated ALKBH5 overexpression vectors and ALKBH5 shRNA were commercially supplied by GeneChem (Shanghai, China). The two kinds of adenovirus (2 μL, 5×10^{13} TU/mL) were injected intrathecally into rats respectively 10 days prior to SCI surgery.

Animal Treatment and Grouping

The 72 rats were randomly divided into six groups: the Control group ($n=12$), the SCI group ($n=12$), the SCI + LD-LPS group ($n=12$), the SCI + sh-ALKBH5 group ($n=12$), the SCI + ALKBH5 group ($n=12$), the SCI + LD-LPS + ALKBH5 group ($n=12$). Rats in each group were treated as follows:

The Control group: rats were subjected to a similar SCI surgery process (but without the SCI construction).

The SCI group: SCI construction on rats was implemented.

The SCI + LD-LPS group: rats were pre-treated with LD-LPS (0.2 mg/kg) by intraperitoneal injection 72 h prior to the SCI construction.

The SCI + sh-ALKBH5 group: rats were injected intrathecally with Adenovirus-mediated ALKBH5 shRNA (10 days before SCI construction), and then experienced SCI construction.

The SCI + ALKBH5 group: after intrathecally injection of Adenovirus-mediated ALKBH5 overexpression vectors (10 days before SCI construction), rats were subjected to SCI construction.

The SCI + LD-LPS + ALKBH5 group: rats were sequentially subjected to the injection of Adenovirus-mediated ALKBH5 overexpression vectors (10 days before SCI construction), the pre-treatment of LD-LPS (72 h prior to the SCI construction), and then the construction of SCI.

After 3 days of SCI construction, rats were euthanized to gather the spinal cord tissues. The spinal cord tissues were preserved at -80 °C for subsequent detection.

Assessment of Motor Function

To assess nerve damage, the motor function of rats was evaluated using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale after 2 days of SCI construction. The BBB score ranged from 0 to 21. A high BBB score instructed a favorable motor function and mild nerve damage. The BBB scoring criteria had been reported previously [16].

Hematoxylin and Eosin (H&E) Staining and Nissl Staining

The spinal cord tissues of rats were immersed in 4% paraformaldehyde (Huzhen Industrial, Shanghai, China) for 24 h for fixation. Before preparing tissue sections, the spinal cord tissues were embedded in paraffin. The tissue sections, which had been routinely dewaxed and rehydrated, were stained with hematoxylin solution (Huzhen Industrial, Shanghai, China) for 5 min and then with eosin solution (Huzhen Industrial, Shanghai, China) for 1 min. The residual staining solution was rinsed away by water. After routine dehydration and transparency, the dried sections were enclosed into neutral gum and placed under a microscope (Olympus Corporation, Tokyo, Japan) for observation.

For the examination of neuronal survival, the sections were stained with Nissl staining solution (Huzhen Industrial, Shanghai, China) for 40 min at a temperature of 60 °C, before being captured under the microscope.

Immunohistochemistry

The spinal cord tissues of rats, which had been routinely dewaxed and rehydrated, were immersed in 3% hydrogen peroxide for a 10-min treatment. After being blocked with 0.5% Triton X-100 (Huzhen Industrial, Shanghai, China) for 20 min at room temperature, the blockage of the sections was achieved by 5% bovine serum albumin (Yuanye Biotechnology, Shanghai, China) treatment for 30 min. The sections were probed with primary antibodies (for 12 h treatment at 4 °C), including rabbit anti-ALKBH5 (1:100, ab195377, Abcam, Cambridge, UK) and anti-Nrf2 (1:100, ab313825, Abcam, Cambridge, UK), Subsequently, the corresponding horseradish peroxidase labeled goat anti-rabbit secondary antibody (a dilution of 1:5000, ab205718, Abcam, Cambridge, UK) (for 2 h treatment at room temperature). After counterstaining by hematoxylin, the sections were routinely dehydrated and observed under a light microscope (Olympus Corporation, Tokyo, Japan) for the expression of ALKBH5 and Nrf2.

PC12 Cell Culture, Transfection and Treatment

Rat PC12 cells were commercially provided by Tongpai Biotechnology (Shanghai, China), and cultivated in Dulbecco's modified Eagle Medium (DMEM) (Xinfan Biotechnology, Shanghai, China) suspended with 10% fetal bovine serum (FBS) (Xinfan Biotechnology, Shanghai, China) at 37 °C and 5% CO_2.

ALKBH5 overexpression vectors, the corresponding negative control (NC) vectors, siRNAs targeting ALKBH5 and Nrf2, and NC siRNA were all purchased from GeneChem (Shanghai, China). PC12 cells, which had been plated into the 6-well plates with 1×10^5 cells in 1 mL serum-free DMEM were transfected with these vectors and siRNAs using Lipofectamine 3000 reagent (Thermo Fisher Scientific, San Jose, CA USA). In the transfection process, PC12 cells were divided into different groups: the ALKBH5 group, the NC group, the siALKBH5 group, the Nrf2 group, and the siNC group. After 8 h of transfection, the PC12 cells were cultured in DMEM supplemented with 10% FBS.

LD-LPS at different final concentrations (0.1, 0.2, 0.3 and 0.4 μg/mL) were adopted to treat PC12 cells for 24 h in order to determine the optimal dose. In the subsequent experiments, the optimal dose of LD-LPS (0.2 μg/mL) was chosen to pre-treat PC12 cells for 24 h prior to oxygen and glucose deprivation/reoxygenation (OGD/R). In the OGD/R treatment, PC12 cells were first subjected to OGD treatment for 4 h, followed by reoxygenation treatment for 24 h. PC12 cells that did not receive any treatment were designated as the control group.

Cell Counting kit-8 (CCK-8) Assay

PC12 cells were plated into the 96-well plates with 1×10^4 cells/100 μL medium per well and then treated under the respective conditions at 37 °C and 5% CO_2. After incubating for 2 h at 37 °C with 10 μL CCK-8 solution (Huzhen Industrial, Shanghai, China), the PC12 cells were placed under a microplate reader (Biotek, Winooski, VT, USA) to read the OD values.

Enzyme-Linked Immunosorbent Assay (ELISA)

Spinal cord tissues and PC12 cells were collected and then homogenized on ice for 10 min using a homogenizer. The supernatant was obtained by centrifuging the homogenate for 5 min at 4 °C and 10,000 rpm. Malondialdehyde (MDA) level and superoxidedismutase (SOD) activity in the supernatant were monitored using MDA assay kit (Xinfan Biotechnology, Shanghai, China) and SOD activity assay kit (Xinfan Biotechnology, Shanghai, China). The detection process was executed following the commercial kit instructions.

Cell Morphology Observation

After receiving the appropriate treatment, PC12 cells from various treatment groups were placed under an electron microscope (Olympus Corporation, Tokyo, Japan) to observe any morphological changes.

Flow Cytometry

Apoptosis of PC12 cells from different treatment groups was assayed by flow cytometry. PC12 cells were treated with fluorescein isothiocyanate-labeled Annexin V (Biolab Technology, Beijing, China) for 15 min in a dark environment, followed by staining with propidium iodide (Biolab Technology, Beijing, China) for 5 min.The apoptotic cells were evaluated by a FACSort™ flow cytometer (Becton Dickinson, San Jose, CA, USA), and qualified with the CellQuest™ software system (Becton Dickinson, San Jose, CA, USA).

Methylated RNA Immunoprecipitation (Me-RIP) Assay

The level of Nrf2 m6A modification was assessed using Me-RIP assay. Total RNA that had been extracted from spinal cord tissues and PC12 cells using Trizol reagent (Tongwei Biological, Shanghai, China) were isolated with the PolyATtract® mRNA Isolation System (Kanglang Biotechnology, Shanghai, China). Antibodies against m6A (1:100, ab208577, Abcam, Cambridge, UK) and IgG (1:100, ab182931, Abcam, Cambridge, UK) were separately added into immunoprecipitation buffer containing protein A/G magnetic beads (AmyJet Scientific Technology, Wuhan, China) a 1-hour incubation. Subsequently, the immunoprecipitation buffer containing protease and RNase inhibitors was incubated with the purified mRNA and the magnetic beads-antibody complex for 12 h at a temperature of 4 °C. Elution buffer (Guyan Industrial, Shanghai, China) was adopted to elute RNA, and the phenol-chloroform extraction method was employed to purify RNA. The enrichment of Nrf2 m6A was qualified by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Dot-Blot Experiment

The Dot-Blot experiment was conducted to measure the Nrf2 m6A level. PC12 cells were collected for total RNA extraction using Trizol reagent. After being purified by the PolyATtract® mRNA Isolation System, the isolated RNA was subjected to ultraviolet irradiation for 7 min to denature mRNA denaturation. Subsequently, the RNA was spotted onto Amersham Hybond-N membranes (Limin Industrial, Shanghai, China). The ultraviolet cross-linking was followed by sequentially performing the phosphate buffered saline-Tween 20 washing was sequentially performed. RNA was blocked in 5% skimmed milk (Huzhen Industrial, Shanghai, China) before being probed with anti-m6A antibody (1:1000, ab208577, Abcam, Cambridge, UK) for 12 h at a temperature of 4 °C. After being treated with a horseradish peroxidase-conjugated secondary antibody (1:3000, ab205718, Abcam, Cambridge, UK), Chemilum HRP Substrate (Beinuo Biotechnology, Shanghai, China) was responsible for visualizing the dots.

RNA Pull-Down Assay

The binding between ALKBH5 and Nrf2 was verified by RNA pull-down assay. Pierce Magnetic RNA-Protein Pull-down Kit, commercially supplied by Thermo Fisher Scientific (San Jose, CA USA), was adopted for RNA pull-down assay. The experimental procedure was strictly executed in accordance with the manufacturer's instructions. The prepared PC12 cell lysate was treated with biotinylated RNA and streptavidin-coated magnetic beads for a 2-h pull-down at 4 °C with rotation.Proteins that had been bound were retrieved by boiling in loading buffer. The Nrf2 protein that was bound was identified by Western blot analysis.

Actinomycin D Experiment

After 24 h of transfection, actinomycin D (2 μg/mL) was added into the culture medium to treat cells for a specific time (0, 60, 120, 180 and 240 min, respectively). At each time point, PC12 cells were gathered for total RNA isolation by Trizol reagent. The level of Nrf2 mRNA was examined by qRT-PCR.

qRT-PCR

Spinal cord tissues and PC12 cells were collected and then added to Trizol reagent for the extraction of total RNA. To synthesize the cDNA template, a reverse transcription reaction was performed using a PrimeScript RT Kit (Takara, Shiga, Japan) under the reaction conditions of 15 min at 37 °C and 5 s at 85 °C. The qRT-PCR was executed using the ABI Prism 7500 Real Time PCR system along with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The conditions included 5 min at 94 °C, followed by 39 cycles of 30 s at 95 °C and 30 s at 58 °C, and finally 5 min at 72 °C. The primers used were as follows: ALKBH5 sense primer, GCAAGTTCCAGTTCAA GCCC, and antisense primer, CATCAGCAGCATACCCA CTGA.Nrf2: sense - CACATCCAGACAGACACCAGT, antisense - CTACAAATGGGAATGTCTCTGC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense - GG AGAGTGTTTCCTCGTCCC, antisense - ATGAAGGGGT CGTTGATGGC. GAPDH played a role as a control gene. The relative mRNA expression for ALKBH5 and Nrf2 was determined by the $2^{-\Delta\Delta CT}$ method.

Western Blot

Spinal cord tissues were minced and then homogenized in radio-immuno precipitation assay (RIPA) lysis buffer (Huzhen Industrial, Shanghai, China) for total protein extraction. PC12 cells treated under the relevant conditions were gathered and dispersed into RIPA lysis buffer for the acquisition of total proteins. BCA Protein Quantification Kit (Huzhen Industrial, Shanghai, China) was recruited for the content determination of the total proteins. The separation of total proteins was achieved by treatment with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Afterwards, the proteins were loaded onto polyvinylidene fluoride membranes through electrotransfer method, followed by blockage in 5% skimmed milk for 1 h at room temperature. Overnight incubation of proteins was executed at 4 °C by using primary antibodies (a dilution of 1:1000). Rabbit anti-ALKBH5 (ab195377), anti-Nrf2 (ab313825), anti-Bax (ab32503), and anti-GAPDH (ab181602) primary antibodies were all commercially provided by Abcam (Cambridge, UK). Rabbit anti-cleaved caspase-3 (C-caspase-3) (9661) was provided by Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase labeled goat anti-rabbit secondary antibody (a dilution of 1:5000, ab205718), purchased from Abcam (Cambridge, UK), was adopted for 2 h treatment of the proteins. The visualization of the reactive blots was finished by the enhanced chemiluminescent reagent (Huzhen Industrial, Shanghai, China) treatment. The reactive blots were qualified by the Image J software (NIH, Bethesda, MD, USA) by taking GAPDH as a reference.

Dihydroethidium (DHE) Staining

The content of reactive oxygen species (ROS) in PC12 cells and spinal cord tissues of rats was examined by DHE staining. PC12 cells were plated into 6-well plates (1×10^5 cells/mL medium per well). Before cell inoculation, a coverslip was placed on the bottom of each well. After cultured under the relevant conditions, PC12 cells were stained by DHE staining solution (Biolab Technology, Beijing, China) for 15 min at 37 °C, and then by Hoechst staining solution (Beyotime, Shanghai, China) for 5 min in the dark. Spinal cord tissues of rats were prepared as frozen tissue sections (4 μm). Under a dark condition, the sections were incubated with DHE staining solution for 30 min at 37 °C. Hoechst staining solution was dropped onto the sections to stain the nuclei of cells for 5 min. Finally, PC12 cells on the coverslip and the spinal cord tissue sections were dried, sealed in neutral resin, and observed under a fluorescence microscopy (Olympus Corporation, Tokyo, Japan).

The Terminal Transferase Uridyl Nick End Labeling (TUNEL) Assay

A coverslip was placed on the bottom of the 6-well plates, and then 1×10^5 PC12 cells in 1 mL medium were grown into each well. Following the relevant treatment, PC12 cell apoptosis was scrutinized using a commercial TUNEL assay kit (Zeye Biotechnology, Shanghai, China). Regarding spinal cord tissues, the prepared tissue sections underwent 0.1% Triton X-100 (Zeye Biotechnology, Shanghai, China) treatment for 10 min at room temperature, followed by TUNEL staining with the commercial kit. Staining of nuclei was carried out by 4'-6-diamidino-2-phenylindole (DAPI) solution (Beyotime, Shanghai, China) treatment. The apoptotic cell observation was performed under the fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Statistical Analysis

All experiments in this study were conducted independently at least three times. The data was presented as mean ± standard deviation and analyzed using SPSS 23.0 software (IBM, Armonk, NY, USA). Statistical graphs with error bars were created based on mean ± standard deviation. The normally distributed data that had been verified by the Shapiro-Wilk test were statistically analyzed by one-way analysis of variance along with post-hoc Tukey's test. Graphs were drawn by GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was defined as a statistically significant difference.

Results

LD-LPS Alleviated Spinal Cord Tissue Inflammation, Neuronal Injury, and Oxidative Stress in Rats with SCI

The flowchart of animal experiment was shown in Fig. 1A. After 2 days of LD-LPS treatment, rats with SCI (the SCI group) showed severely impaired motor function, as they possessed prominently lower BBB score than the control rats (the Control group) ($P < 0.001$). LD-LPS treatment effectively improved the motor function of rats with SCI, as evidenced by the higher BBB score in rats of the SCI + LD-LPS group compared to the SCI group ($P < 0.001$) (Fig. 1B).

H&E (Fig. 1C) and Nissl staining (Fig. 1D) were performed to observe spinal cord tissue damage and neuronal survival. Normal spinal cord tissues along with a significant number of surviving neurons, were observed in the control rats (the Control group). However, severe spinal cord tissue damage and inflammatory response, as well as a much reduction in survival neurons, occurred in spinal cord tissues of rats with SCI (the SCI group). By LD-LPS treatment, the spinal cord tissue damage and inflammatory response were mitigated, and an increase in survival neurons was discovered in spinal cord tissues of SCI rats (the SCI + LD-LPS group vs. the SCI group).

To assess oxidative stress, we conducted ELISA on homogenates of spinal cord tissues. We detected elevated levels of MDA but decreased SOD activity in rats with SCI compared to control rats ($P < 0.001$). Conversely, after LD-LPS treatment, the decreased MDA level but intensified SOD activity were monitored in spinal cord tissues of rats with SCI (the SCI + LD-LPS group vs. the SCI group) ($P < 0.001$) (Fig. 1E and F). Moreover, DHE staining of spinal cord tissues was conducted to monitor ROS. ROS was rarely detected in spinal cord tissues of the control rats (the Control group). However, a large amount of ROS was observed in rats with SCI (the SCI group). LD-LPS treatment reduced ROS content in spinal cord tissues of rats with SCI, comparatively (the SCI + LD-LPS group vs. the SCI group) (Fig. 1G). Thus, the mitigating effect of LD-LPS on spinal cord tissue damage, neuronal injury, and oxidative stress in rats with SCI was revealed based on these results.

LD-LPS Relieved Neuronal Apoptosis in Spinal Cord Tissues of Rats with SCI

TUNEL staining was adopted to examine neuronal apoptosis in spinal cord tissues of rats. A large amount apoptotic neurons were found in spinal cord tissues of rats with SCI (the SCI group vs. the Control group), which was reduced by LD-LPS treatment (the SCI + LD-LPS group vs. the SCI group) (Fig. 2A). Western blot showed a distinct elevation in the expression of Bax and C-caspase-3 proteins in spinal cord tissues of rats with SCI (the SCI group vs. the Control group) ($P < 0.001$). Actually, the up-regulated Bax and C-caspase-3 proteins in spinal cord tissues of rats with SCI were decreased by LD-LPS treatment (the SCI + LD-LPS group vs. the SCI group) ($P < 0.001$) (Fig. 2B). It was implied that LD-LPS treatment attenuated neuronal apoptosis in spinal cord tissues of rats with SCI.

LD-LPS Treatment Down-Regulated ALKBH5 But Up-Regulated Nrf2 and its m6A Methylation in Spinal Cord Tissues of Rats with SCI

Immunohistochemistry showed the expression of ALKBH5 and Nrf2 proteins in spinal cord tissues of rats. Rats with SCI expressed higher ALKBH5 protein but lower Nrf2 protein in spinal cord tissues than the control rats ($P < 0.001$). Conversely, LD-LPS treatment led to a reduction in ALKBH5 protein expression but an elevation in Nrf2 protein expression in spinal cord tissues of rats with SCI, when compared between the SCI + LD-LPS group and the SCI group ($P < 0.001$) (Fig. 3A and B). qRT-PCR showed a similar trend in the expression of ALKBH5 and Nrf2 mRNAs in spinal cord tissues of rats in the three groups, with statistically significant differences (Fig. 3C). The RIP assay results revealed a lower Nrf2 m6A modification level in the spinal cord tissues of rats with SCI compared to the Control group ($P < 0.001$). However, the Nrf2 m6A modification level was significantly increased with LD-LPS treatment in the SCI + LD-LPS group compared to the SCI group ($P < 0.001$) (Fig. 3D). This suggested that LD-LPS treatment in rats with SCI could decrease ALKBH5 expression while increasing Nrf2 expression and its m6A methylation in spinal cord tissues.

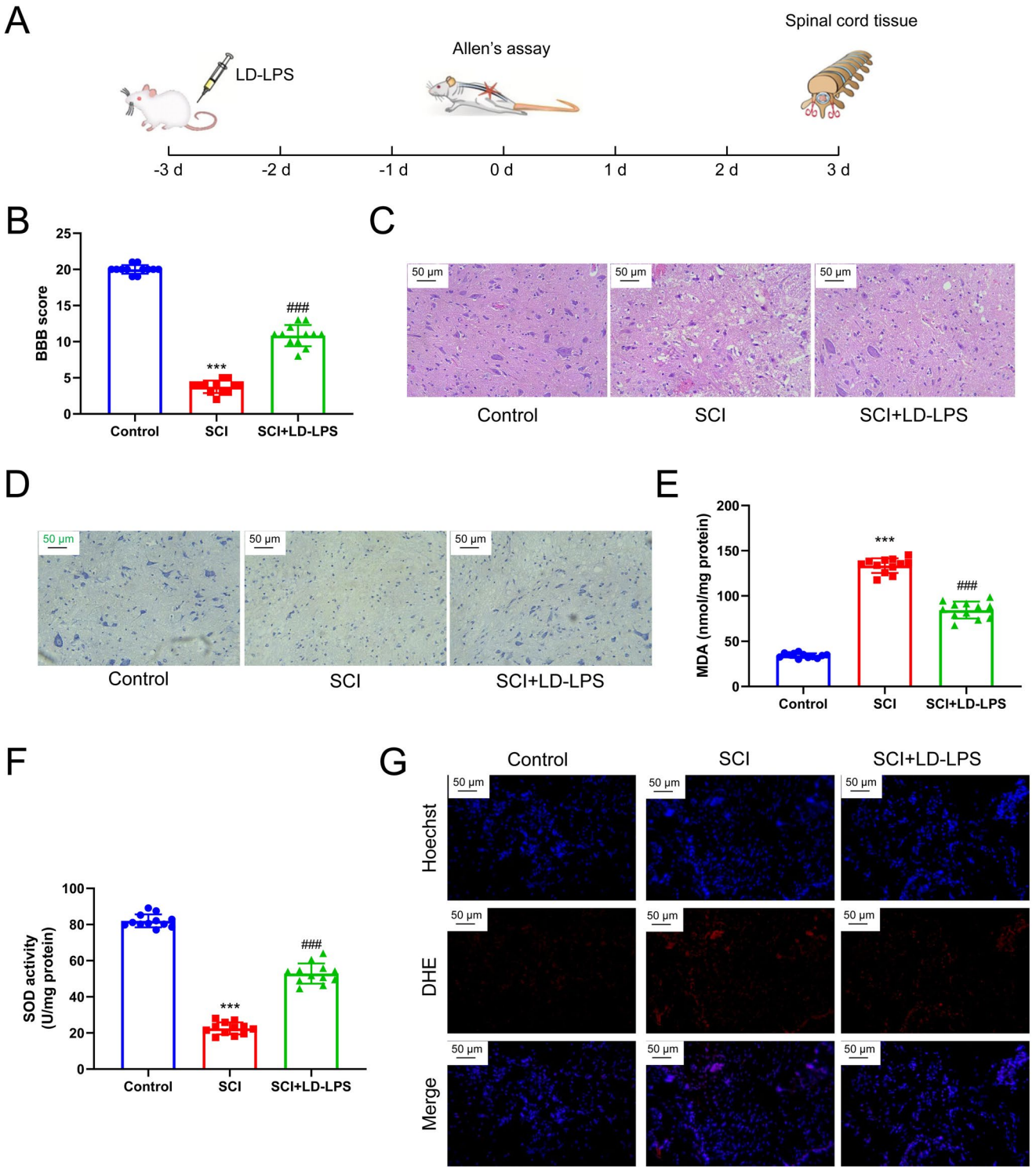

Fig. 1 LD-LPS treatment relieved spinal cord tissue inflammation, neuronal injury, and oxidative stress in rats with SCI. (**A**) The flowchart of animal experiment. (**B**) LD-LPS treatment improved motor function of rats with SCI, as evaluated by BBB score. (**C**) H&E instructed the relief of LD-LPS on spinal cord tissue damage and inflammation in rats with SCI. (**D**) Nissl staining showed the favorable effect of LD-LPS on neuronal survival in spinal cord tissues of rats with SCI. (**E-F**) ELISA exhibited the suppression of LD-LPS on MDA level and promotion on SOD level in spinal cord tissues of rats with SCI. (**G**) DHE staining showed that, LD-LPS treatment reduced ROS content in spinal cord tissues of rats with SCI. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group

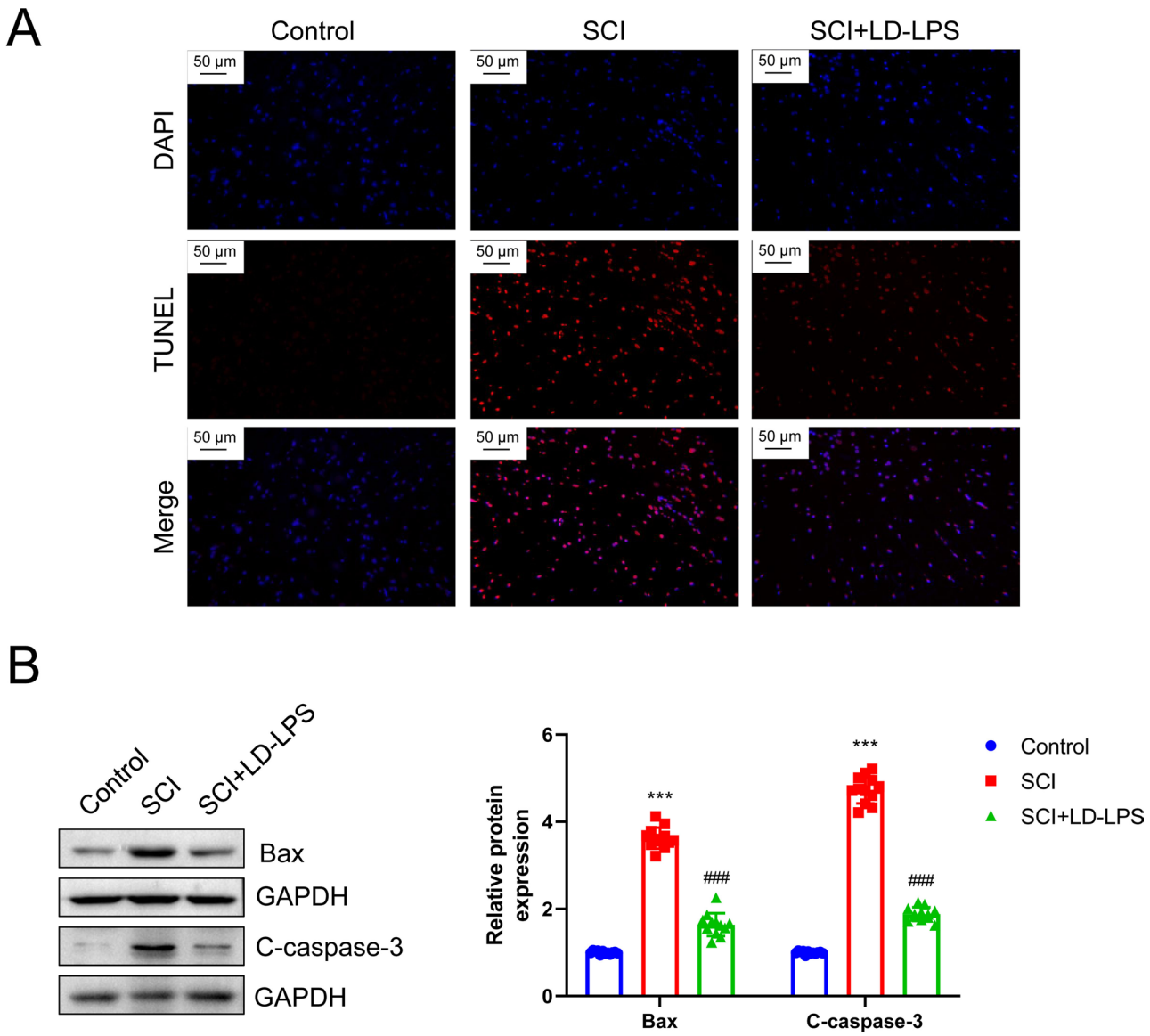

Fig. 2 LD-LPS treatment attenuated neuronal apoptosis in spinal cord tissues of rats with SCI. (**A**) TUNEL staining indicated that, LD-LPS treatment mitigated neuronal apoptosis in spinal cord tissues of rats with SCI. (**B**) Western blot illustrated that LD-LPS treatment reduced the expression of Bax and C-caspase-3 proteins in spinal cord tissues of rats with SCI. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group

LD-LPS Might Alleviate Oxidative Stress and Apoptosis in the OGD/R-induced PC12 Cells by Down-Regulating ALKBH5

An in vitro study was conducted by exposing PC12 cells to LPS. The appropriate concentration of LPS for PC12 cells was determined using the CCK-8 assay. LPS at 0.1 and 0.2 μg/mL was non-toxic to PC12 cells, as the viability of PC12 cells treated with these concentrations was similar to that of untreated PC12 cells. However, at concentrations of 0.3 and 0.4 μg/mL, LPS significantly impaired the viability of PC12 cells compared to untreated cells ($P<0.01$) (Fig. 4A). Therefore, LPS at 0.2 μg/mL was considered to be the optimal concentration for treating PC12 cells.

For the purpose of mechanistic study, ALKBH5 overexpression vectors and NC vectors were separately transfected into PC12 cells. Transfection with ALKBH5 overexpression vectors distinctly intensified the expression of ALKBH5 protein in PC12 cells compared to the Control group ($P<0.001$). The results showed that the ALKBH5 overexpression vectors successfully increased the expression of ALKBH5 protein in PC12 cells. Furthermore, since the ALKBH5 protein levels in PC12 cells were comparable between the NC group and the Control group, the PC12

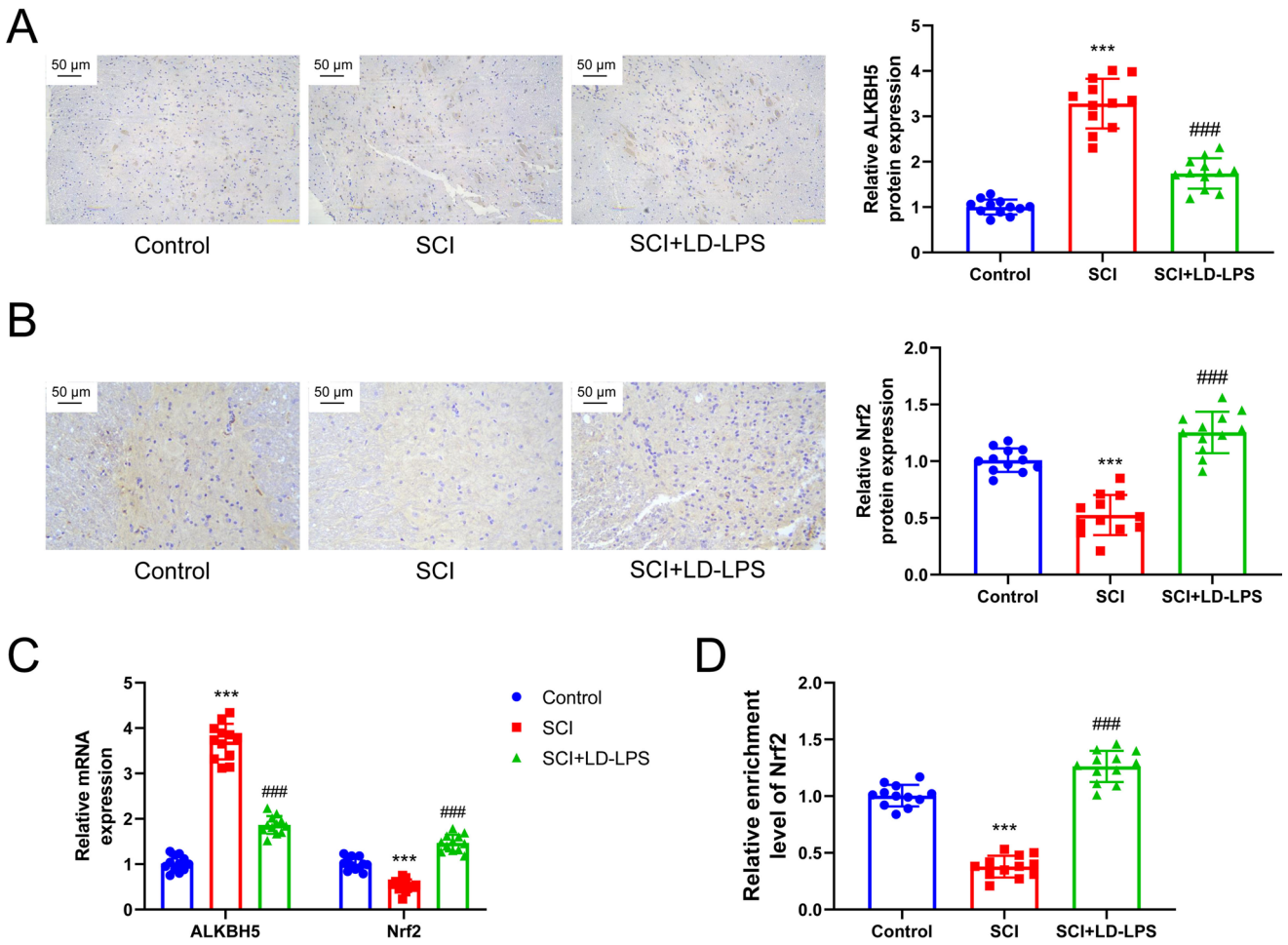

Fig. 3 LD-LPS treatment decreased ALKBH5 but increased Nrf2 and its m6A methylation in spinal cord tissues of rats with SCI. (**A**-**B**) Immunohistochemistry showed the down-regulated ALKBH5 and the up-regulated Nrf2 in spinal cord tissues of the LD-LPS treated SCI rats. (**C**) qRT-PCR results suggested the down-regulated ALKBH5 mRNA and the up-regulated Nrf2 mRNA in spinal cord tissues of the LD-LPS treated SCI rats. (**D**) Me-RIP assay revealed the enhanced Nrf2 m6A methylation in spinal cord tissues of the LD-LPS treated SCI rats. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group

cells from the NC group were not included in the subsequent study (Fig. 4B).

In the following, LPS at 0.2 μg/mL was adopted to treat the ALKBH5 overexpression vector transfected- and the OGD/R-induced PC12 cells. Oxidative stress factors in PC12 cells were scrutinized by ELISA. Referred to the Control group, lower MDA level and higher SOD activity were monitored in PC12 cells of the LD-LPS group ($P<0.01$, $P<0.001$). Conversely, the increased MDA level and decreased SOD activity were found in those of the ALKBH5 group and the OGD/R group, as compared with the Control group ($P<0.001$). In comparison to the OGD/R group, PC12 cells of the OGD/R + LD-LPS group showed the decreased MDA level and enhanced SOD activity ($P<0.001$). Those of the OGD/R + ALKBH5 group displayed higher MDA level and lower SOD activity, comparatively (the OGD/R + ALKBH5 group vs. the OGD/R group) ($P<0.001$). When compared to PC12 cells of the OGD/R + LD-LPS group, those of the OGD/R + LD-LPS + ALKBH5 group exhibited higher MDA level and lower SOD activity ($P<0.001$) (Fig. 4C and D).

Based on DHE staining, the attenuated ROS content in the LD-LPS group, but the enhanced ROS content in the ALKBH5 group and the OGD/R group were found, as matched to PC12 cells of the Control group. In contrast to PC12 cells of the OGD/R group, the reduced ROS content in the OGD/R + LD-LPS group but the intensified ROS content in the OGD/R + ALKBH5 group was observed. PC12 cells of the OGD/R + LD-LPS + ALKBH5 group showed the increased ROS content than those of the OGD/R + LD-LPS group (Fig. 4E). These data indicated that LD-LPS might attenuate oxidative stress in the OGD/R-induced PC12 cells by down-regulating ALKBH5.

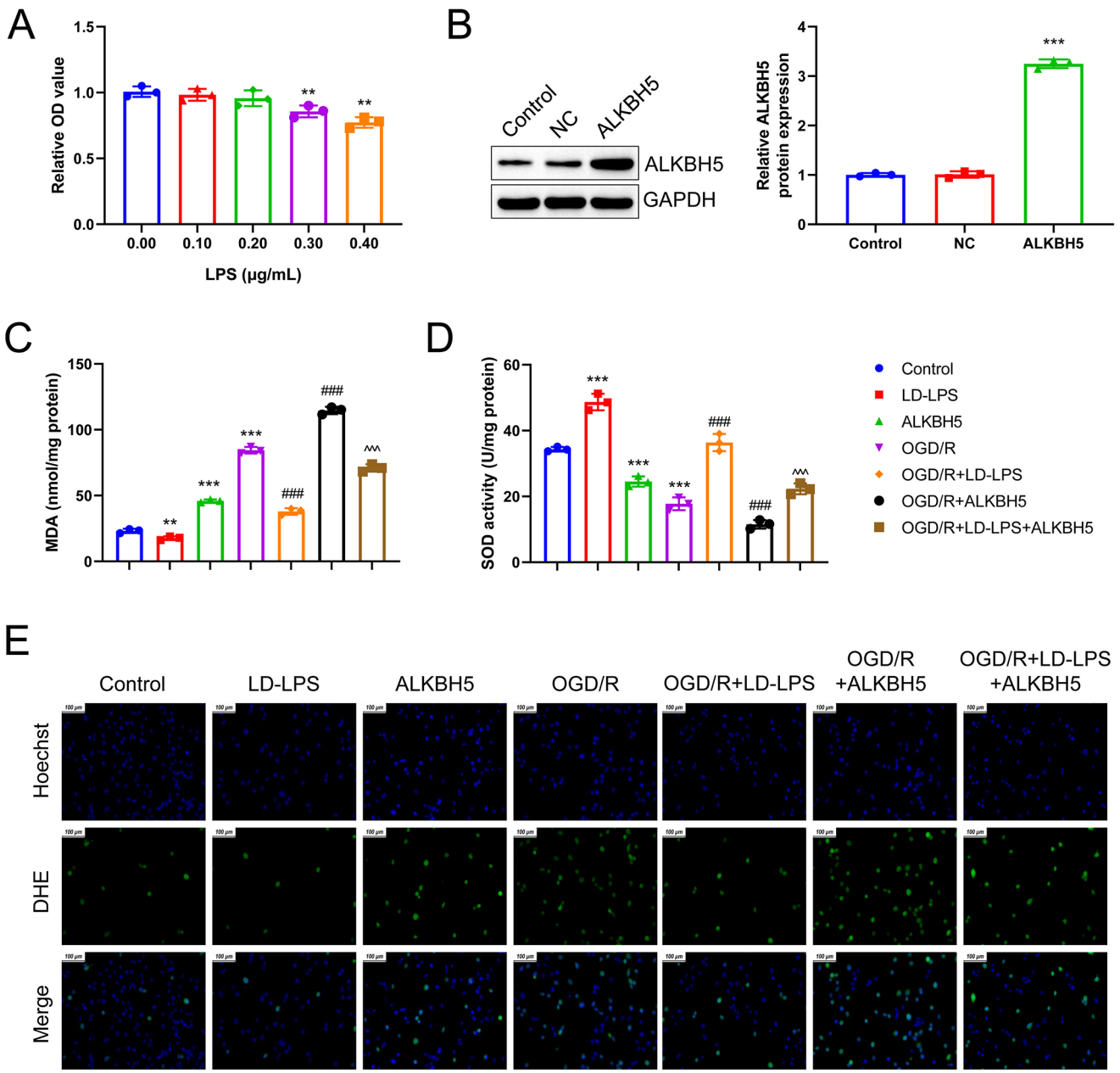

Fig. 4 LD-LPS relieved oxidative stress in the OGD/R-induced PC12 cells by down-regulating ALKBH5. (**A**) CCK-8 assay identified that LPS at 0.2 μg/mL was the optimal concentration for treating PC12 cells. ** $P<0.01$ vs. PC12 cells without LPS treatment. (**B**) Western blot instructed the effectively up-regulated ALKBH5 protein in PC12 cells by transfection of ALKBH5 overexpression vectors. *** $P<0.001$ vs. the Control group and the NC group. (**C**-**D**) Through ELISA, ALKBH5 up-regulation counteracted the suppression of LD-LPS on oxidative stress in the OGD/R-induced PC12 cells. ** $P<0.01$ and *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the OGD/R group. ^^^ $P<0.001$ vs. the OGD/R+LD-LPS group. (**E**) By DHE staining, LD-LPS treatment reduced ROS content in the OGD/R-induced PC12 cells, which was reversed by ALKBH5 up-regulation

LD-LPS Might Attenuate Apoptosis in the OGD/R-Induced PC12 Cells by Suppressing ALKBH5

The morphology of PC12 cells in each group was observed under an electron microscope. As shown in Fig. 5A, PC12 cells of the Control group showed normal spindle-like morphology with adherent growth. LD-LPS treatment had no obvious effect on the spindle-like morphology of PC12 cells (LD-LPS group vs. the Control group). However, ALKBH5 overexpression or OGD/R induction significantly disrupted the spindle-like morphology of PC12 cells and led to significant cell clustering, especially with OGD/R induction (the ALKBH5 group vs. the Control group; the OGD/R group vs. the Control group). The disruption of the spindle-like

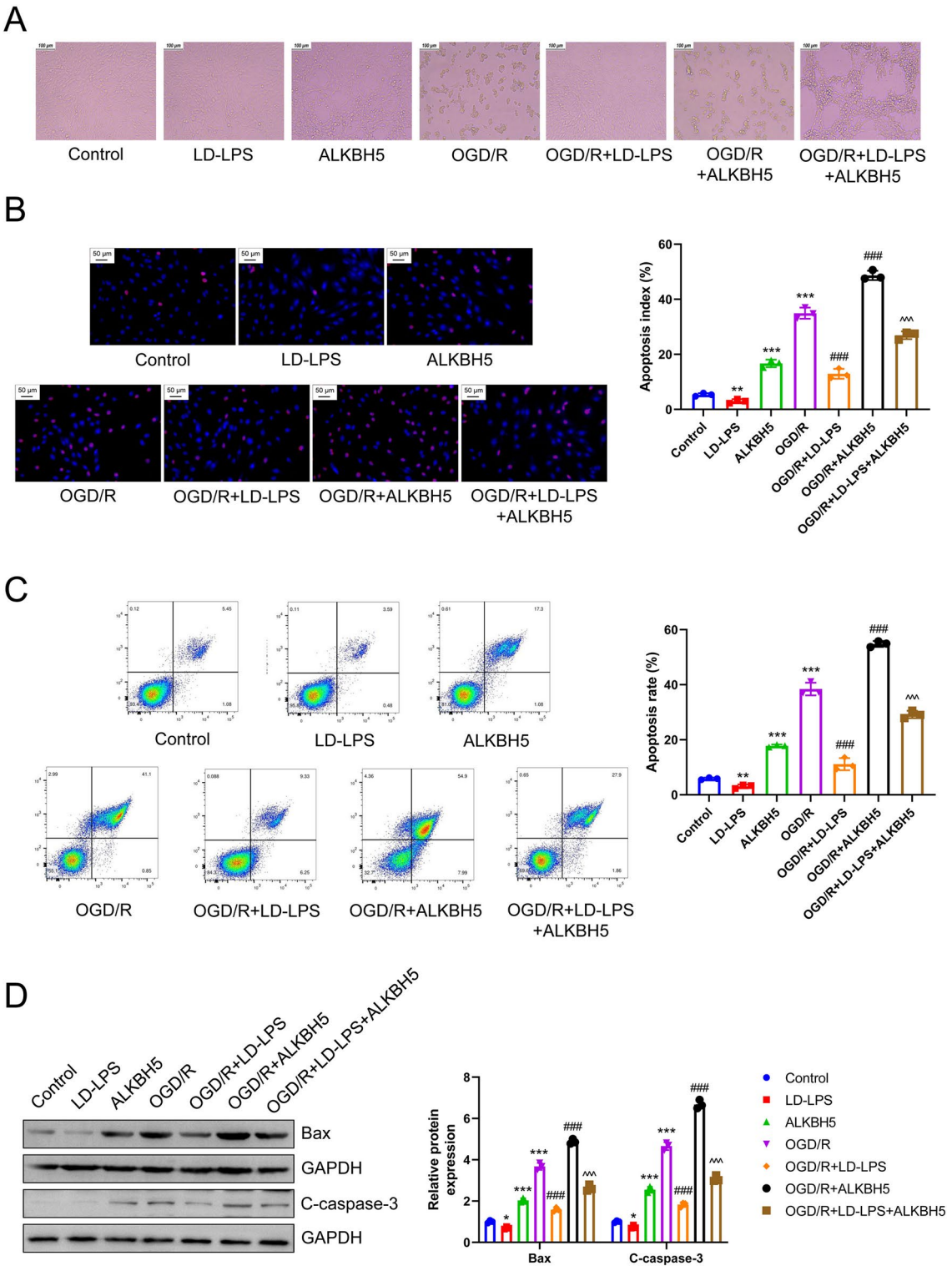

Fig. 5 LD-LPS suppressed apoptosis in the OGD/R-induced PC12 cells by inhibiting ALKBH5 expression. (**A**) Under an electron microscope, LD-LPS treatment helped to improve the spindle-like morphology of the OGD/R-induced PC12 cells and alleviate cell clustering, but was reversed by ALKBH5 overexpression. (**B**-**C**) TUNEL and flow cytometry suggested that, ALKBH5 up-regulation abrogated the inhibition of LD-LPS treatment on the apoptosis of the OGD/R-induced PC12 cells. (**D**) By Western blot, LD-LPS treatment reduced the expression of Bax and C-caspase-3 proteins in the OGD/R-induced PC12 cells, which was counteracted by ALKBH5 overexpression. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the OGD/R group. ^^^ $P<0.001$ vs. the OGD/R + LD-LPS group

morphology and cell clumping caused by OGD/R induction was alleviated by LD-LPS treatment (the OGD/R+LD-LPS group vs. the OGD/R group), but were exacerbated by ALKBH5 overexpression (the OGD/R+ALKBH5 group vs. the OGD/R group). When matched to the OGD/R+LD-LPS group, PC12 cells of the OGD/R+LD-LPS+ALKBH5 group exhibited more severe disruption of the spindle-like morphology and cell clumping.

TUNEL staining (Fig. 5B) and flow cytometry (Fig. 5C) were performed to analyze apoptosis of PC12 cells. In comparison to the Control group, the apoptosis of PC12 cells was attenuated in the LD-LPS group ($P<0.01$), but intensified in the ALKBH5 group and the OGD/R group ($P<0.001$). Relative to the OGD/R group, PC12 cells of the OGD/R+LD-LPS group showed the relieved apoptosis but those of the OGD/R+ALKBH5 group displayed the enhanced apoptosis ($P<0.001$). Exacerbated apoptosis was monitored in PC12 cells of the OGD/R+LD-LPS+ALKBH5 group, as compared to the OGD/R+LD-LPS group ($P<0.001$).

Based on results from Western blot (Fig. 5D), PC12 cells of the LD-LPS expressed lower Bax and C-caspase-3 proteins than those of the Control group ($P<0.05$). Conversely, higher Bax and C-caspase-3 proteins were observed in PC12 cells of the ALKBH5 group and the OGD/R group, when compared to the Control group ($P<0.001$). The increased Bax and C-caspase-3 proteins induced by OGD/R was decreased by LD-LPS treatment (the OGD/R+LD-LPS group vs. the OGD/R group) ($P<0.001$), but further enhanced by ALKBH5 overexpression (the OGD/R+ALKBH5 group vs. the OGD/R group) ($P<0.001$). PC12 cells of the OGD/R+LD-LPS+ALKBH5 group showed the intensified expression of Bax and C-caspase-3 proteins than those of the OGD/R+LD-LPS group ($P<0.001$). Therefore, all of these results implied that LD-LPS might inhibit apoptosis in the OGD/R-induced PC12 cells via blocking ALKBH5 expression.

LD-LPS Might Enhance Nrf2 Expression and its m6A Methylation in the OGD/R-induced PC12 Cells by Down-Regulating ALKBH5

The expression of Nrf2 protein, mRNA, and its m6A methylation in PC12 cells were appraised by Western blot (Fig. 6A), qRT-PCR (Fig. 6B), and Me-RIP assay (Fig. 6C), separately. The increased Nrf2 protein, mRNA and m6A modification levels in PC12 cells of the LD-LPS group was observed (the LD-LPS group vs. the Control group) ($P<0.001$), whereas these were decreased in PC12 cells of the ALKBH5 group and the OGD/R group (the ALKBH5 group and the OGD/R group vs. the Control group) ($P<0.001$). Matched to PC12 cells of the OGD/R group, higher Nrf2 protein, mRNA and m6A modification levels of the OGD/R+LD-LPS group ($P<0.001$), but lower Nrf2 protein, mRNA and m6A modification levels of the

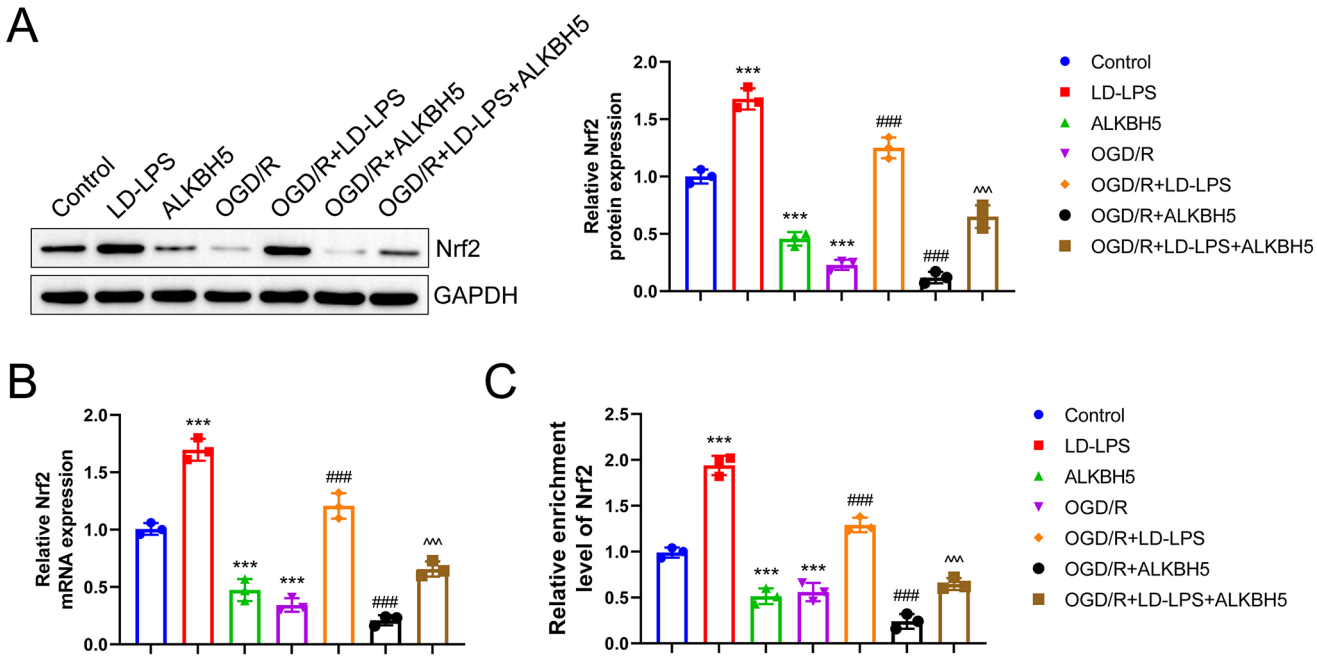

Fig. 6 LD-LPS facilitated Nrf2 expression and its m6A methylation in the OGD/R-induced PC12 cells by repressing ALKBH5. (**A-B**) Western blot along with qRT-PCR indicated that ALKBH5 up-regulation abrogated the enhancement of LD-LPS on protein and mRNA expression of ALKBH5 in the OGD/R-induced PC12 cells. (**C**) Me-RIP assay revealed that ALKBH5 up-regulation abolished the promotion of LD-LPS on Nrf2 m6A methylation in the OGD/R-induced PC12 cells. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the OGD/R group. ^^^ $P<0.001$ vs. the OGD/R+LD-LPS group

OGD/R + ALKBH5 group were monitored ($P < 0.001$). As referred to the OGD/R + LD-LPS group, PC12 cells of the OGD/R + LD-LPS + ALKBH5 group presented a distinct reduction in Nrf2 protein, mRNA and m6A modification levels ($P < 0.001$). It was noteworthy that the data between the different groups in Fig. 6B and C showed a similar trend of results. This was attributed to the fact that the m6A methylation level of Nrf2 mRNA directly regulated the stability and degradation of Nrf2 mRNA. Therefore, LD-LPS treatment was found to increase Nrf2 expression and its m6A methylation in OGD/R-induced PC12 cells by inhibiting ALKBH5.

ALKBH5 Suppressed Nrf2 m6A Methylation in PC12 Cells

The influence of ALKBH5 on Nrf2 m6A methylation was verified. ALKBH5 expression at mRNA and protein levels in PC12 cells was up-regulated by ALKBH5 overexpression vectors-transfection (the ALKBH5 group vs. the NC group) ($P < 0.001$), but was down-regulated by ALKBH5 siRNA transfection (the si-ALKBH5 group vs. the si-NC group) ($P < 0.001$) (Fig. 7A and B).

Interestingly, ALKBH5 up-regulation pronouncedly reduced Nrf2 m6A methylation and mRNA expression (the

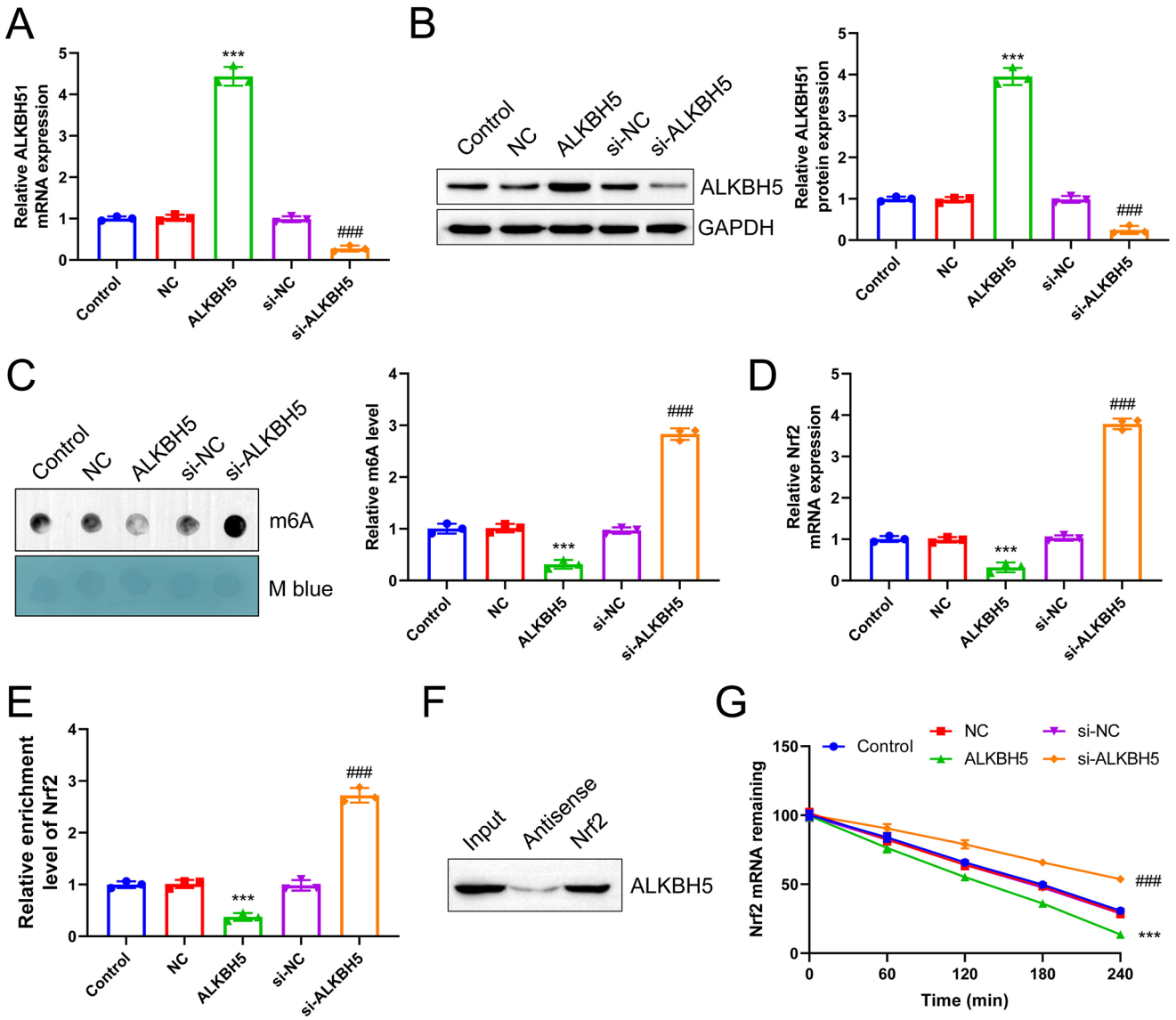

Fig. 7 ALKBH5 blocked Nrf2 m6A methylation in PC12 cells. (**A**-**B**) Western blot showed the effectively regulated ALKBH5 mRNA and protein expression in PC12 cells. (**C**) Dot-blot experiment displayed the repression of ALKBH5 on Nrf2 m6A methylation in PC12 cells. (**D**) qRT-PCR instructed the inhibition of ALKBH5 on Nrf2 mRNA expression in PC12 cells. (**E**) Me-RIP assay illustrated the blockage of ALKBH5 on Nrf2 m6A methylation in PC12 cells. (**F**) RNA-pull down assay demonstrated the effectiveness of ALKBH5 on pulling down Nrf2 protein in PC12 cells. (**G**) ALKBH5 markedly facilitated the degradation of Nrf2 mRNA in the actinomycin D-treated PC12 cells. *** $P < 0.001$ vs. the NC group. ### $P < 0.001$ vs. the si-NC group

ALKBH5 group vs. the NC group) ($P<0.001$) as shown by dot-blot experiment (Fig. 7C), qRT-PCR (Fig. 7D), and MeRIP assay (Fig. 7E). Conversely, opposite results were discovered by ALKBH5 silencing (the si-ALKBH5 group vs. the si-NC group) ($P<0.001$). A prominent pull-down influence of ALKBH5 on Nrf2 protein in PC12 cells was monitored by RNA-pull down assay (Fig. 7F). Concurrently, in the PC12 cells treated with actinomycin D, the up-regulation of ALKBH5 significantly enhanced the degradation of Nrf2 mRNA within 240 min ($P<0.001$). Conversely, silencing ALKBH5 clearly impeded the degradation of Nrf2 mRNA at the same time point ($P<0.001$) (Fig. 7G). Based on these results, the suppression role of ALKBH5 on Nrf2 m6A methylation in PC12 cells was verified.

ALKBH5 Might Enhance Oxidative Stress in the OGD/R-induced PC12 Cells by Suppressing Nrf2 m6A Methylation

siRNAs against ALKBH5 and Nrf2 were utilized to transfect PC12 cells. According to the results of Western blot, ALKBH5 siRNA and Nrf2 siRNA significantly reduced the expression of proteins for ALKBH5 and Nrf2 in PC12 cells ($P<0.001$) (Fig. 8A and D).

ELISA displayed higher MDA level and lower SOD activity in the OGD/R-induced PC12 cells (the OGD/R group vs. the Control group) ($P<0.001$). In contrast to the OGD/R group, PC12 cells of the OGD/R + si-ALKBH5 group had lower MDA level and higher SOD activity ($P<0.001$), while those of the OGD/R + si-Nrf2 group exhibited higher MDA level and lower SOD activity ($P<0.001$). Intriguingly, PC12 cells of the OGD/R + si-ALKBH5 + si-Nrf2 group had higher MDA level and lower SOD activity than those of the OGD/R + si-ALKBH5 group ($P<0.001$) (Fig. 8E and F). From DHE staining, the intensified ROS content was monitored in PC12 cells of the OGD/R group, as referred to the Control group. Compared to the OGD/R group, PC12 cells of the OGD/R + si-ALKBH5 group showed the reduced ROS content, but those of the OGD/R + si-Nrf2 group displayed the exacerbated ROS content. The increased ROS content in PC12 cells of the OGD/R + si-ALKBH5 + si-Nrf2 group was observed when matched to the OGD/R + si-ALKBH5 group (Fig. 8G). Thereby, in the OGD/R-induced PC12 cells, the suppression of oxidative stress by ALKBH5 silencing was reversed by the loss of Nrf2. This suggested that ALKBH5 might facilitate oxidative stress in the OGD/R-induced PC12 cells via blocking Nrf2 m6A methylation.

ALKBH5 Might Exacerbate Apoptosis of the OGD/R-induced PC12 Cells by Blocking Nrf2 m6A Methylation

Under electron microscope, the normal spindle-like morphology with adherent growth was observed in PC12 cells of the Control group, whereas severe disruption in spindle-like morphology and obvious cell clustering was found in those of the OGD/R group. In contrast to the OGD/R group, PC12 cells of the OGD/R + si-ALKBH5 group showed the improved spindle-like morphology and relieved cell clustering, but those of the OGD/R + si-Nrf2 group exhibited the intensified damage in spindle-like morphology and the exacerbated cell clustering. Relative to the OGD/R + si-ALKBH5 group, PC12 cells of the OGD/R + si-ALKBH5 + si-Nrf2 group presented the enhanced spindle-like morphology damage and cell clustering (Fig. 9A).

By TUNEL staining (Fig. 9B and C) and flow cytometry (Fig. 9D and E), the increased apoptosis occurred in the OGD/R-induced PC12 cells (the OGD/R group vs. the Control group) ($P<0.001$). ALKBH5 silencing relieved the OGD/R-induced PC12 cell apoptosis (the OGD/R + si-ALKBH5 group vs. the OGD/R group) ($P<0.001$), but the loss of Nrf2 intensified it (the OGD/R + si-Nrf2 group vs. the OGD/R group) ($P<0.001$). In comparison to the OGD/R + si-ALKBH5 group, the enhanced apoptosis was discovered in PC12 cells of the OGD/R + si-ALKBH5 + si-Nrf2 group ($P<0.001$).

Western blot was adopted to research the expression of Bax and C-caspase-3 proteins in PC12 cells from different treatment groups. OGD/R induction caused a much elevation in the expression of Bax and C-caspase-3 proteins in PC12 cells (the OGD/R group vs. the Control group) ($P<0.001$). ALKBH5 silencing down-regulated the expression of Bax and C-caspase-3 proteins in the OGD/R-induced PC12 cells (the OGD/R + si-ALKBH5 group vs. the OGD/R group) ($P<0.001$), but Nrf2 knockdown up-regulated it (the OGD/R + si-Nrf2 group vs. the OGD/R group) ($P<0.001$). In fact, PC12 cells of the OGD/R + si-ALKBH5 + si-Nrf2 group expressed higher Bax and C-caspase-3 proteins than those of the OGD/R + si-ALKBH5 group ($P<0.001$) (Fig. 9F). Hence, ALKBH5 was implied to enhance the apoptosis of the OGD/R-induced PC12 cells through suppressing Nrf2 m6A methylation.

LD-LPS Might Relieve Neuronal Damage in Rats with SCI by Down-Regulating ALKBH5

By in vivo rescue experiments, the role of ALKBH5 in LD-LPS mitigation of SCI was validated. The flowchart of the animal experiment was shown in Fig. 10A. BBB score evaluation (Fig. 10B), Nissl staining (Fig. 10C), ELISA

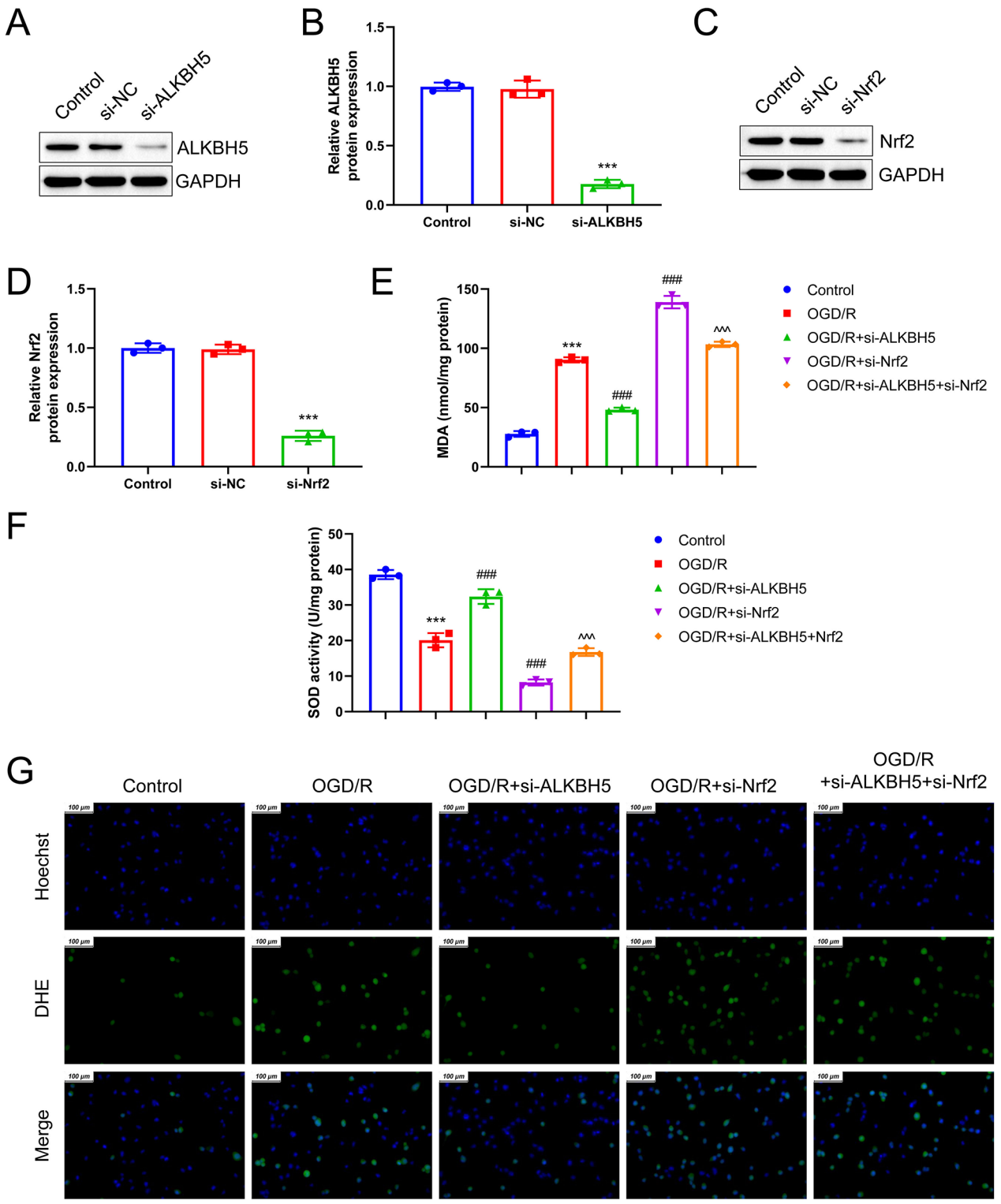

Fig. 8 ALKBH5 enhanced oxidative stress in the OGD/R-induced PC12 cells through blocking Nrf2 m6A methylation. (**A-D**) Western blot presented the inhibition of siRNAs against ALKBH5 and Nrf2 on ALKBH5 and Nrf2 protein expression in PC12 cells. (**E-F**) ELISA suggested the suppression on oxidative stress by ALKBH5 silencing was reversed by the loss of Nrf2 in the OGD/R-induced PC12 cells. (**G**) By DHE staining, the suppression of ALKBH5 silencing on ROS content in the OGD/R-induced PC12 cells was abolished by the loss of Nrf2. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the OGD/R group. ^^^ $P<0.001$ vs. the OGD/R + si-ALKBH5 group

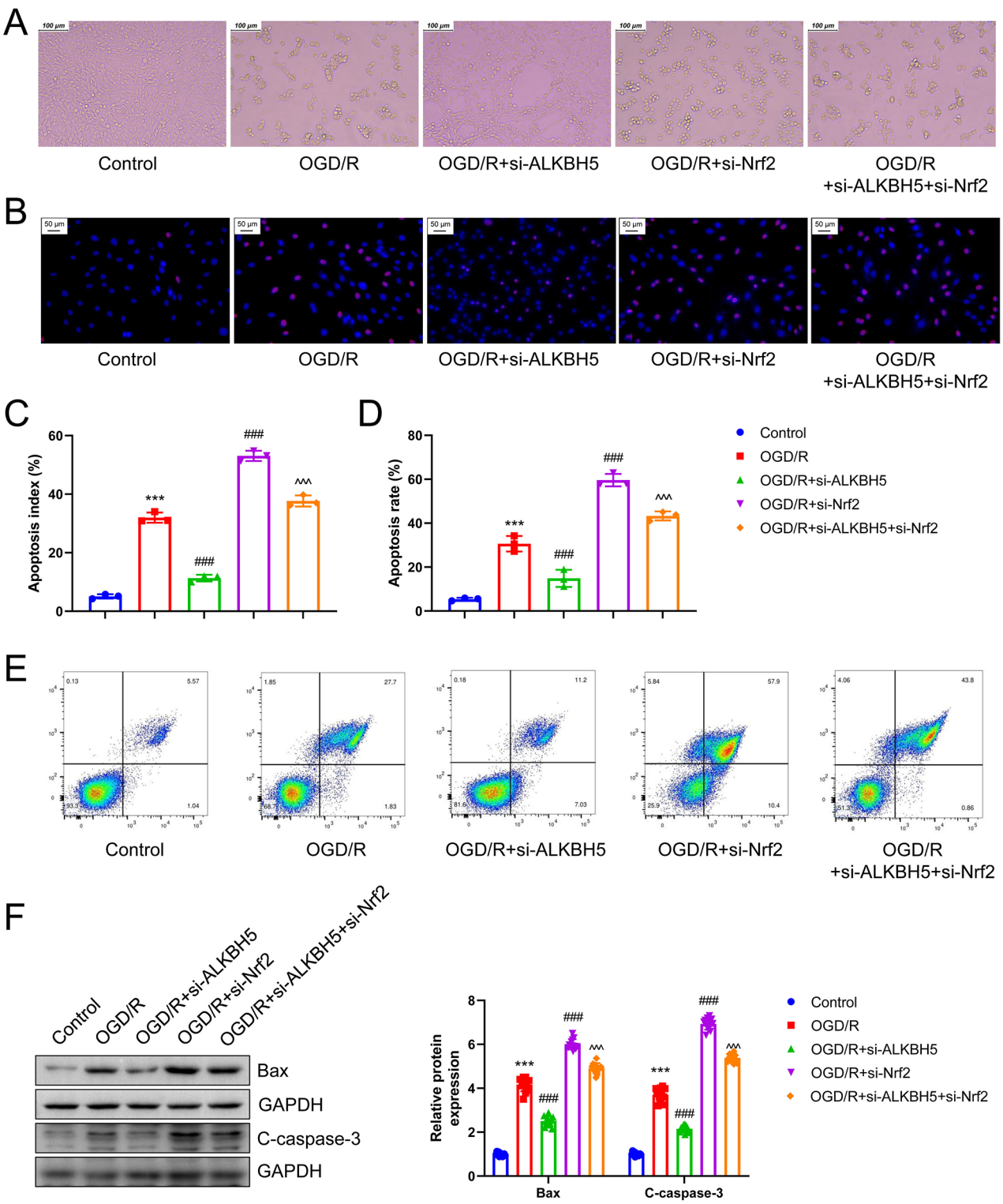

Fig. 9 ALKBH5 facilitated apoptosis of the OGD/R-induced PC12 cells through suppressing Nrf2 m6A methylation. (**A**) Under electron microscope, ALKBH5 silencing was conductive to improve the spindle-like morphology and relieve cell clustering of the OGD/R-induced PC12 cells, which was eliminated by Nrf2 silencing. (**B-C**) TUNEL staining revealed that, the inhibition of ALKBH5 silencing on the OGD/R-induced PC12 cell apoptosis was reversed by the loss of Nrf2. (**D-E**) Flow cytometry indicated that, ALKBH5 silencing suppressed the OGD/R-induced PC12 cell apoptosis, but this influence was abrogated by the loss of Nrf2. (**F**) Western blot showed the repression of ALKBH5 silencing on the expression of Bax and C-caspase-3 proteins in the OGD/R-induced PC12 cells, which was abolished by the loss of Nrf2. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the OGD/R group. ^^^ $P<0.001$ vs. the OGD/R + si-ALKBH5 group

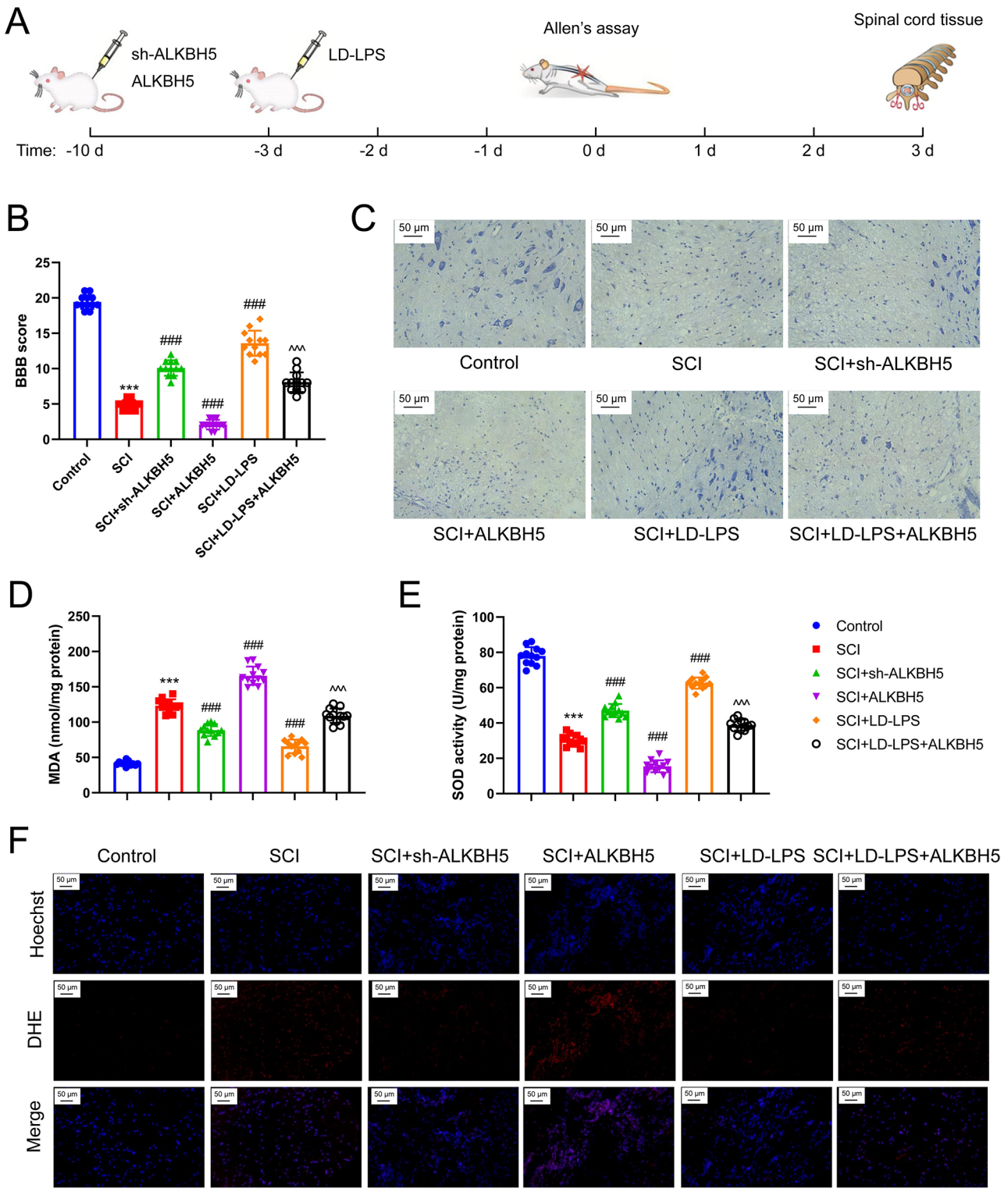

Fig. 10 LD-LPS attenuated neuronal damage in rats with SCI by down-regulating ALKBH5. (**A**) The flowchart of the animal experiment. (**B**) ALKBH5 reversed the elevation of LD-LPS on BBB score in rats with SCI. (**C**) Nissl staining revealed that ALKBH5 abrogated the neuroprotective effect of LD-LPS on neuronal survival in the spinal cords of rats with SCI. (**D**-**E**) ELISA results demonstrated that ALKBH5 counteracted the inhibitory effect of LD-LPS on oxidative stress in the spinal cord tissues of rats with SCI. (**F**) By DHE staining, ALKBH5 abrogated the relief of LD-LPS on ROS content in spinal cord tissues of rats with SCI. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group. ^^^ $P<0.001$ vs. the SCI + LD-LPS group

(Fig. 10D and E), and DHE staining (Fig. 10F) were carried out to investigate neurologic function, neuronal survival, MDA content and SOD activity, and ROS content, respectively. Relative to control rats, rats with SCI (the SCI group) showed lower BBB score, less survival neurons, higher MDA level, lower SOD activity, and increased ROS content in spinal cord tissues ($P<0.001$). In SCI rats, ALKBH5 silencing led to an elevation in BBB score, increase in survival neurons, reduction in MDA level, enhancement in SOD activity, and decline in ROS content in spinal cord tissues (the SCI+sh-ALKBH5 group vs. the SCI group) ($P<0.001$). However, ALKBH5 up-regulation showed an opposite result, comparatively (the SCI+ALKBH5 group vs. the SCI group) ($P<0.001$). LD-LPS treatment showed similar effects to ALKBH5 silencing on rats with SCI, which resulted in the increased BBB score, reduced survival neurons, declined MDA level, enhanced SOD activity, and attenuated ROS content in spinal cord tissues (the SCI+LD-LPS group vs. the SCI group) ($P<0.001$). Importantly, as referred to the SCI+LD-LPS group, rats of the SCI+LD-LPS+ALKBH5 group had lower BBB score, less survival neurons, higher MDA level, lower SOD activity, and exacerbated ROS content in spinal cord tissues ($P<0.001$). These data showed the exacerbating effect of ALKBH5 on neuronal damage in rats with SCI, and the relief of LD-LPS on neuronal damage in rats with SCI was abrogated by ALKBH5. Thus, LD-LPS might attenuate neuronal damage in rats with SCI by blocking the expression of ALKBH5.

LD-LPS Might Attenuate Neuronal Apoptosis in Rats with SCI via Repressing the Expression of ALKBH5

Neuronal apoptosis and expression of Bax and C-caspase-3 proteins in spinal cord tissues of rats were examined by TUNEL staining (Fig. 11A) and Western blot (Fig. 11B), respectively. Rats with SCI exhibited the increased apoptosis and expression of Bax and C-caspase-3 proteins in spinal cord tissues (the SCI group vs. the Control group) ($P<0.001$). As referred to the SCI group, the reduced apoptosis and expression of Bax and C-caspase-3 proteins in the SCI+sh-ALKBH5 group but the elevated apoptosis and expression of Bax and C-caspase-3 proteins in the SCI+ALKBH5 group were monitored in spinal cord tissues of rats ($P<0.001$). Rats of the SCI+LD-LPS group displayed lower apoptosis and expression of Bax and C-caspase-3 proteins in spinal cord tissues than those of the SCI group ($P<0.001$). Compared to the SCI+LD-LPS group, the intensified apoptosis and expression of Bax and C-caspase-3 proteins was discovered in spinal cord tissues of rats in the SCI+LD-LPS+ALKBH5 group ($P<0.001$). It was illustrated that LD-LPS might relieve neuronal apoptosis in rats with SCI by blocking ALKBH5 expression.

LD-LPS Might Enhance Nrf2 m6A Methylation by Down-Regulating ALKBH5 in Rats with SCI

Western blot (Fig. 12A), qRT-PCR (Fig. 12B), and Me-RIP assay (Fig. 12C) were used to detect ALKBH5 and Nrf2 protein expression, Nrf2 mRNA expression, and its m6A methylation. The results showed higher ALKBH5 protein expression but lower Nrf2 mRNA, protein, and m6A methylation in spinal cord tissues of rats with SCI, compared to the Control group ($P<0.001$). In comparison to the SCI group, rats of the SCI+sh-ALKBH5 group displayed lower ALKBH5 protein, but higher Nrf2 mRNA, protein and m6A methylation in spinal cord tissues ($P<0.001$). Conversely, higher ALKBH5 protein, as well as lower Nrf2 mRNA, protein and m6A methylation occurred in spinal cord tissues of rats in the SCI+ALKBH5 group, as matched to the SCI group ($P<0.001$). Similar to ALKBH5 silencing, LD-LPS treatment decreased ALKBH5 protein, but enhanced Nrf2 mRNA, protein and m6A methylation in spinal cord tissues of rats with SCI (the SCI+LD-LPS group vs. the SCI group) ($P<0.001$). However, in contrast to rats in the SCI+LD-LPS group, those in the SCI+LD-LPS+ALKBH5 group had higher ALKBH5 protein, but lower Nrf2 mRNA, protein and m6A methylation in spinal cord tissues ($P<0.001$). In spinal cord tissues of rats with SCI, ALKBH5 was found to reduce Nrf2 expression and its m6A methylation. Additionally, ALKBH5 abolished the enhanced Nrf2 expression and its m6A methylation caused by LD-LPS treatment. The data from this study suggested that LD-LPS might alleviate neuronal damage in rats with SCI by enhancing Nrf2 m6A methylation through the inhibition of ALKBH5 expression.

Discussion

This study emphasized the effectiveness of LD-LPS on improving motor function and mitigating neuronal apoptosis and oxidative stress in rats after SCI. Bax and C-caspase-3 proteins are key pro-apoptotic proteins [17]. In this study, LD-LPS was shown to decrease the levels of Bax and C-caspase-3 proteins in the spinal cord tissues of rats with spinal cord injury (SCI) and in OGD/R-induced PC12 cells. It is well known that SOD and MDA are important indicators of oxidative stress. SOD has antioxidant activity, whereas MDA is a major metabolite of oxidative stress [18, 19]. The overproduction of ROS can trigger oxidative stress injury in brain tissues and neurons, and can also trigger apoptosis by damaging lipids, proteins, DNA and subcellular organelles [20]. This study revered the suppression of LD-LPS on neuronal oxidative stress after SCI, as it increased SOD but decreased MDA and ROS content in spinal cord tissues of rats with SCI and the OGD/R-induced PC12 cells. LD-LPS

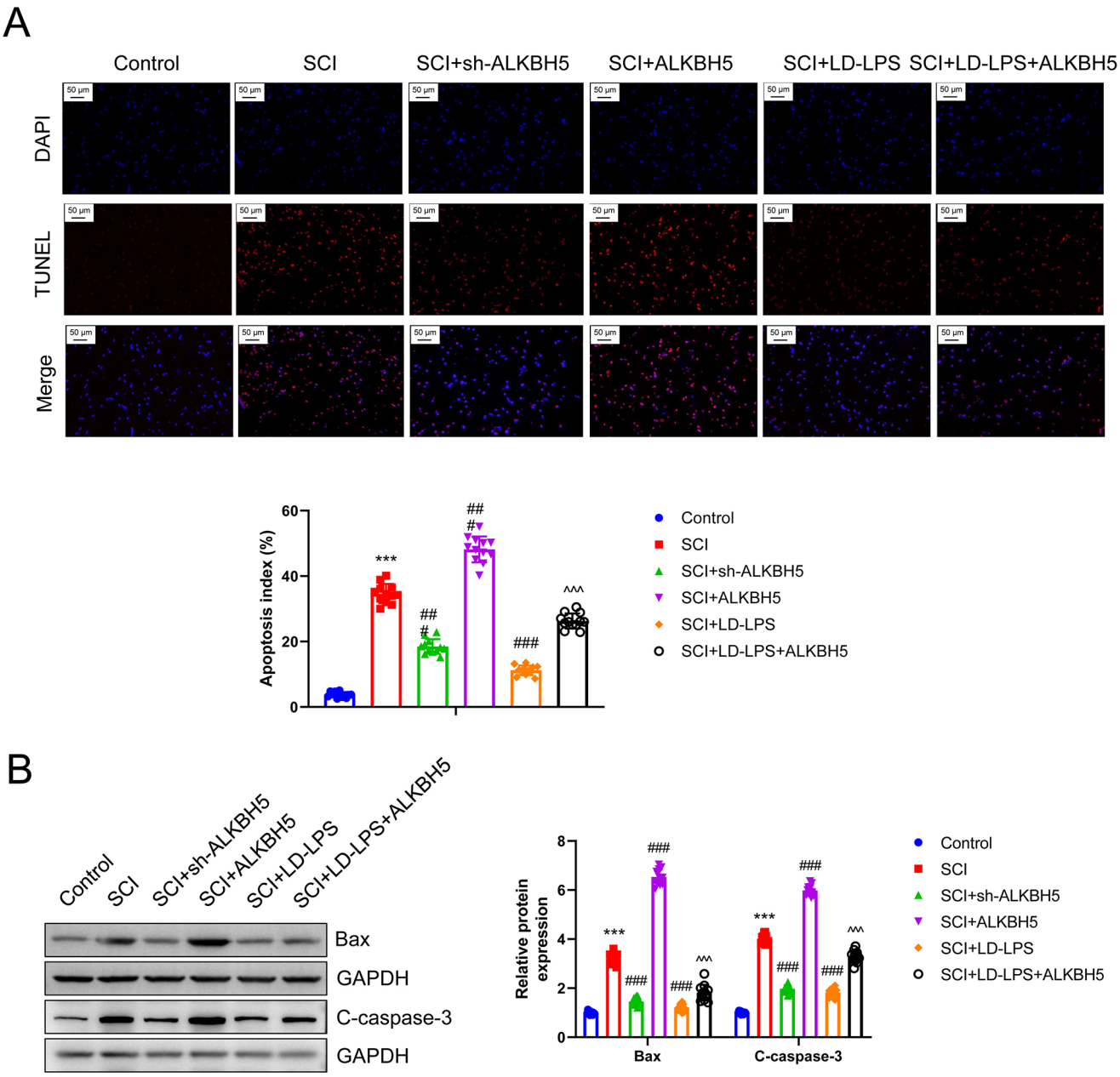

Fig. 11 LD-LPS relieved neuronal apoptosis in rats with SCI by blocking ALKBH5 expression. (**A**) By TUNEL staining, ALKBH5 abrogated the relief of LD-LPS on neuronal apoptosis in spinal cord tissues of rats with SCI. (**B**) Western blot indicated that, the suppression of LD-LPS on the expression of Bax and C-caspase-3 proteins in spinal cord tissues of rats with SCI was reversed by ALKBH5 overexpression. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group. ^^^ $P<0.001$ vs. the SCI+LD-LPS group

has been found to potentially improve spinal cord function and rehabilitation in rats with SCI by facilitating neuroprotective phenotypic switching of microglia/macrophages [21]. In our previous study, LD-LPS has been implied to exert neuroprotective effects after SCI, for example, it activated neuronal autophagy and attenuated neuronal apoptosis by activating Nrf2 via enhancing the expression of long non-coding RNA MALAT1 [8]. LD-LPS treatment alleviates neuronal inflammation and oxidative stress after SCI by activating the PI3K/AKT/Nrf2 pathway via blocking miR-429 expression [9]. Unlike these previous studies, this paper firstly instructed that LD-LPS might attenuate neuronal apoptosis and oxidative stress after SCI by facilitating Nrf2 m6A methylation via down-regulating ALKBH5.

As a conserved RNA m6A demethylase, ALKBH5 has been monitored to be involved in multiple disease progression through regulating the m6A demethylation of multiple genes [22, 23]. In the field of neurological injuries, ALKBH5 has been discovered to be a regulator of axonal regeneration; specifically, ALKBH5 up-regulation can restrict axonal

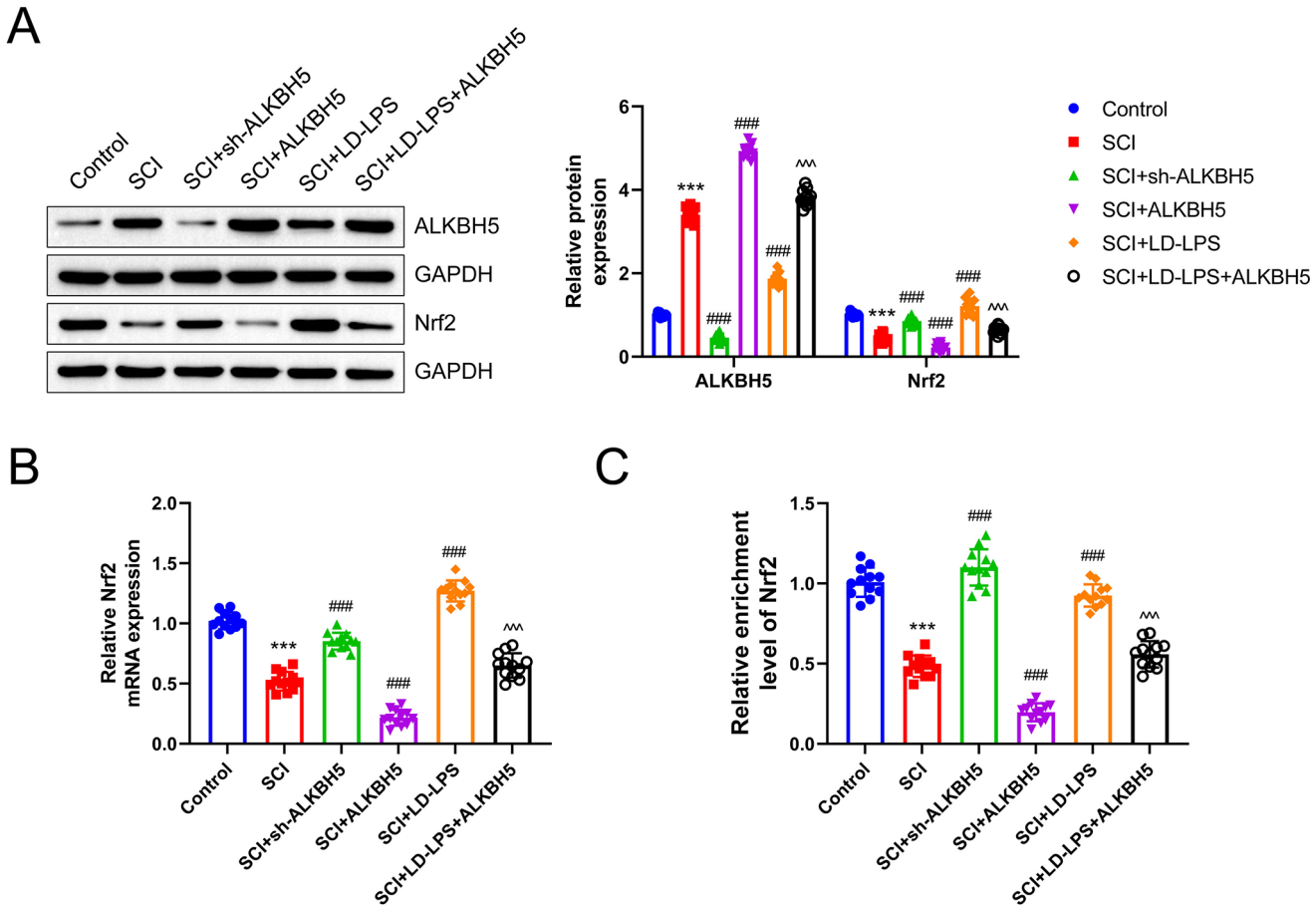

Fig. 12 LD-LPS enhanced Nrf2 m6A methylation by down-regulating ALKBH5 in spinal cord tissues of rats with SCI. (**A**-**B**) Western blot and qRT-PCR exhibited the promotion on Nrf2 expression by LD-LPS was reversed by ALKBH5 in spinal cord tissues of rats with SCI. (**C**) Me-RIP assay instructed@ the induction on Nrf2 m6A methylation by LD-LPS was abolished by ALKBH5 in spinal cord tissues of rats with SCI. *** $P<0.001$ vs. the Control group. ### $P<0.001$ vs. the SCI group. ^^^ $P<0.001$ vs. the SCI+LD-LPS group

regeneration in an m6A-dependent manner, while the loss of ALKBH5 leads to the enhancement in neuronal survival and axonal regeneration [24]. On the other hand, ALKBH5 may also enhance the expression of apoptosis-promoting proteins through m6A demethylation, thereby exacerbating neuronal damage in cerebral ischemia-reperfusion injury [22]. This work implied the up-regulated ALKBH5 in spinal cord tissues of rats with SCI. Similar results were found in the OGD/R-induced PC12 cells. ALKBH5 overexpression exacerbated neuronal apoptosis and oxidative stress in the OGD/R-induced PC12 cells. Thus, the role of ALKBH5 on neuronal damage was consistent with these above previous studies. Moreover, this work showed that LD-LPS treatment hindered the expression of ALKBH5 in spinal cord tissues of rats with SCI and the OGD/R-induced PC12 cells. Importantly, in rats with SCI and the OGD/R-induced PC12 cells, the enhanced neuronal survival and the blocked neuronal apoptosis and oxidative stress by LD-LPS was counteracted by ALKBH5 overexpression. This suggested that LD-LPS might attenuate neuronal damage and oxidative stress after SCI by hindering the expression of ALKBH5. As far as we know, this mechanism by which LD-LPS alleviated SCI was elucidated for the first time.

After SCI, the enhanced Nrf2 signaling can suppress NLRP3 inflammasome activation to induce the attenuation of neuroinflammation and oxidative stress [25], and the recovery of neurological function is largely attributed to the antioxidant properties of Nrf2 [26]. Therefore, Nrf2 is proposed to be an ideal target for the therapy of SCI [27]. In this work, the reduced Nrf2 expression in spinal cord tissues of rats with SCI and the OGD/R-induced PC12 cells was up-regulated by LD-LPS treatment, suggesting that LD-LPS might relieve neuronal apoptosis and oxidative stress after SCI by inducing the expression of Nrf2. It has been reported that the ALKBH5-mediated m6A methylation can result in post-transcriptional repression of Nrf2 and knockdown of ALKBH5 increases the expression of Nrf2 protein [15]. In a study related to ischaemic stroke, Nrf2 m6A methylation modification has been researched to protect neurons from microglia-induced inflammatory injury by promoting the

conversion of microglia from M1 pro-inflammatory phenotype to M2 anti-inflammatory phenotype [28]. The present paper not only revealed the suppression effect of ALKBH5 on Nrf2 expression, but also instructed the inhibition of ALKBH5 on Nrf2 m6A methylation. More importantly, ALKBH5 silencing attenuated neuronal apoptosis and oxidative stress in the OGD/R-induced PC12 cells, which was abolished by the loss of Nrf2. Thus, all of the evidence implied that LD-LPS might attenuate neuronal apoptosis and oxidative stress after SCI by enhancing Nrf2 m6A methylation modification through blocking the expression of ALKBH5. To our knowledge, this molecular pathway uncovering LD-LPS mitigation of neuronal damage after SCI represents a groundbreaking discovery. This finding will establish a reliable molecular mechanism supporting the clinical use of LD-LPS in treating SCI.

Conclusion

This work uncovered the neuroprotective effects of LD-LPS after SCI. LD-LPS treatment relieved neuronal apoptosis and oxidative stress in rats with SCI. Importantly, LD-LPS treatment led to a decrease in ALKBH5 expression, an increase in Nrf2 expression, and its m6A methylation modification in spinal cord tissues of rats with SCI. Mechanistic findings have shown that LD-LPS could reduce neuronal apoptosis and oxidative stress after SCI by increasing Nrf2 m6A methylation through the inhibition of ALKBH5. These findings suggest that LD-LPS treatment may be a promising therapeutic approach for SCI.

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Data Availability All data generated or analyzed in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate The approval of the Animal Ethics Committee of The First People's Hospital of Yunnan province, Affiliated Hospital of Kunming University of Science and Technology, was obtained before the animal experiments being performed.

Competing Interests The authors declare no competing interests.

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