Article

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Hyperbaric Oxygen Therapy for Spinal Cord Injury: Involvement of the HIF-1alpha/HMOX1 Pathway and Ferroptosis

Xinguo Sun[1], Zitong Wu[1], Xiao Liu[2], Chunqiang Wang[1], Hongen Liu[1,*]

[1]Department of Neurosurgery, Binzhou People's Hospital, 256600 Binzhou, Shandong, China
[2]Medical Department, Binzhou People's Hospital, 256600 Binzhou, Shandong, China
*Correspondence: liuhongen_he@163.com (Hongen Liu)
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Background: Spinal cord injury (SCI) represents a severe neurological condition with limited effective treatments. Hyperbaric oxygen (HBO) therapy has demonstrated efficacy in alleviating SCI, yet its precise molecular mechanisms remain poorly elucidated. Previous genome-wide transcriptome analysis indicated that HBO downregulates mRNA expression of Heme oxygenase 1 (HMOX1) and Ferritin light chain 1 (FTL1), suggesting a potential link to ferroptosis, a novel form of iron-dependent regulated cell death. Notably, hypoxia-inducible factor-1alpha (HIF-1alpha), a key regulator of cellular response to hypoxia, is upregulated following SCI, and this upregulation is attenuated by HBO intervention. Given the involvement of the HIF-1alpha/HMOX1 axis in regulating ferroptosis, we hypothesized that the therapeutic effect of HBO in SCI may be mediated through modulation of this pathway.

Methods: An SCI-induced mouse model was established and treated with HBO for 7 days. Nissl staining and hematoxylin-eosin staining were performed for histopathological assessment. Real-time quantitative polymerase chain reaction and Western blotting were used to quantify HIF-1alpha/HMOX1 expression and ferroptosis-related proteins (Glutathione Peroxidase 4 (GPX4), Solute Carrier Family 7 Member 11 (SLC7A11)) in spinal cord tissues. Primary rat spinal neurons were treated with Erastin (ferroptosis inducer), HBO, or HIF-1alpha overexpression plasmid. Malondialdehyde (MDA) and Fe^{2+} levels were measured *in vitro* employing thiobarbituric acid reactive substances (TBARS) kit and colorimetric assay.

Results: HBO therapy increased Nissl bodies and reduced spinal cord edema and cavitation in SCI mice. It diminished HIF-1alpha/HMOX1 expression and elevated GPX4/SLC7A11 levels in the mouse model and in Erastin-induced neurons. HBO therapy also abrogated Erastin-induced upregulation of MDA and Fe^{2+}. Notably, HIF-1alpha overexpression reversed the protective effects of HBO on ferroptosis.

Conclusion: HBO therapy shows ameliorative effects against SCI, which are associated with the downregulation of the HIF-1alpha/HMOX1 signaling axis and the consequent suppression of ferroptosis. These findings elucidate a novel and promising molecular mechanism underlying HBO's therapeutic effect, providing a potential target for future SCI treatment strategies.

Keywords: hyperbaric oxygen; spinal cord injury; ferroptosis; hypoxia-inducible factor-1alpha; heme oxygenase 1

Introduction

Spinal cord injury (SCI) is a common condition affecting the central nervous system in clinical practice [1], which can also cause a variety of secondary injuries, including lung infection and respiratory failure [2]. Therefore, exploring treatment methods and the mechanisms of SCI is also a key direction in neuroscience research. Reportedly, programmed cell death (PCD) plays an important role in the development of the nervous system [3,4]. Some mechanisms of PCD in the pathology of SCI have been reviewed, contributing to understanding the etiology of SCI [3].

Hyperbaric oxygen (HBO) therapy is a safe, non-invasive physical modality that involves breathing pure oxygen in a barometric chamber at pressures exceeding atmospheric levels [5,6]. HBO therapy has been reported to reduce tissue damage and infection expansion [7]. The value of HBO has also been demonstrated in Alzheimer's disease [8]. Importantly, HBO treatment can alleviate SCI through multiple mechanisms, including rapid improvement of tissue hypoxia, as well as mitigation of apoptosis, oxidative stress, inflammation and spinal cord edema [9,10]. However, the molecular mechanism of HBO in SCI still needs to be further explored.

Through genome-wide transcriptome analyses, HBO has been proven to repress the mRNA levels of Heme oxygenase 1 (HMOX1) and Ferritin light chain 1 (FTL1) [11]. Given that upregulation of HMOX1 can promote ferroptosis, and FTL1 is related to iron metabolism [12,13], both

genes can serve as ferroptosis-related markers. Collectively, the mechanism of HBO may be related to ferroptosis. Ferroptosis is a PCD mode, in which increased iron ions induce massive lipid peroxidation and cause cell death [14]. Ferroptosis is involved in various neurological diseases such as stroke and degeneration [15]. Inhibition of ferroptosis can promote neural repair and exert neuroprotective effects [15,16]. The involvement of ferroptosis in the pathophysiological process of SCI has been directly documented, and its inhibition represents a new therapeutic strategy for SCI [17].

Furthermore, upregulation of HMOX1 promotes ferroptosis in acute leukemia cells [18]. In an endometriosis-related study, HMOX1 expression is augmented in early embryonic ferroptosis, and HMOX1 knockdown inhibits ferroptosis by maintaining mitochondrial function [19]. In addition, Hypoxia-inducible factor (HIF)-1alpha/Heme oxygenase 1 (HO-1) is highly expressed during ferroptosis [20], and HIF-1alpha is upregulated in SCI [21]. Notably, HBO treatment has been reported to reduce HIF-1alpha expression [22]. Therefore, this study investigates whether the protective effect of HBO in SCI is related to the regulation of the HIF-1alpha/HMOX1 axis and the inhibition of ferroptosis, with the aim of identifying a novel mechanistic link for HBO therapy in neural repair.

Materials and Methods

Ethics Committee and Animals

A total of 18 C57BL/6 J mice (female, about 8 weeks old, 20–25 g) and pregnant Sprague-Dawley (SD) rats (n = 6) were purchased from Hangzhou Medical College (Experimental Animal Production License: SCXK (Zhe) 2024-0002). The study protocols were approved by the Ethics Committee of Zhejiang Provincial Laboratory Animal Center for Experimental Animals Welfare (Approval No. ZJCLA-IACUC-20010873). Mice and rats were housed in a specific pathogen-free (SPF) animal room, with *ad libitum* access to food and water, and maintained under appropriate light, temperature and humidity.

SCI Model and HBO Treatment

Mice were randomly divided into three groups (n = 6 per group): Sham group, SCI group, and SCI+HBO group. SCI was induced in accordance with previous literature [23]. Mice were deeply anesthetized by intraperitoneal injection of 1% sodium pentobarbital (P3761, Sigma-Aldrich, St. Louis, USA) and placed in a prone position on the operating table to fully expose the spine, followed by T9/T10 laminectomy. After fixation of the spine, a MASCIS impactor (diameter: 2 mm, weight: 12.5 g, height: 1 cm) (Model III, W. M. Keck Center, Urbana, USA) was used to induce a spinal cord contusion injury. The Sham group adopted the same procedure without spinal cord contusion. Six hours after SCI modeling, mice in the SCI+HBO group were placed in an experimental HBO room (volume: 0.196 m^3, ProOx-820, TOW-INT TECH, Shenzhen, China) for treatment (100% oxygen for 60 minutes) once a day for 7 days [11]. The pressure in the chamber was kept at 2.0 atmospheres absolute (ATA, ≈203 kPa). Afterwards, mice were euthanized by intraperitoneal injection of 1% sodium pentobarbital (P3761, Sigma-Aldrich, St. Louis, USA) at an overdose of 150 mg/kg body weight. Spinal cord tissues intended for histological sectioning and staining were harvested and paraffin-embedded for subsequent analysis.

All mice received comprehensive supportive care immediately after surgery and continuing daily until sacrifice to minimize suffering and standardize recovery conditions. Care protocols included: (1) Bladder expression manually twice daily until restoration of voluntary urination; (2) Subcutaneous injection of an analgesic (buprenorphine, 0.05 mg/kg, B9275, Sigma-Aldrich, St. Louis, USA) every 8–12 hours for the first 72 hours; (3) Subcutaneous injection of an antibiotic (enrofloxacin, 5 mg/kg, 17849-5G-F, Sigma-Aldrich, St. Louis, USA) once daily for 5 days to prevent urinary tract infection; (4) Subcutaneous administration of warm lactated Ringer's solution (1 mL daily for 3 days, L4263, Sigma-Aldrich, St. Louis, USA) to prevent dehydration; and (5) Soft food placed on the cage floor for easy access.

Rat Primary Spinal Cord Neuron Extraction and Processing

Primary spinal cord neurons were obtained from rat embryos (embryonic days 14–15 from SD rats), according to previous reports [24]. Briefly, pregnant Sprague-Dawley rats were deeply anesthetized via intraperitoneal injection of sodium pentobarbital (1%, P3761, Sigma-Aldrich, St. Louis, USA) and euthanized by cervical dislocation. Uterine horns were aseptically removed and transferred to ice-cold, sterile D-Hanks' buffer. Embryos were carefully dissected from the uterine tissues, and their spinal cords were rapidly micro-dissected from the vertebral canals under a stereomicroscope in a laminar flow hood and then digested with 0.05% trypsin (15400054, Gibco, Grand Island, USA) to prepare a single cell suspension (1×10^6 cells/mL). Subsequently, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (11965092, Gibco, Grand Island, USA) with 10% fetal bovine serum (FBS) (16140089, Gibco, Grand Island, USA) for 4 hours at 37 °C in a 5% CO_2 incubator (51033770, Thermo Scientific, Waltham, USA). Afterwards, the medium was changed to complete medium for rat spinal cord neurons (CM-R144, Procell, Wuhan, China). Neurons from multiple litters were pooled before plating, and three independent cell preparations were performed as biological replicates. For the induction of ferroptosis *in vitro*, Erastin (50 μM) (S7242, Selleck, Shanghai, China) was used to treat neuronal cells (except for the normal control group) for 6 hours to establish a ferroptosis model prior to subsequent interventions [25]. The exper-

imental groups were designed as follows: Control (Con): Neurons without any treatment; Model (Mod): Neurons treated only with 50 µM Erastin for 6 hours; HBO treatment (HBO): Neurons pretreated with Erastin (50 µM, 6 hours) followed by exposure to hyperbaric cell incubator (OxyCure 3000, OxyHeal® Health Group, San Diego, USA) at 280 kPa for 60 min. The chamber was maintained at 37 ℃, with an oxygen-containing atmosphere supplemented with 1.79% CO_2 to achieve a 5 kPa partial pressure of CO_2 for physiological pH maintenance [24]; HIF-1alpha: Neurons were first transfected with the HIF-1alpha overexpression plasmid for 48 hours and then treated with Erastin (50 µM, 6 hours); HBO + NC / HBO + HIF-1alpha: Neurons were first transfected with the negative control plasmid (pEX-3) or the HIF-1alpha overexpression plasmid for 48 hours, treated with Erastin (50 µM, 6 hours) and exposed to HBO as abovementioned. All cells were confirmed to be free of mycoplasma contamination.

Cell Transfection

For gene overexpression, primary neurons at 70–80% confluence underwent transfection. The HIF-1alpha overexpression plasmid (pEX-3-HIF-1alpha) and the empty vector control (pEX-3) were prepared at a concentration of 1 µg/µL. The HIF-1alpha full-length sequence (**Supplementary File 1**) was cloned into pEX-3 vector (C05003, GenePharma, Suzhou, China) using Lipofectamine 3000 (L3000015, Invitrogen, Waltham, USA) according to the manufacturer's instructions. 2.5 µg of plasmid DNA in each well of a 6-well plate and 5 µL of Lipofectamine 3000 reagent were separately diluted in 125 µL of Opti-MEM® I Reduced Serum Medium. The diluted DNA was then combined with the diluted Lipofectamine 3000 reagent (total DNA to reagent ratio of 1:2 [w/v]), mixed gently, and incubated at room temperature for 15 minutes to form transfection complexes. The complexes were added dropwise for 48-hour incubation with cells to allow sufficient protein expression before subsequent interventions (Erastin treatment and/or HBO exposure).

Immunofluorescence

To confirm successful isolation of primary spinal cord neurons, cells were fixed on day 7 *in vitro* and subjected to immunofluorescence staining for the established neuronal marker (β-III-tubulin (Rabbit, 10068-1-AP, 1:800, Proteintech, Wuhan, China)). Secondary antibody was Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488, ab150081, 1:1000, abcam, Cambridge, USA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime, Shanghai, China). Fluorescent images were captured using a fluorescence microscope, and the percentage of β-III-tubulin-positive cells in total DAPI-positive cells was quantified. Cultures consistently exhibited >90% β-III-tubulin-positive neurons, confirming high neuronal purity and successful isolation. The cells were authenticated to be free of mycoplasma contamination.

Nissl Staining

The Nissl bodies in the anterior horn of the spinal cord in each group were observed using Nissl staining solution (C0117, Beyotime, Shanghai, China). Briefly, tissues from each group were routinely deparaffinized using xylene (534056, Sigma-Aldrich, St. Louis, USA) and graded ethanol (E7023, Sigma-Aldrich, St. Louis, USA), and subsequently exposed to Nissl staining solution for 5 minutes and 95% ethanol for approximately 5 seconds. The staining was observed under a microscope (100× magnification, VH lens, KEYENCE, Osaka, Japan).

Hematoxylin and Eosin Staining

Hematoxylin and eosin (HE) kit (C0105) was purchased from Beyotime (Shanghai, China) to perform histopathological analysis. Spinal cord tissue sections were routinely deparaffinized (similar to the Nissl staining procedure) and stained with hematoxylin for 10 minutes and eosin for 1 minute. After mounting, the tissue changes were observed under a microscope with a magnification of 100×.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

HIF-1alpha and HMOX1 expression levels in mouse spinal cord tissue and rat neurons were detected using RT-qPCR. TRIzol solution (T9424, Sigma-Aldrich, St. Louis, USA) was used for isolating the total RNAs, which were then converted into cDNAs utilizing a cDNA synthesis kit (K1072, ApexBio Technology, Houston, USA). The qPCR reactions were conducted in the QuantStudio 5 system (A28575, Applied Biosystems, Waltham, USA), using SYBR Green qPCR Mix (D7260, Beyotime, Shanghai, China). The quantitative real-time PCR conditions were as follows: denaturation at 95 ℃ for 5 minutes, followed by 40 cycles of 95 ℃ for 10 seconds, 60 ℃ for 30 seconds, and 72 ℃ for 30 seconds. The relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method [26]. All PCR reactions were performed in triplicate technical replicates (three identical reactions per biological sample) to ensure assay reliability and reduce pipetting variability. The primers were available (5′-3′): HIF-1alpha (mouse): Forward: TCTCGGCGAAGCAAAGAGTC, Reverse: AGCCATCTAGGGCTTTCAGATAA; HIF-1alpha (rat): Forward: CCTATGTAGTTGTGGAAGTTTATGC, Reverse: ACTAGGCAATTTTGCTAAGAATG. HMOX1 (mouse): Forward: CCTCACAGATGGCGTCACTT, Reverse: TGGGGGCCAGTATTGCATTT; HMOX1 (rat): Forward: AAGCTGAGGATCAGTGACGC, Reverse: TGGGGTTAAAGTCCCGCTTC. Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) (mouse): Forward: GCTGGTGAAAAGGACCTCT, Reverse: CACAGGACTAGAACACCTGC; HPRT1 (rat): Forward: TTGTTGGATATGCCCTTGACT, Reverse: CCGCTGTCTTTTAGGCTTTG.

Western Blot Assay

The expression levels of ferroptosis defense-related proteins Glutathione Peroxidase 4 (GPX4) and Solute Carrier Family 7 Member 11 (SLC7A11) in mouse spinal cord tissue and rat neurons were determined by Western blot assay. Total proteins were harvested using Radioimmunoprecipitation Assay (RIPA) lysis buffer (P0013B, Beyotime, Shanghai, China). Protein content was measured by Enhanced Bicinchoninic Acid (BCA) Protein Assay Kit (P0010S, Beyotime, Shanghai, China). 30 µg of protein per lane was separated on 10% Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and then transferred onto Polyvinylidene Difluoride (PVDF) membranes (FFP24, Beyotime, Shanghai, China). Membranes were blocked with 5% non-fat milk in Tris-Buffered Saline with Tween-20 (TBST) for 1 h at room temperature. After washing three times with TBST (10 min each). Membranes were blotted with primary antibodies GPX4 (Rabbit, ab125066, 17 kDa, 1:1000, Abcam, Cambridge, UK), SLC7A11 (Rabbit, PA1-16893, 55 kDa, 1:1000, Thermo Fisher ,Waltham, USA) and HPRT1 (Rabbit, ab10479, 25 kDa, 1:2000, Abcam, Cambridge, UK) overnight at 4 ℃. After washing three times with TBST, membranes were incubated with secondary antibody Goat Anti-Rabbit IgG H&L (ab205718, 1:3000, Abcam, Cambridge, UK) for 1 h at room temperature. BeyoECL Plus (P0018M, Beyotime, Shanghai, China) was used to expose the protein bands.

Thiobarbituric Acid Reactive Substances and Fe^{2+} Level Assays

The OxiSelect thiobarbituric acid reactive substances (TBARS) Assay Kit (malondialdehyde (MDA) Quantitation) (STA-330-T, Cell Biolabs, San Diego, USA) was applied to detect lipid peroxidation status in neuronal cells. For sample preparation, neurons were resuspended in 1× phosphate-buffered saline (PBS) (1×10^7 cells/mL, PB180327, Procell, Wuhan, China) with 1× butylated hydroxytoluene (BHT, HY-Y0172, MedChemExpress, Monmouth Junction, USA) to prevent oxidation, homogenized on ice, and centrifuged at 10,000 ×g for 5 min. The resulting supernatant was collected and analyzed according to the manufacturer's protocol, and absorbance at 532 nm was measured using a microplate reader (VICTOR Nivo, UNIV-bio, Shanghai, China).

Ferrous iron Colorimetric Assay Kit (E-BC-K773-M, Elabscience, Wuhan, China) was exploited for Fe^{2+} level analysis. Neuronal pellets were homogenized in the extraction solution provided with the kit (0.1 g: 0.9 mL), centrifuged at 12,000 ×g for 10 min, and the supernatant was analyzed according to the manufacturer's instructions. The absorbance at 593 nm was measured using a microplate reader. Results of both assays were normalized to total protein content. TBARS levels are presented as pmol/mg protein, and Fe^{2+} levels are expressed as percentage (%) relative to the standard Fe^{2+} concentration, normalized to total protein content.

Statistical Analysis

Data were analyzed by GraphPad 8.0 software (GraphPad Software, San Diego, USA) and presented as mean ± standard deviation. The sample size (n) for each experiment represents the number of independent biological replicates, where three individual mice per group were used for *in vivo* assays and three independent primary neuronal cultures for *in vitro* assays. Normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test. Comparison between groups was performed by one-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test. $p < 0.05$ was regarded as statistically significant. All histological assessments and data analyses were performed by two independent researchers who were blinded to the experimental groups.

Results

HBO Treatment was Related to Attenuated Spinal Cord Tissue Injury in Mice, in Which HIF-1alpha/HMOX1-Mediated Ferroptosis Was Implicated

To investigate the mechanism of HBO treatment in SCI, we constructed a mouse model of SCI. In SCI mice, decreased Nissl bodies and spinal cord histiocytic edema and cavitation were observed, whereas the subsequent HBO treatment increased Nissl bodies and alleviated tissue damage (Fig. 1A,B), indicating its efficacy in preserving spinal cord tissue integrity.

Given the critical role of the hypoxic response and iron metabolism in secondary injury, we investigated the HIF-1alpha/HMOX1 axis. As anticipated, SCI triggered a substantial upregulation of both HIF-1alpha and HMOX1 at the transcriptional level, while HBO partially suppressed their expressions in SCI mice ($p < 0.001$, Fig. 2A,B). Importantly, HBO increased the expression levels of GPX4 and SLC7A11 proteins in spinal cord tissues of SCI mice ($p < 0.01$, Fig. 2C), revealing its potential anti-ferroptotic effect.

HBO Treatment Modulated Ferroptosis-Related Proteins in Rat Spinal Cord Neurons In Vitro, a Process in Which HIF-1alpha/HMOX1 Participated

As shown in Fig. 3A, the vast majority of cells were positive for the neuronal marker β-III-tubulin, confirming successful isolation of enriched neuronal cultures. The high purity of the isolated neurons minimizes interference from non-neuronal cells and guarantees the accuracy of subsequent experimental results. To further validate the ferroptosis-related mechanism of HBO treatment, primary spinal cord neurons from rats were treated with Erastin to induce ferroptosis *in vitro*. Erastin led to a significant ac-

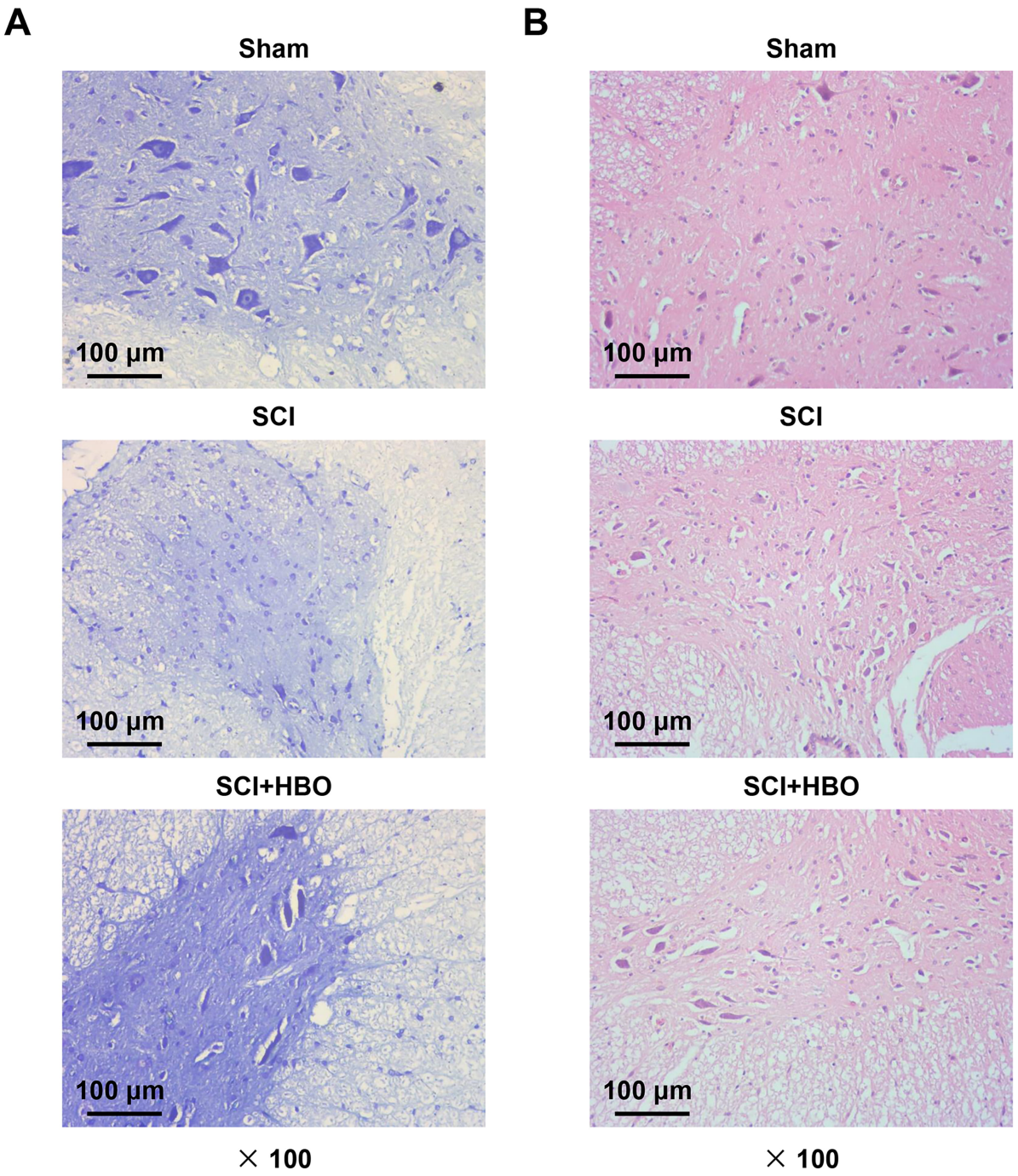

Fig. 1. Hyperbaric oxygen (HBO) treatment increased Nissl bodies and alleviated tissue damage in spinal cord injury (SCI) mice. (A) Nissl bodies were observed through Nissl staining of the anterior horn of the mouse spinal cord in the Sham, SCI, and SCI+HBO groups. (B) Spinal cord tissue injury was assessed using hematoxylin and eosin staining. Scale bar = 100 μm, magnification = 100×. n = 3.

cumulation of MDA and Fe^{2+} in the Mod group, whereas these effects were inhibited by HBO intervention ($p < 0.001$, Fig. 3B,C). Mechanistically, consistent with the results of *in vivo* experiments, HBO treatment promoted the expression levels of GPX4 and SLC7A11 proteins in the Mod group ($p < 0.05$, Fig. 3D). In addition, the mRNA levels of HIF-1alpha and HMOX1 were markedly increased in the Mod group, but were later downregulated by HBO intervention ($p < 0.001$, Fig. 3E,F), supporting the association between the HIF-1alpha/HMOX1 axis and ferroptosis.

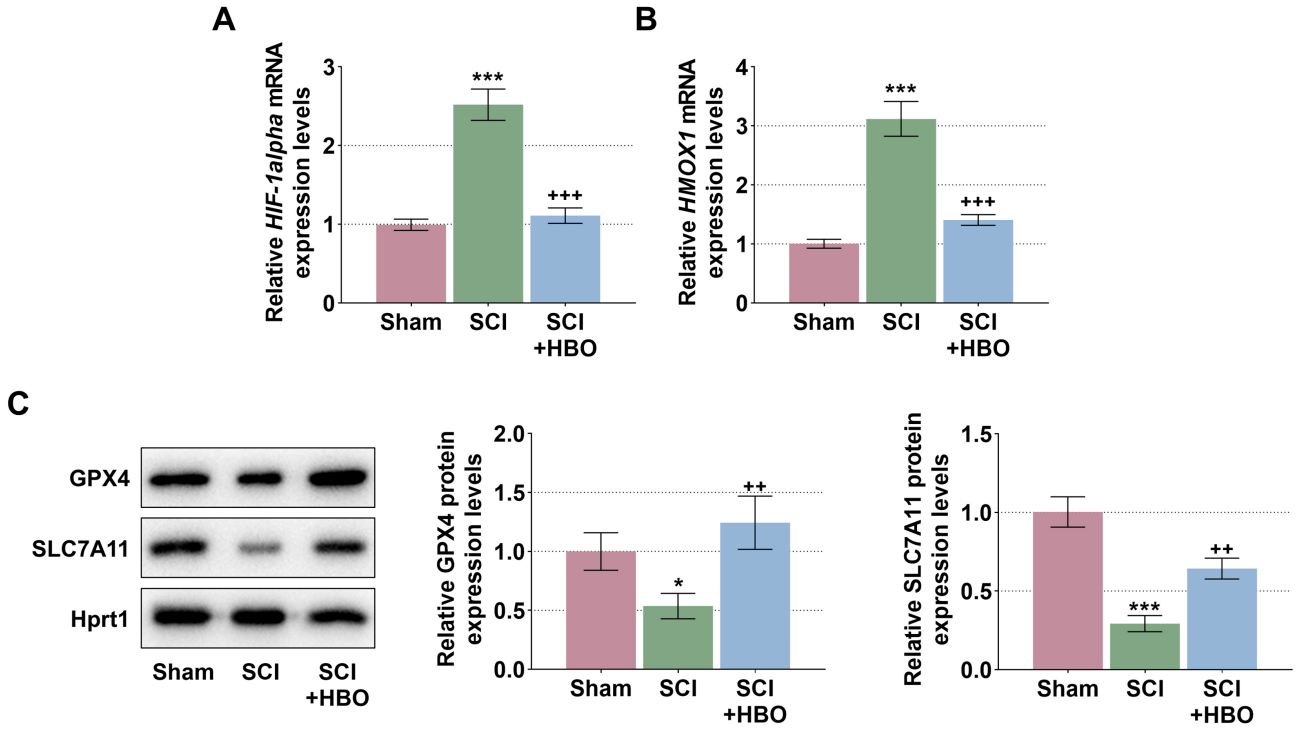

Fig. 2. HBO decreased HIF-1alpha and HMOX1 levels, and increased GPX4 and SLC7A11 expression levels in SCI mice. (A,B) HIF-1alpha/HMOX1 expression in spinal cord tissue was determined using real-time quantitative polymerase chain reaction (RT-qPCR). (C) Expression levels of ferroptosis-related proteins GPX4 and SLC7A11 in spinal cord tissues of mice in Sham, SCI, and SCI+HBO groups were analyzed by Western blot assay. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Sham; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. SCI. n = 3. HIF-1alpha, hypoxia-inducible factor-1alpha; HMOX1, Heme oxygenase 1; GPX4, Glutathione Peroxidase 4; SLC7A11, Solute Carrier Family 7 Member 11.

HIF-1alpha Partially Reversed the Regulation of Ferroptosis by HBO in Rat Spinal Cord Neurons In Vitro

To establish a causal role of HIF-1alpha in the effects of HBO, a genetic rescue experiment was performed. HIF-1alpha overexpression plasmids were transfected into neuronal cells ($p < 0.001$, Fig. 4A). HIF-1alpha overexpression reversed the inhibiting effects of HBO treatment on MDA and Fe^{2+} contents in the Erastin-induced ferroptosis cell model ($p < 0.001$, Fig. 4B,C). Importantly, GPX4 and SLC7A11 protein expression levels were remarkably decreased in the HBO+HIF-1alpha group compared with the HBO+NC group ($p < 0.01$, Fig. 4D). Notably, the HIF-1alpha group (a critical control) showed elevated MDA and Fe^{2+} levels, as well as decreased GPX4 and SLC7A11 expression compared with the Mod group ($p < 0.01$, Fig. 4B–D), demonstrating that HIF-1alpha overexpression alone promoted ferroptosis independent of HBO administration. Taken together, these results demonstrated that HIF-1alpha was functionally required for HBO to exert its inhibitory effects on ferroptosis, and overexpression of HIF-1alpha could counteract the protective role of HBO against neuronal ferroptosis.

Discussion

The pathophysiological mechanism of SCI is complex, involving primary injury and secondary injury [2]. Secondary injury, including apoptosis, necrosis, and inflammatory response, can further deteriorate SCI [27,28]. HBO is widely used in clinical treatment of diabetic foot ulcers, hypoxic-ischemic encephalopathy and other diseases, presenting a good curative effect [7]. HBO is characterized by non-invasiveness, harmlessness, operational simplicity, minimal side effects, and antibacterial activity [29]. This study confirmed that HBO can effectively improve the histomorphological damage after SCI (manifested as attenuation of spinal cord histiocytic edema and cavitation, and restoration of reduced Nissl bodies), and this protective effect was closely related to its multi-target regulation of the microenvironment. It is noteworthy that while the involvement of the HIF-1alpha/HMOX1 axis in ferroptosis regulation has been documented in other contexts, our study presents the first evidence linking HBO-mediated neuroprotection in SCI to the modulation of the HIF-1alpha/HMOX1 axis and a corresponding suppression of ferroptosis. This finding established a novel mechanistic connection within the SCI therapeutic landscape.

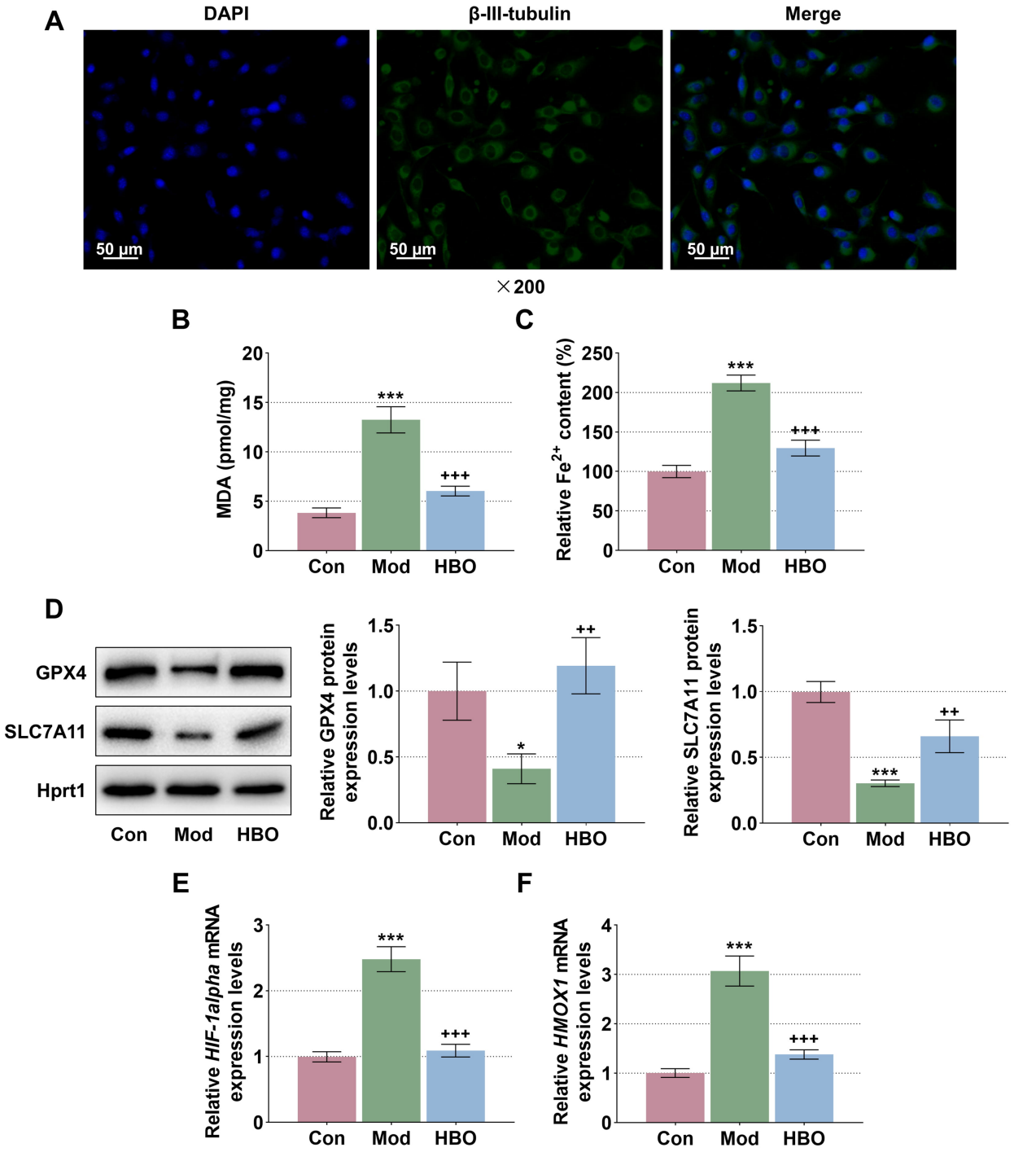

Fig. 3. HBO correlates with ferroptosis through HIF-1alpha/HMOX1 in a rat spinal cord neuron model *in vitro*. (A) Identification and purity assessment of primary rat spinal cord neurons. (B) The lipid peroxidation status of neurons in Con (control), Mod (model) (Erastin, 50 μM) and HBO (280 kPa, 60 min) groups was detected with the thiobarbituric acid reactive substances (TBARS) kit. (C) Detection of Fe^{2+} content in each group. (D) The expression levels of GPX4 and SLC7A11 were analyzed by Western blot assay. (E,F) HIF-1alpha/HMOX1 expression was determined using RT-qPCR. All groups were treated with Erastin. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Con; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. Mod. n = 3.

HBO has also been reported to provide a good microenvironment for cells in SCI, thereby restoring spinal cord function [30]. Herein, we established a SCI mouse model and observed reduced Nissl bodies accompanied by spinal cord histiocytic edema and cavitation, as well as increased HIF-1alpha/HMOX1 expression and decreased GPX4 and SLC7A11 protein levels, implying successful modeling and the inducing effect of SCI on ferroptosis. Pre-

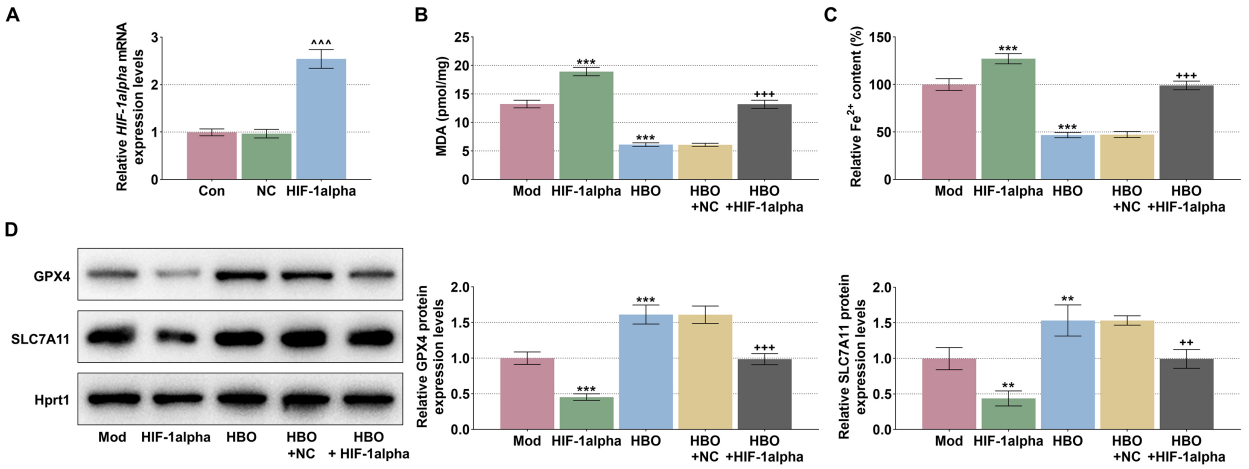

Fig. 4. HIF-1alpha partially reversed the regulation of ferroptosis by HBO *in vitro*. (A) Transfection efficiency of HIF-1alpha overexpression plasmid in rat spinal cord neurons was determined using RT-qPCR. (B) The lipid peroxidation status of neuronal cells in the Mod, HIF-1alpha, HBO, HBO+NC, and HBO+HIF-1alpha groups was detected with the TBARS kit. (C) Detection of Fe^{2+} content in each group. (D) The effects of HIF-1alpha and HBO on GPX4 and SLC7A11 expressions were analyzed by Western blot assay. $^{++}p < 0.01$ vs. HBO+NC, $^{+++}p < 0.001$ vs. HBO+NC; $^{**}p < 0.01$ vs. Mod, $^{***}p < 0.001$ vs. Mod; $^{\wedge\wedge\wedge}p < 0.001$ vs. NC (negative control). n = 3.

vious studies have documented that HBO promotes the expression of antioxidant genes in endothelial cells and regulates the expression of immune genes such as Integrin Subunit Alpha M (ITGAM), Interleukin-6 (IL-6), and Protein Tyrosine Phosphatase Receptor Type C (PTPRC) in keloids [31,32]. Importantly, we found that HBO attenuated nerve and tissue damage in SCI mice, which was associated with the regulation of the ferroptosis-related HIF-1alpha/HMOX1 pathway, a mechanism that was also validated *in vitro* in rat spinal cord neurons.

HIF-1alpha has been widely investigated in several SCI studies. For instance, Li *et al*. [33] pointed out that HIF-1alpha activation reduces inflammatory factors in SCI rats. Ni *et al*. [34] also explained that HIF-1alpha is mediated by Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) to reduce the glycolytic process. HIF-1alpha can promote functional recovery in SCI rats by promoting the migration of bone marrow stromal cells [35]. HIF-1alpha expression is significantly elevated in SCI rats [36], while HBO has been confirmed to reduce its expression [22], thereby inhibiting tumor growth in non-small cell lung cancer [37]. In the present study, HBO inhibited HIF-1alpha expression in SCI mouse spinal cord tissue and in a rat spinal cord neuron injury model.

In addition, HMOX1, an antioxidant-related gene, also presents upregulated levels in SCI and dampens neuronal apoptosis [38,39]. However, in SCI, tissue damage and blood breakdown products can lead to increased iron ion concentrations, thus inducing ferroptosis [14]. Many studies have shown that modulation of ferroptosis-related molecular mechanisms promotes recovery from SCI [17,40]. The HIF-1alpha/HMOX1 pathway is activated during ferroptosis and plays a detrimental role in diabetic nephropathy [20]. Wu *et al*. [41] confirmed that the HIF-1alpha/HO-1 signaling pathway can mediate ferroptosis. Herein, our results unveiled that HBO downregulated the HIF-1alpha/HMOX1 axis to activate the GPX4/SLC7A11 defense pathway and prevent ferroptosis in SCI. Moreover, HMOX1 deficiency can restore Fe^{2+} levels and GPX4 expression [42]. However, the precise mechanism linking the deficiency of HMOX1, a heme-degrading enzyme, to the upregulation of GPX4 and SLC7A11, including the associated molecular pathway, remains poorly understood and warrants further investigation. In this study, Erastin, a classic inducer of ferroptosis, was used to treat rat spinal cord neurons for modeling [25]. *In vitro* experiments further suggested that the therapeutic effect of HBO may be related to ferroptosis, potentially involving the downregulation of HIF-1alpha/HMOX1 and activation of the GPX4/SLC7A11 pathway. Therefore, we believe that HBO promoted nerve repair after SCI through the establishment of a pleiotropic regulatory network that included antioxidant, anti-apoptotic, and anti-ferroptotic effects. Notably, the mechanistic link between HMOX1 downregulation and GPX4/SLC7A11 upregulation remains speculative and requires further experimental validation. Future studies should further analyze the crosstalk between these different mechanistic nodes to more comprehensively clarify the therapeutic mechanism of HBO.

Of note, the roles of HIF-1alpha and its downstream target gene HMOX1 are complex. A previous study showed that in ischemia models, HMOX1 stabilizes HIF-1alpha through its product carbon monoxide, thereby promoting metabolic reprogramming to adapt to hypoxia, which has

been revealed as a key mechanism for its tissue-protective effect [43]. Accordingly, under specific ischemic conditions, the HIF-1alpha/HMOX1 axis can form a positive feedback loop and exert a key protective function. However, in the context of moderate to severe SCI accompanied by extensive hemorrhage and iron ion release, as simulated in this study, the microenvironment differs fundamentally. Excessive or sustained activation of the HIF-1alpha/HMOX1 axis may shift towards a pro-injury pathway. This significant functional divergence may depend on the nature of the injury (ischemic vs. hemorrhagic contusion), the severity and duration of hypoxia, cell type, and the specific activation pattern of downstream pathways, all of which need further investigation. However, the current data do not include dynamic expression changes at sequential time points post-injury, which prevents a precise spatiotemporal analysis of its functional transition. Therefore, the dynamic transition between the protective and detrimental roles of the HIF-1alpha/HMOX1 axis remains to be further verified through systematic time-course and regional detection in future studies.

Several limitations need to be addressed in this study. First, the experimentally detected indicators are limited, and the mechanistic links among HBO treatment, the HIF-1alpha/HMOX1 axis, and ferroptosis remain to be fully elucidated. Second, the primary neuron ferroptosis model induced by Erastin is a simplified system that cannot replicate the complex pathological microenvironment *in vivo* after SCI, such as the intertwined state of multiple factors like ischemia, inflammation, and excitotoxicity. Third, sex-dependent differences may influence the pathophysiology of SCI and the therapeutic response. Fourth, although the functional involvement of HIF-1alpha overexpression is supported by experimental evidence, the lack of HIF-1alpha loss-of-function experiments and independent manipulation targeting HMOX1 limits further analysis of the causal relationship of this regulatory axis. Future studies are needed to validate the role of the HIF-1alpha/HMOX1 axis in mediating the effects of HBO on ferroptosis in both male and female animals, thus providing a more comprehensive assessment of its therapeutic potential.

Conclusion

In summary, we demonstrate that the persistent elevation of the HIF-1alpha/HMOX1 axis after SCI is associated with ferroptosis and contributes to the secondary injury progression. HBO treatment may play a protective role in SCI by inhibiting ferroptosis, which correlates with reduced HIF-1alpha/HMOX1 axis. Our study provides new experimental evidence supporting the therapeutic potential of HBO and establishes a direct link between HBO and the suppression of ferroptosis in SCI with the involvement of the HIF-1alpha/HMOX1 pathway. These findings reveal a novel mechanism underlying the therapeutic effects of HBO in SCI.

Availability of Data and Materials

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

Author Contributions

XGS and ZTW designed the research study; XL performed the research; CQW and HEL collected and analyzed the data. XGS and ZTW have been involved in drafting the manuscript, and all authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity are addressed.

Ethics Approval and Consent to Participate

The study protocols were approved by the Ethics Committee of Zhejiang Provincial Laboratory Animal Center for Experimental Animals Welfare (ZJCLA-IACUC-20010873). The study was conducted in accordance with the ARRIVE guidelines 2.0.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638208.112.

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