Article

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Mechanism Underlying Hyperbaric Oxygen's Effect on Nitric Oxide in an *in Vitro* Model of Traumatic Brain Injury

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Background: Hyperbaric oxygen (HBO) therapy functions as a possible therapeutic option for traumatic brain injury (TBI). The aim of this study is to detect the mechanism of HBO on TBI.
Methods: Neurons and astrocytes isolated from healthy neonatal rat cortices were co-cultured, a TBI model was established, and cells were cultured under HBO conditions. Neuron/astrocyte viability and glutamate transporter-1 (GLT-1) expression in neuron/astrocyte co-cultures were assessed by immunofluorescence. Tumor necrosis factor (TNF)-α/tumor necrosis factor receptor 1 (TNFR1)/nitric oxide (NO)/neuronal nitric oxide synthase (nNOS)/interleukin (IL)-1β/inducible nitric oxide synthase (iNOS)/GLT-1 levels in neuron/astrocyte co-cultures were detected using quantitative real-time polymerase chain reaction (qRT-PCR), colorimetry, and western blotting. To identify the key role of the target gene TNF receptor 1 (*TNFR1*) in HBO therapy, *TNFR1* was silenced or overexpressed. After transfection, the cellular functions and the levels of related factors were re-examined.
Results: HBO (2 atmospheric absolute (ATA) for 30/60 min) attenuated the effect of TBI-induced on decrease of neuronal viability, increase of astrocyte viability, up-regulation of TNF-α, IL-1β, NO, nNOS, iNOS, and TNFR1 levels, down-regulation of GLT-1 levels, and reduce of GLT-1-positive astrocytes in neuron/astrocyte co-cultures ($p < 0.05$). TNFR1 knockdown and HBO (2 ATA for 60 min) enhanced neuronal viability, decreased astrocyte viability, and down-regulated TNF-α, IL-1β, NO, nNOS, iNOS, and TNFR1 levels in TBI-induced neuron/astrocyte co-cultures ($p < 0.01$). *TNFR1* overexpression reversed the above role of HBO in TBI-induced neuron/astrocyte co-cultures. HBO (2 ATA for 60 min) up-regulated GLT-1 levels in TBI-induced neuron/astrocyte co-cultures ($p < 0.05$).
Conclusions: HBO inhibits TNFR1 expression to down-regulate NO content in TBI in an *in vitro* model.

Keywords: hyperbaric oxygen; glutamate transporter-1; TNFR1; nitric oxide; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is characterized by external force strike-induced structural or physiological cerebral dysfunction, greatly resulting in disability or death worldwide [1]. TBI is commonly categorized into primary injury and secondary injury [2]. Primary brain injury caused by trauma leads to tissue damage and neuronal loss hours or days after which secondary brain injury occurs, which is associated with oxidative stress, calcium overload, neuroinflammation, glutamate excitotoxicity, and cell death [3]. Although neuroprotective agents have been identified through decades of preclinical and clinical research, the therapeutic strategies for TBI are limited to symptomatic relief and surgical intervention due to the complex pathophysiological processes [4]. Thus, emphasis should be placed on novel treatment approaches for TBI.

The first stage of TBI is a mechanical injury of brain tissue that directly causes neuronal death [5]. Astrocytes express receptors that can recognize endogenous molecules released from damaged cells and tissues, which allow astrocytes to be quickly activated upon injury [6]. Astrocyte activation can induce the generation of nitric oxide (NO), and neurons can also synthesize NO [7,8]. NO, and its derivatives are commonly recognized as inflammatory mediators, and NO is up-regulated in TBI, which then induces an inflammatory reaction, neuronal apoptosis, and cytotoxic reaction [9]. A better understanding of NO homeostasis in TBI is essential for the treatment of TBI.

Release of damage-associated molecular pattern (DAMP) arising from mechanic damage of brain tissues is a main pathophysiological process that regulates NO homeostasis during TBI [10]. DAMPs enhance the production of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, which can be resynthesized or produced promptly through cleavage of the extracellular domain of TNF-α by the matrix metalloprotease, TNF-α-converting enzyme (TACE) [11]. Soluble TNF-α can bind to TNF receptor 1 (TNFR1) receptors to activate signaling pathways

dependent on TNFR1 [9]. TNFR1 is a cellular receptor of TNF-α, expressed in neurons, astrocytes, and microglia [11,12]. Glutamate is the primary ligand activating the influx of Ca^{2+} and cytotoxicity in TBI [13,14]. Glutamate transporter glutamate transporter-1 (GLT-1) expression during TBI is repressed, which renders glutamate unclearable from cells [15]. TNFR1 promotes the synthesis of glutamate, and glutamate promotes the influx of Ca^{2+} to activate neuronal nitric oxide synthase (nNOS) in neurons, thereby facilitating NO production [9]. Equally important, novel TNFR1 inhibitors were identified as potential therapeutic candidates for TBI [16].

Hyperbaric oxygen (HBO) therapy, referring to the inhalation of 100% oxygen under a pressure greater than 1 atmospheric absolute (ATA), functions as a possible therapeutic option for TBI through various mechanisms, such as attenuating microgliosis and TNF-α expression [17–19]. HBO therapy inhibits the formation of NO in ischemia/reperfusion testes by repressing dimethylarginine dimethylaminohydrolase and inducible nitric oxide synthase (iNOS) [20]. HBO treatment reduces glutamate content in acute brain injury [21]. Therefore, we speculate HBO up-regulates GLT-1 by repressing TNFR1 expression, thereby reducing NO content in TBI *in vitro* models.

Herein, we established a TBI *in vitro* model by scalpel injury and investigated how HBO treatment affects NO levels through TNFR1.

Materials and Methods

Animals

Healthy female Sprague-Dawley (SD) embryonic rats (n = 10, 17 days old, approximately 5 g body weight) were maintained under standard conditions (humidity of 45–65%, temperature of 20 °C, and circadian rhythm of 12 h). The Experimental Animal Welfare Committee of Zhejiang Center of Laboratory Animals (No. ZJCLA-IACUC-20010753) approved all experiments, which were performed according to the guidelines of the China Council on Animal Care and Use.

Neuron/Astrocyte Collection and Co-Cultures

Healthy embryonic rats were anesthetized using 1% pelltobarbitalum natricum (40 mg/kg, P3761, Sigma-Aldrich, St. Louis, MO, USA) at 40 mg/kg and euthanized by cervical dislocation, and cortices were used to prepare neuron/astrocyte primary cultures.

In brief, blood vessels and pia maters were removed, followed by dissection and digestion (34 °C, 45 min) of cerebral cortices using papain (100 units, 40.4 mg/mL; HY-P1645, MedChemExpress, Monmouth Junction, NJ, USA), L-cysteine (0.0036–0.0042 g/mL; HY-Y0337, MedChemExpress, Princeton, NJ, USA), and DNase I (4 mg/mL; HY-108882, MedChemExpress, Princeton, NJ, USA). After treating with 20% fetal bovine serum (FBS, 12483020, Thermo Fisher, Waltham, MA, USA) and 10× Earle's balanced salt solution (C0213, Beyotime, Shanghai, China), cells were filtered (40-µm filter) to harvest a single-cell suspension of neurons or astrocytes [22].

After resuspension in Dulbecco's modified Eagle's medium (DMEM, A4192101, Thermo Fisher, Waltham, MA, USA) supplemented with 1% penicillin–streptomycin (30-2300, American Type Culture Collection, Manassas, VA, USA), 10% Ham's F12 (PM150810, Procell, Wuhan, China), and 10% FBS, cells (5×10^4 cells/mL) were seeded into poly-L-lysine (P4707, Sigma-Aldrich, St. Louis, MO, USA)-coated 24-well dishes, 96-well plates, and flasks, and grown (37 °C, 5% CO_2, 2 weeks) in an incubator (BMSH356545, Sigma-Aldrich, St. Louis, MO, USA) for 10–12 days. Subsequently, neurons were seeded on the top of the confluent monolayer of astrocytes [23]. Cells, distinguished by the multipolar or triangular neuronal morphology, were tested for mycoplasma and were free of contamination.

In Vitro Model of Traumatic Brain Injury (TBI)

An *in vitro* scratch neuron/astrocyte co-culture injury model was used. In brief, 22 size scalpel blade cuts (20 times) were used to induce cellular injury in neuron/astrocyte co-cultures 10 times perpendicularly in both directions about 2 mm apart [24]. Next, injured cells were separated from the surrounding uninjured cells in the cell co-culture, incubated (72 h, 37 °C, 5% CO_2), and collected.

HBO Therapy

HBO therapy was carried out after TBI [25]. An infant HBO chamber (YLC0.5/1A, 701 Institute of China State Shipbuilding Corporation, Wuhan, China) was used to perform HBO treatment (30/60 min) in the presence of 100% oxygen and 2 ATA pressure, with 85% or higher oxygen in the cabin. Cells were exposed to HBO conditions for 72 h after TBI and then incubated (37 °C, 5% CO_2).

Cell Transfection

The sequence of the *TNFR1* overexpression plasmid is listed in **Supplementary file 1**. Plasmids overexpressing *TNFR1* (C05002), small interfering RNA against *TNFR1* (si*TNFR1*, 5′-AUUAAACCGAUGGAGAAGGTT-3′), and small interfering RNA negative control (siNC, A06001, 5′-ACGUGACACGUUCGGAGAATT-3′) were synthesized by Genepharma (Shanghai, China). The pcDNA3.1 vector (V79020, Thermo Fisher, Waltham, MA, USA) was utilized to establish for *TNFR1* overexpression. The empty vector was negative control (NC). Neuron/astrocyte co-cultures at about 80% confluence were transfected with NC, plasmids overexpressing *TNFR1*, siNC or si*TNFR1*, and co-transfected with NC and siNC, or NC and si*TNFR1* using Lipofectamine™ 3000 (L3000150, Thermo Fisher, Waltham, MA, USA) for 48 h at 37 °C. Next, TBI was induced after cell transfection (48 h).

Immunofluorescence

Neuron/astrocyte co-cultures were seeded on poly-L-lysine-coated glass coverslips. After transfection, TBI and HBO treatment (30, 60 min), cell fixation (1 h, 4% paraformaldehyde, P0099, Beyotime, Shanghai, China) was followed by antigen retrieval and permeabilization (15 min) using phosphate-buffered saline (PBS, C0221A, Beyotime, Shanghai, China)/0.1% Triton X-100 (P0096, Beyotime, Shanghai, China). After washing with PBS thrice, blocking was performed (1 h, room temperature (RT)) employing normal goat serum (R37624, Thermo Fisher, Waltham, MA, USA). Cells were probed (overnight, 4 °C) with microtubule-associated protein 2 (MAP-2) (ab183830, 1:1000, Abcam, Cambridge, UK), glial fibrillary acidic protein (GFAP) (ab7260, 1:5000, Abcam, Cambridge, UK) or GLT-1 (ab235202, a concentration of 15 µg/mL, Abcam, Cambridge, UK). The PBS-washed (thrice) cells were incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 488, ab150077, 1:1000), donkey anti-rabbit IgG H&L (Alexa Fluor® 647, ab150075, 1:1000) or donkey anti-goat IgG H&L (Alexa Fluor® 488, ab150129, 1:1000) (Abcam) for 1 h at RT. Color development (10 min, RT) by 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, C1002, Beyotime, Shanghai, China) was performed after washing cells with PBS. Cells were then mounted on glass slides with FluorSave reagent (345789, Sigma-Aldrich, St. Louis, MO, USA), followed by imaging using a fluorescence microscope (×200 magnification, TE2000, Nikon, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After transfection, TBI, and HBO treatment (30, 60 min), total RNA was extracted from neuron/astrocyte co-cultures using the RNA extraction kit (R1200, Solarbio, Beijing, China). RNA purity and concentration were assessed by a ultraviolet (UV) spectrophotometer (DR6000, HACH, Shanghai, China). After complementary DNA synthesis using the Primescript™ RT reagent kit (RR037A, TaKaRa, Osaka, Japan), qRT-PCR was conducted using the SYBR Premix Ex Taq II (RR820A, TaKaRa) on a StepOne Plus real-time PCR system (4376600, Thermo Fisher, Waltham, MA, USA) under conditions of 5 min (95 °C), 10 s (95 °C), 20 s (60 °C) and 20 s (72 °C) for 45 cycles, and 10 min (72 °C). The relative expression of genes was calculated with *GAPDH* as the internal reference using the $2^{-\Delta\Delta ct}$ method [26]. The primer sequences were as follows: *TNF-α*, forward (F): 5′-CAGACCCTCACACTCAGATCATC-3′ and reverse (R): 5′-AGCCTTGTCCCTTGAAGAGAAC-3′; interleukin-1β (*IL-1β*), F: 5′-CCTATGTCTTGCCCGTGGAG-3′ and R: 5′-CACACACTAGCAGGTCGTCA-3′; *TNFR1*, F: 5′-TGGCTCATGATCGGGCTTAC-3′ and R: 5′-AGAAAGAATCCTGCGTGGCA-3′; *GAPDH*, F: 5′-TCCCTCAAGATTGTCAGCAA-3′ and R: 5′-AGATCCACAACGGATACATT-3′.

Detection of NO Content

After transfection, TBI, and HBO treatment (30, 60 min), the NO assay kit (nitrate reductase method; A012-1-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure NO levels in neuron/astrocyte co-cultures. NO levels were measured in cells following the manufacturer's instructions.

Western Blotting Analysis

Following the lysis of neuron/astrocyte co-cultures (radioimmunoprecipitation assay (RIPA) lysis buffer), protein concentration was measured with a bicinchoninic acid assay (BCA) Protein Assay Kit (7780S, CST, Boston, MA, USA). Proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, P0012A, Beyotime, Shanghai, China) were transferred onto polyvinylidene difluoride (PVDF) membranes (88518, Thermo Fisher, Waltham, MA, USA), blocked with 5% non-fat milk (2 h, RT), incubated with primary antibodies (overnight, 4 °C), washed in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, T1081, Solarbio, Beijing, China), and incubated in secondary antibodies (1 h, RT). The primary (Abcam) and secondary (Sigma-Aldrich, St. Louis, MO, USA) antibodies used were nNOS (ab76067, 161 kDa, 1:1000), iNOS (ab178945, 131 kDa, 1:1000), TNFR1 (ab223352, 50 kDa, 1:1000), GLT-1 (ab41621, 62 kDa, 1 µg/mL), GAPDH (ab8245, 37 kDa, 1:1000), goat anti-rabbit IgG (AP132, 1:3000) or goat anti-mouse IgG (AP124, 1:3000). Proteins were developed using chemiluminescent substrate (34577, Thermo Fisher, Waltham, MA, USA) and visualized using the Odyssey® M Imaging System (LI-COR Biosciences, Lincoln, NE, USA). ImageJ software (version 2.0.0, National Institutes of Health, Bethesda, MD, USA) was used to determine the band intensities.

Statistical Analysis

Data represented as mean ± standard deviation were processed in GraphPad 8.0 software (GraphPad Software, San Diego, CA, USA). The independent sample *t*-test was applied for two-group comparisons, and one-way analysis of variance was for multi-group comparisons. Tukey test was applied for post hoc comparisons. $p < 0.05$ demonstrated statistical significance.

Results

HBO Attenuated the TBI-Induced Decrease of Neuronal Viability and Increase of Astrocyte Viability in Neuron/Astrocyte Co-Cultures

To investigate how HBO affects TBI, scalpel injury was applied to mimic an *in vitro* TBI model of neuron/astrocyte co-cultures that received HBO treatment for 30/60 min, with pressure reaching 2 ATA. MAP-2 immunofluorescent staining was performed to assess neuron

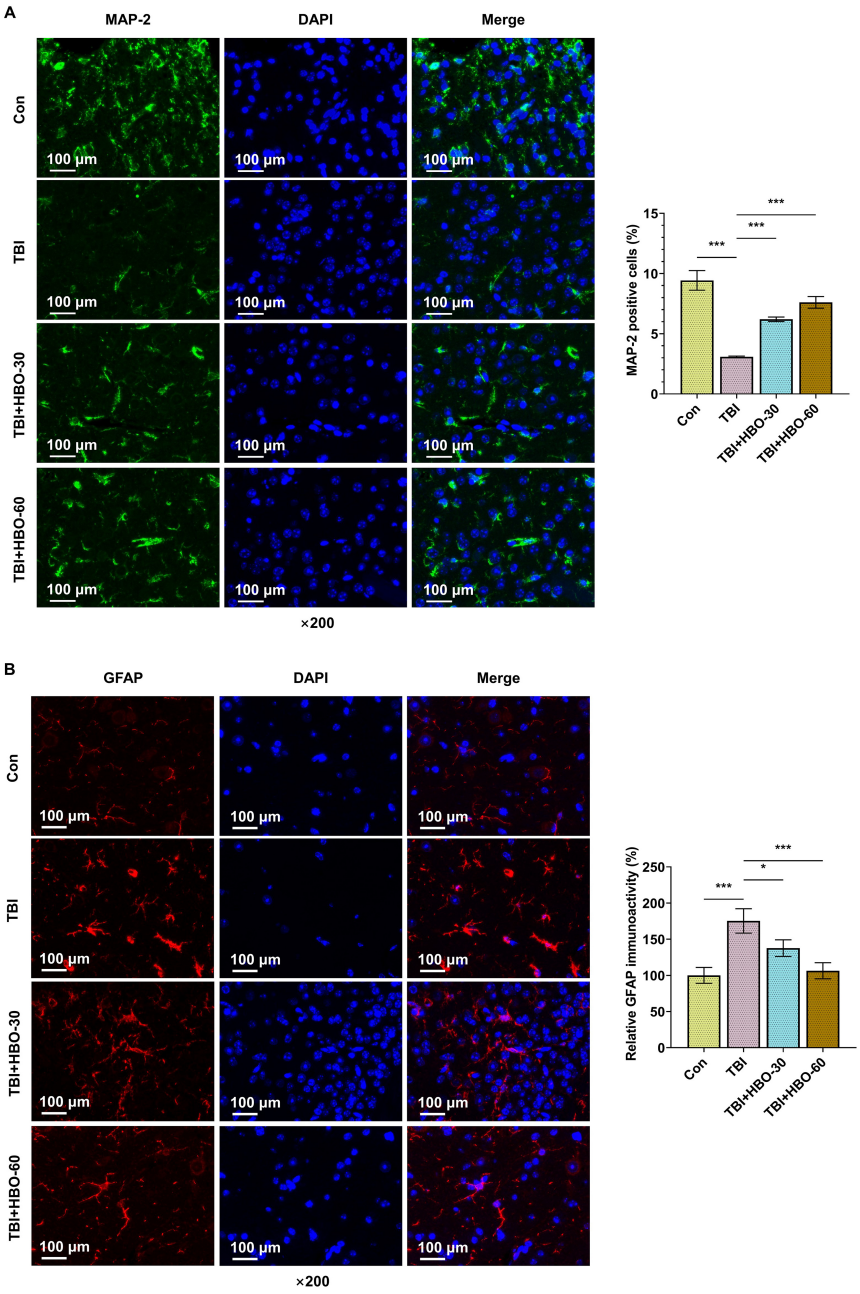

Fig. 1. HBO reversed the effect of TBI-induced decrease of neuronal viability and increase of astrocyte viability in neuron/astrocyte co-cultures. (A,B) Con group: neuron/astrocyte co-cultures; TBI group: neuron/astrocyte co-cultures were subjected to TBI through scalpel injury; TBI + HBO-30 and TBI + HBO-60 group: neuron/astrocyte co-cultures were subjected to TBI through scalpel injury, and TBI-induced co-cultures received HBO treatment (2 ATA) for 30/60 min. (A) Neuronal viability in neuron/astrocyte co-cultures (MAP-2 immunofluorescent staining). Scale bar = 100 µm, magnification, ×200. (B) Astrocyte viability in neuron/astrocyte co-cultures (GFAP immunofluorescent staining). Scale bar = 100 µm, magnification, ×200. $^{*}p < 0.05$, $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. HBO, hyperbaric oxygen; TBI, traumatic brain injury; ATA, atmospheric absolute; MAP-2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; Con, control.

viability in neuron/astrocyte co-cultures, where we found a decrease in the number of neurons by TBI (Fig. 1A, $p < 0.001$). However, HBO treatment for both 30 and 60 min increased neuronal number in TBI-induced neuron/astrocyte co-cultures (Fig. 1A, $p < 0.001$). Furthermore, GFAP immunofluorescent staining was conducted to evaluate astrocyte viability in neuron/astrocyte co-cultures [27]. In TBI-induced neuron/astrocyte co-cultures, astro-

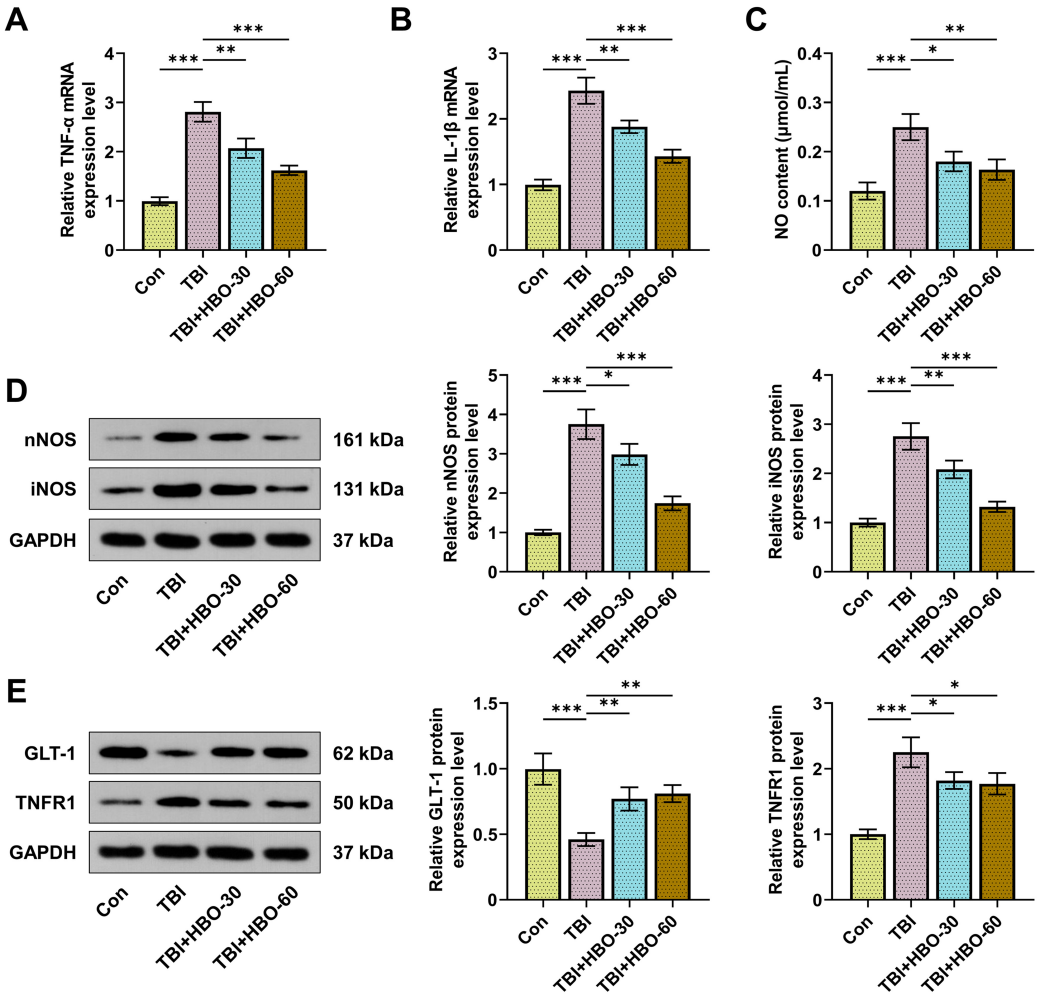

Fig. 2. HBO attenuated TBI-induced up-regulation of TNF-α, IL-1β, NO, nNOS, iNOS, and TNFR1 levels and down-regulation of GLT-1 levels in neuron/astrocyte co-cultures. (A–E) Neuron/astrocyte co-cultures underwent TBI through scalpel injury, and TBI-induced co-cultures received HBO treatment (2 ATA) for 30 or 60 min or not. (A,B) The expressions of inflammatory factors, including *TNF-α* and *IL-1β*, in neuron/astrocyte co-cultures (qRT-PCR, *GAPDH* as an internal control). (C) NO levels in neuron/astrocyte co-cultures (colorimetry). (D) nNOS and iNOS expression in neuron/astrocyte co-cultures (western blotting, GAPDH as an internal control). (E) TNFR1 and GLT-1 expression in neuron/astrocyte co-cultures (western blotting, GAPDH as a loading control). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. TNF-α, tumor necrosis factor-α; HBO, hyperbaric oxygen; TBI, traumatic brain injury; IL-1β, interleukin-1β; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNFR1, tumor necrosis factor receptor 1; GLT-1, glutamate transporter-1; ATA, atmospheric absolute; qRT-PCR, quantitative real-time polymerase chain reaction; NO, nitric oxide; mRNA, messenger RNA; Con, control.

cyte number was increased (Fig. 1B, $p < 0.01$), while HBO treatment (30/60 min) reduced GFAP immunoactivity (Fig. 1B, $p < 0.05$). Therefore, HBO reversed the effect of TBI-induced decrease of neuronal viability and increase of astrocyte viability in neuron/astrocyte co-cultures.

HBO Attenuated the TBI-Induced Up-Regulation of TNF-α, IL-1β, NO, nNOS, iNOS, and TNFR1 Levels and Down-Regulation of GLT-1 Levels in Neuron/Astrocyte Co-Cultures

TBI promoted *TNF-α* and *IL-1β* mRNA expression in neuron/astrocyte co-cultures (Fig. 2A,B, $p < 0.001$), which was reversed by HBO treatment for 30 or 60 min (Fig. 2A,B, $p < 0.01$). NO levels in neuron/astrocyte co-cultures were increased upon TBI (Fig. 2C, $p < 0.001$), whereas HBO treatment (30 or 60 min) down-regulated NO levels in TBI-treated neuron/astrocyte co-cultures (Fig. 2C, $p < 0.05$). TBI increased nNOS and iNOS protein expression in neuron/astrocyte co-cultures (Fig. 2D, $p < 0.001$). At the same time, HBO treatment for both 30 and 60 min inhibited nNOS and iNOS protein expression in TBI-induced neuron/astrocyte co-cultures (Fig. 2D, $p < 0.05$). TBI elevated TNFR1 protein levels while reducing GLT-1 protein levels in neuron/astrocyte co-cultures (Fig. 2E, $p < 0.001$),

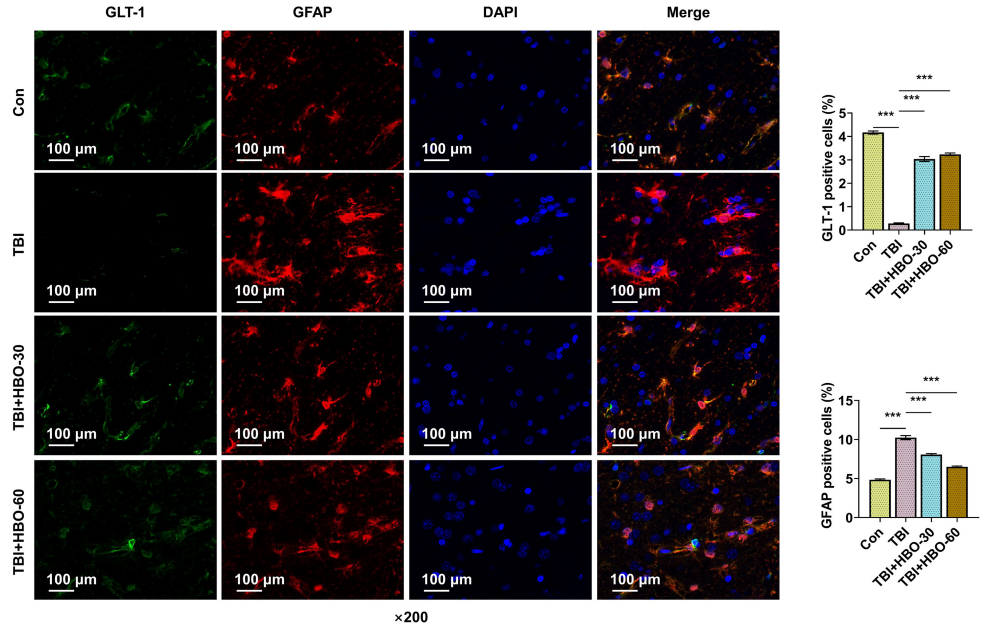

Fig. 3. HBO attenuated the TBI-induced decrease in GLT-1-positive astrocytes in neuron/astrocyte co-cultures. Neuron/astrocyte co-cultures underwent TBI through scalpel injury, and TBI-induced co-cultures received HBO treatment (2 ATA) for 30 or 60 min or not. GLT-1 expression in astrocytes in neuron/astrocyte co-cultures (GFAP and GLT-1 immunofluorescent staining). Scale bar = 100 μm, magnification, ×200. $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. HBO, hyperbaric oxygen; TBI, traumatic brain injury; GLT-1, glutamate transporter-1; ATA, atmospheric absolute; GFAP, glial fibrillary acidic protein; Con, control.

which was counteracted by HBO treatment (30 or 60 min) (Fig. 2E, $p < 0.05$). Taken together, HBO attenuated TBI-induced up-regulation of TNF-α, IL-1β, NO, nNOS, iNOS, and TNFR1 levels and down-regulation of GLT-1 levels in neuron/astrocyte co-cultures.

HBO Attenuated TBI-Induced Decrease of GLT-1-Positive Astrocytes in Neuron/Astrocyte Co-Cultures

Immunofluorescence was performed for GLT-1 quantification in astrocytes in neuron/astrocyte co-cultures. We found that TBI decreased GLT-1-positive cells while increasing GFAP-positive cells (Fig. 3, $p < 0.001$), indicating TBI reduced GLT-1-positive astrocytes. Nevertheless, HBO treatment for both 30 and 60 min increased GLT-1-positive neurons/astrocytes but decreased GFAP-positive astrocytes in TBI-treated neuron/astrocyte co-cultures (Fig. 3, $p < 0.001$), which suggested HBO attenuated TBI-induced decrease of GLT-1-positive astrocytes in neuron/astrocyte co-cultures. HBO treatment for 60 min with the most pronounced effect was used in subsequent experiments.

HBO Enhanced Neuronal Viability in TBI-Induced Neuron/Astrocyte Co-Cultures by Down-Regulating TNFR1

TNFR1 mRNA expression in neuron/astrocyte co-cultures was promoted by transfection of plasmids over-expressing *TNFR1* (Fig. 4A, $p < 0.001$) and repressed by si*TNFR1* transfection (Fig. 4A, $p < 0.001$). *TNFR1* knockdown increased neuronal density in TBI-induced neuron/astrocyte co-cultures (Fig. 4B, $p < 0.001$), where HBO treatment enhanced neuronal viability, which was reversed by *TNFR1* overexpression (Fig. 4B, $p < 0.001$). Accordingly, HBO enhanced neuronal viability in TBI-induced neuron/astrocyte co-cultures by down-regulating *TNFR1*.

HBO Decreased Astrocyte Viability and Dwindled TNF-α, IL-1β, and NO in TBI-Induced Neuron/Astrocyte Co-Cultures by Down-Regulating TNFR1

Astrocyte viability in TBI-induced neuron/astrocyte co-cultures was decreased upon both TNFR1 knockdown and HBO treatment (Fig. 5A, $p < 0.001$). However, TNFR1 overexpression reversed the role of HBO in astrocyte viability in TBI-induced neuron/astrocyte co-cultures (Fig. 5A, $p < 0.001$). *TNF-α* and *IL-1β* mRNA expression in TBI-treated neuron/astrocyte co-cultures was suppressed by both TNFR1 silencing (Fig. 5B,C, $p < 0.001$) and HBO treatment (Fig. 5B,C, $p < 0.001$). TNFR1 overexpression reversed the role of HBO in TNF-α and IL-1β mRNA expression in TBI-treated neuron/astrocyte co-cultures (Fig. 5B,C, $p < 0.01$). In addition, NO content in TBI-induced neuron/astrocyte co-cultures was reduced upon both TNFR1 knockdown (Fig. 5D, $p < 0.001$) and HBO treatment (Fig. 5D, $p < 0.001$). Nevertheless, TNFR1

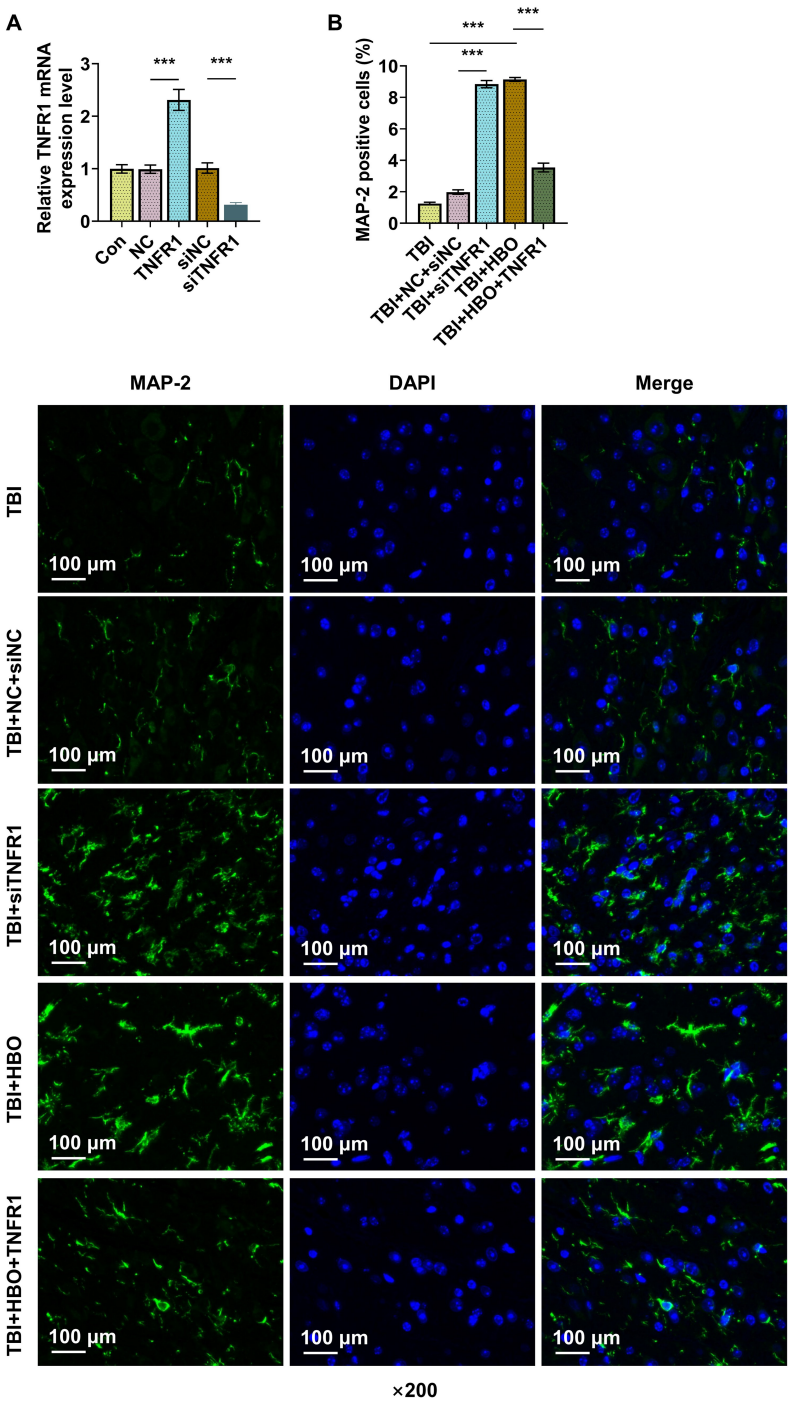

Fig. 4. HBO enhanced neuronal viability in TBI-induced neuron/astrocyte co-cultures by down-regulating TNFR1. (A) *TNFR1* expression in neuron/astrocyte co-cultures transfected with NC, plasmids overexpressing *TNFR1*, siNC or si*TNFR1* or not (qRT-PCR, *GAPDH* as an internal control). (B) TBI-induced neuron/astrocyte co-cultures were pre-transfected with NC and siNC, NC and si*TNFR1*, or plasmids overexpressing *TNFR1*, and then underwent HBO treatment (2 ATA) for 60 min or not. Neuronal viability in neuron/astrocyte co-cultures (MAP-2 immunofluorescent staining). Scale bar = 100 μm, magnification, ×200. $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. HBO, hyperbaric oxygen; TBI, traumatic brain injury; *TNFR1*, tumor necrosis factor receptor 1; NC, negative control; siNC, small interfering RNA negative control; siTNFR1, small interfering RNA against TNFR1; ATA, atmospheric absolute; MAP-2, microtubule-associated protein 2; qRT-PCR, quantitative real-time polymerase chain reaction; mRNA, messenger RNA; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; Con, control.

overexpression reversed the role of HBO in NO content in TBI-induced neuron/astrocyte co-cultures (Fig. 5D, $p < 0.05$). Consequently, HBO decreased astrocyte viability and TNF-α, IL-1β, and NO in TBI-induced neuron/astrocyte co-cultures by down-regulating TNFR1.

HBO Down-Regulated nNOS and iNOS by Inhibiting TNFR1 Expression and Up-Regulated GLT-1 in TBI-Induced Neuron/Astrocyte Co-Cultures

TNFR1 silencing and HBO treatment (Fig. 6A, $p < 0.01$) down-regulated nNOS and iNOS protein expression levels in TBI-treated neuron/astrocyte co-cultures. TNFR1 overexpression reversed the role of HBO in nNOS and iNOS protein expression levels in TBI-treated neuron/astrocyte co-cultures (Fig. 6A, $p < 0.05$). In TBI-induced neuron/astrocyte co-cultures, siTNFR1 transfection suppressed TNFR1 protein expression (Fig. 6B, $p < 0.001$) while barely affecting GLT-1 protein expression (Fig. 6B). In TBI-treated neuron/astrocyte co-cultures, HBO treatment down-regulated TNFR1 protein level while up-regulating GLT-1 protein level (Fig. 6B, $p < 0.001$), and TNFR1 overexpression reversed the above role of HBO in TNFR1 protein level (Fig. 6B, $p < 0.001$). HBO down-regulated nNOS and iNOS by inhibiting TNFR1 expression and up-regulated GLT-1 in TBI-induced neuron/astrocyte co-cultures.

Discussion

Survivors of TBI are often left with severe communicative, behavioral, and cognitive disabilities, and there is no effective intervention or treatment for patients suffering from TBI [17]. Therefore, unraveling the mechanisms underpinning TBI pathogenesis and developing effective methods for treating TBI is critical. HBO therapy has been identified as a possible option for TBI [28,29]. TBI is accompanied by the up-regulation of NO content, which can induce inflammatory reactions, neuronal apoptosis as well as cytotoxic reactions [9]. Herein, the mechanism of HBO therapy in NO content in TBI *in vitro* models was explored.

The impact of HBO therapy on TBI has been suggested in several studies. For example, Lu *et al.* [30] reported that early and intense rehabilitation with HBO therapy relieves functional disorders and improves the prognosis of TBI patients. Xia *et al.* [31] demonstrated HBO therapy plays a neuroprotective role against TBI via nuclear factor-kappa B (NF-κB)/mitogen-activated protein kinases (MAPKs)-C-X-C motif ligand 1 (CXCL1) signaling pathways. He *et al.* [32] revealed that HBO therapy attenuates neuronal apoptosis induced by TBI through protein kinase B (Akt)/glycogen synthase kinase-3β (GSK3β)/β-catenin pathways. Astrocyte activation-mediated neuroinflammation is important in TBI-induced secondary injury [33]. Inflammation contributes to early brain injury and is crucial to recovery after TBI [34]. Excessive and prolonged neuroinflammation reduces neuronal survival after TBI, and promoting neuronal survival can protect against TBI [35,36]. Given the pro-inflammatory roles of TNF-α and IL-1β [37]. We proved that HBO attenuated the TBI-induced decrease in neuronal viability, increase in astrocyte viability, and up-regulation of TNF-α and IL-1β in neuron/astrocyte co-cultures.

NO production in sublethal ischemia is significant for GLT-1 up-regulation induced by preconditioning in neuron/astrocyte co-cultures [38]. NO is up-regulated during TBI, and the synthesis of glutamate activates NO production [9,39]. Furthermore, TBI physically injures the cell membranes of axons, glial cells, and neurons, which leads to the release of glutamate [40]. HBO therapy reduces NO formation in ischemia/reperfusion testes by inhibiting dimethylarginine dimethylaminohydrolase and iNOS [20]. After HBO therapy, glutamate levels are down-regulated in acute brain injury due to porcine intracerebral hemorrhage at high altitudes [21]. The NO synthesis from l-arginine is achieved via the action of the NOS family of enzymes, including three isoforms: iNOS, nNOS, and endothelial NOS (eNOS) [41]. HBO therapy decreases iNOS protein expression in lymphocytes from patients with type 1 diabetes mellitus [42]. Early HBO inhibits iNOS and nNOS expression in chronic constriction injury [43]. GLT-1, as the glutamate transporter in astrocytes, is mainly responsible for the clearance of extracellular glutamate [23]. TBI decreases GLT-1 in the hippocampus [44]. Here, HBO attenuated TBI-induced up-regulation of NO, nNOS, and iNOS, down-regulation of GLT-1, and reduction of GLT-1-positive astrocytes in neuron/astrocyte co-cultures. These results suggest HBO reduces glutamate content by up-regulating GLT-1, thereby down-regulating NO content in an *in vitro* model of TBI.

Soluble TNF-α can bind to TNFR1 receptors to activate TNFR1-dependent signaling pathways [9]. Although there is no report about the relationship between HBO and TNFR1, HBO therapy was demonstrated to suppress TNF-α expression in the early stage after TBI [19]. Inhibition of TNFR1 attenuates lipopolysaccharide-induced inflammation in human nucleus pulposus cells [45]. Suppressing TNFR1 signaling reduces TNF-α-induced inflammation in human vascular endothelial cells [46]. Novel TNFR1 inhibitors were identified as potential therapeutic candidates for TBI [16]. TNFR1 promotes the synthesis of glutamate [9]. In the hypothalamic paraventricular nucleus, TNFR1 activation promotes glutamate signaling and angiotensin II-dependent hypertension [47]. A reduction in NO levels is shown in mice deficient of TNFR1 [48]. The increased expression of TNFR1 in the liver from trichloroethylene-sensitized mice is accompanied by increased iNOS expression in M1-type Kupffer cells [49]. In addition, NO release is inhibited in TNFR1- and IL-1 receptor (IL-1R)-blocked macrophages [50]. However, the TNFR1-related mechanism of HBO in NO content in TBI remains obscure. In

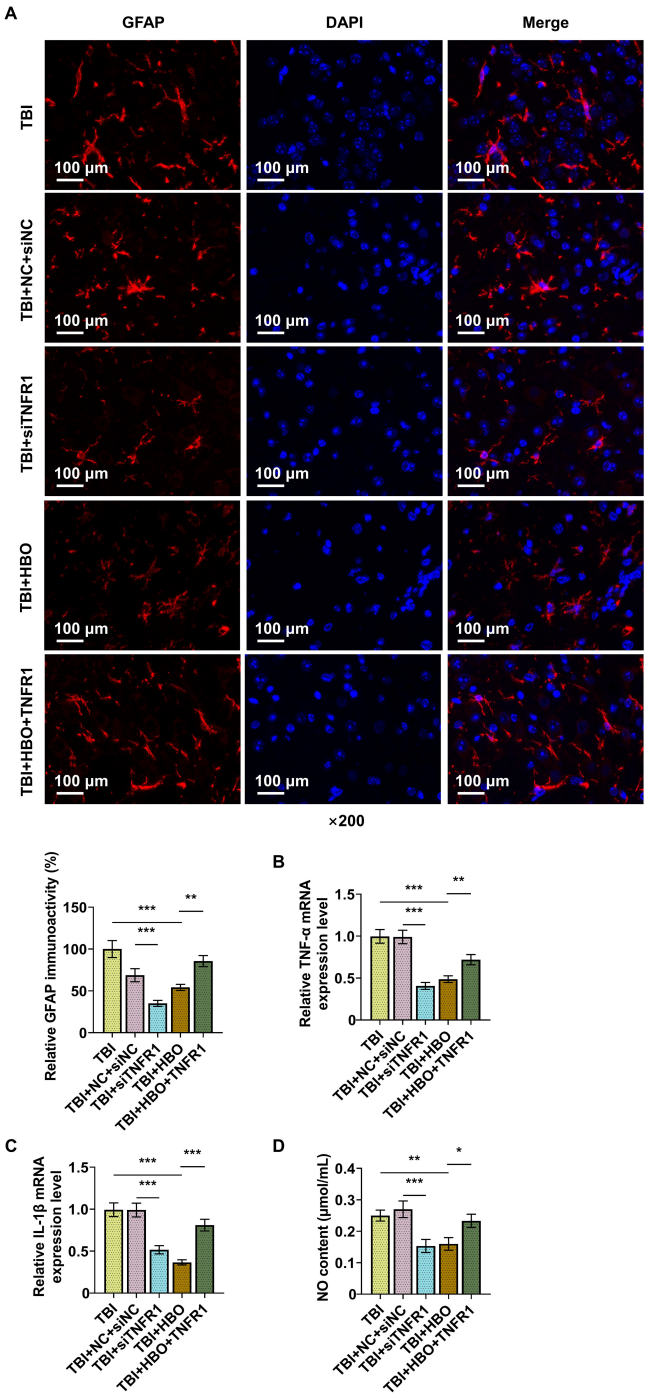

Fig. 5. HBO decreased astrocyte viability and diminished TNF-α, IL-1β, and NO in TBI-induced neuron/astrocyte co-cultures by down-regulating TNFR1. (A–D) TBI-induced neuron/astrocyte co-cultures were pre-transfected with NC and siNC, NC and si*TNFR1*, or plasmids overexpressing *TNFR1*, and then underwent HBO treatment (2 ATA) for 60 min or not. (A) Astrocyte viability in neuron/astrocyte co-cultures (GFAP immunofluorescent staining). Scale bar = 100 μm, magnification, ×200. (B,C) *TNF-*α and *IL-1*β expressions in neuron/astrocyte co-cultures (qRT-PCR, *GAPDH* as an internal control). (D) NO content in neuron/astrocyte co-cultures (colorimetry). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. HBO, hyperbaric oxygen; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; NO, nitric oxide; TBI, traumatic brain injury; TNFR1, tumor necrosis factor receptor 1; NC, negative control; siNC, small interfering RNA negative control; siTNFR1, small interfering RNA against TNFR1; ATA, atmospheric absolute; GFAP, glial fibrillary acidic protein; qRT-PCR, quantitative real-time polymerase chain reaction; mRNA, messenger RNA.

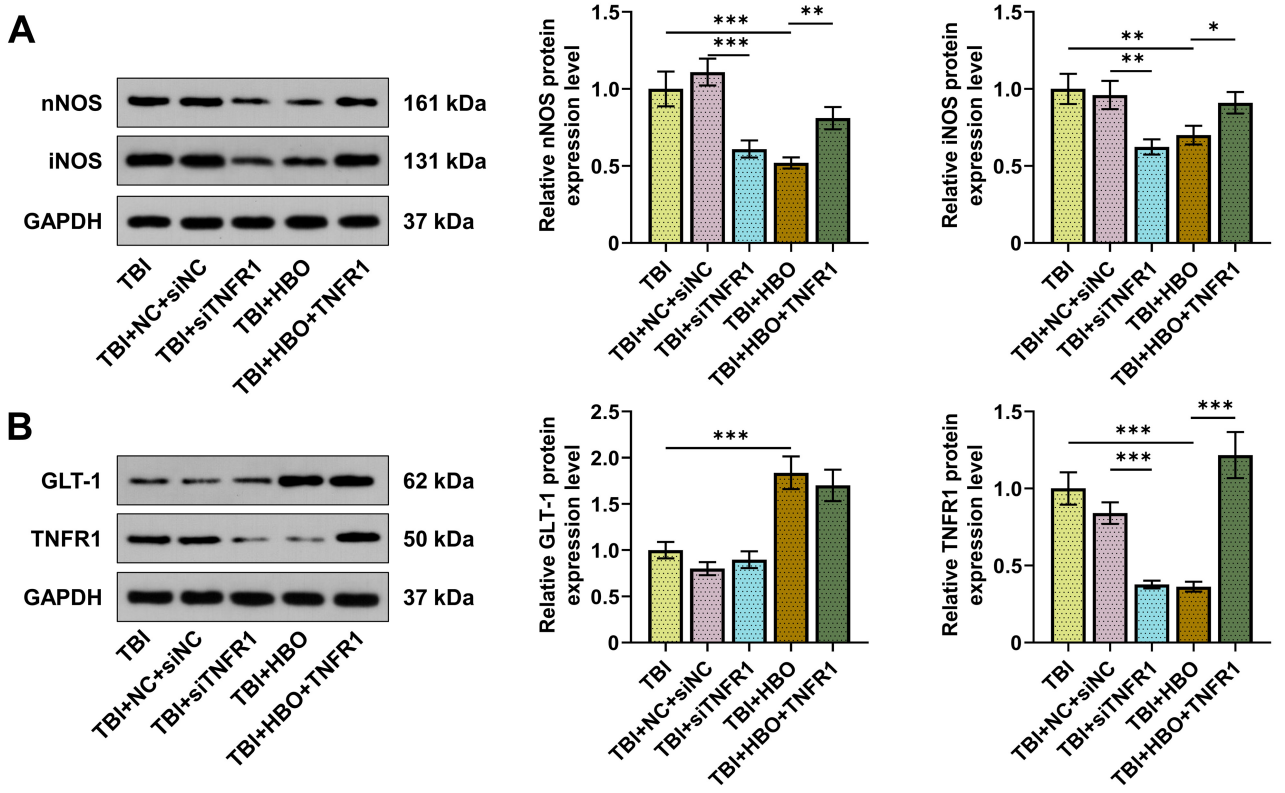

Fig. 6. HBO down-regulated nNOS and iNOS by inhibiting TNFR1 expression and up-regulated GLT-1 expression in TBI-induced neuron/astrocyte co-cultures. (A,B) TBI-induced neuron/astrocyte co-cultures were pre-transfected with NC and siNC, NC and si*TNFR1*, or plasmids overexpressing *TNFR1*, and then underwent HBO treatment (2 ATA) for 60 min or not. (A) nNOS and iNOS expression in neuron/astrocyte co-cultures (western blotting, GAPDH as an internal control). (B) TNFR1 and GLT-1 expression in neuron/astrocyte co-cultures (western blotting, GAPDH as an internal control). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. Compared between groups denoted by horizontal lines. HBO, hyperbaric oxygen; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNFR1, tumor necrosis factor receptor 1; GLT-1, glutamate transporter-1; TBI, traumatic brain injury; NC, negative control; siNC, small interfering RNA negative control; siTNFR1, small interfering RNA against TNFR1; ATA, atmospheric absolute.

the current study, HBO attenuated TBI-induced TNFR1 up-regulation in neuron/astrocyte co-cultures. TNFR1 knock-down enhanced neuronal viability, decreased astrocyte viability, and reduced TNF-α, IL-1β, NO, nNOS, and iNOS levels in TBI-induced neuron/astrocyte co-cultures. Conversely, TNFR1 overexpression reversed the role of HBO in neuronal viability, astrocyte viability, and TNF-α, IL-1β, NO, nNOS, and iNOS levels in TBI-induced neuron/astrocyte co-cultures. These findings suggest that HBO reduces glutamate content by repressing TNFR1 expression, thereby down-regulating NO content in an *in vitro* model of TBI. However, the study did not include *in vivo* experiments, which will be performed in future studies.

Conclusions

In summary, HBO down-regulates TNFR1 to reduce NO content in TBI, which suggests that HBO therapy is a potentially effective method for TBI treatment.

Availability of Data and Materials

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

Author Contributions

Substantial contributions to conception and design: QMZ, SJW. Data acquisition, data analysis and interpretation: HZZ. Drafting the article or critically revising it for important intellectual content: all authors. Final approval of the version to be published: all authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: all authors.

Ethics Approval and Consent to Participate

All the experimental procedures in our study were approved by the Ethics Committee of the Zhejiang Center of

Laboratory Animals (No. ZJCLA-IACUC-20010753) and performed following the guidelines of the China Council on Animal Care and Use.

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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.202537196.75.

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