Original Paper

Effects of MicroRNA-592-5p on Hippocampal Neuron Injury Following Hypoxic-Ischemic Brain Damage in Neonatal Mice - Involvement of PGD2/DP1 and PTGDR

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Key Words

MicroRNA-592 • PTGDR • PGD2/DP signaling pathway • Hypoxic-ischemic brain damage • Hippocampal neurons damage • Proliferation • Apoptosis

Abstract

Background/Aims: This study aimed to explore the effect of microRNA-592-5p (miR-592-5p) on hypoxic-ischemic brain damage (HIBD)-induced hippocampal neuronal injury in a neonatal mouse model relative to the involvement of one target gene, PTGDR, and the PGD2/DP signaling pathway. **Methods:** A total of 30 neonatal mice aged 7 days were randomly selected to establish an HIBD model. Hippocampal neuronal cells were transfected into a control group, a blank group, a negative control (NC) group, an miR-592-5p mimics group, an miR-592-5p inhibitors group, an siRNA-PTGDR group and an miR-592-5p inhibitors + siRNA-PTGDR group. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses were performed to detect the expression levels of miR-592-5p, PTGDR, Bcl-2 and Bax in tissues and cells. Cell proliferation, cell cycle and apoptosis were detected by CCK-8 assay and flow cytometry, respectively. **Results:** The expression levels of miR-592-5p and Bcl-2 decreased, while the expression levels of PTGDR, DP2 and Bax increased in the HIBD group. PTGDR is a target gene of miR-592-5p. Compared with the NC and blank groups, the expression levels of PTGDR, DP2 and Bax decreased, while the expression levels of miR-592-5p and Bcl-2 increased in the miR-592-5p mimics group. The siRNA-PTGDR group showed the same trend as that observed in the miR-592-5p mimics group, except with no difference in miR-592-5p expression. The miR-592-5p inhibitors group showed an opposite gene expression trend compared to that in the miR-592-5p mimics group. The S phase of the cell cycle was prolonged, the G1 phase was reduced, proliferation was increased, and the apoptosis rate was decreased in the siRNA-PTGDR and miR-592-5p mimics groups. Opposite trends for cell cycle, proliferation and apoptosis were observed in the miR-592-5p inhibitors group. **Conclusions:** Our study suggests that miR-592-5p upregulation protects against

hippocampal neuronal injury caused by HIBD by targeting PTGDR and inhibiting the PGD2/DP signaling pathway.

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Introduction

Hypoxic-ischemic brain damage (HIBD) is a common cause of injury in the central nervous system in neonates and is caused by various factors related to neonatal hypoxia and asphyxiation during the perinatal period [1]. Perinatal HIBD can lead to chronic disability and acute mortality, ultimately affecting 1~8 newborns in 1000 births and causing long-lasting neurobehavioral impairments, such as seizures, cognitive retardation, and cerebral palsy in infants and children and even neonatal death [2]. The hippocampus is a crucial component in learning and memory functions and is involved in the integration of information transmitted from outside into the nerve center [3]. Morphological and biochemical experiments have demonstrated that apoptotic and necrotic neuron death occur in the granule layer of the hippocampus after HIBD [4]. Therefore, protection of hippocampal neurons against apoptosis and injury is critical in the prevention and treatment of HIBD.

Recently, increasing evidence has indicated microRNAs (miRNAs) as regulators in a number of neurological disorders, including epilepsy, Alzheimer's disease, and ischemic stroke [5-7]. miRNAs are small non-coding RNAs of approximately 21~25 nucleotides in length that can bind to target mRNAs on the 3'-untranslated region (3'UTR) (which primarily interacts with 5'UTR) [8], resulting in target transcript degradation and/or translational suppression [9]. Several miRNAs have been proven to be modulated by hypoxia and/or oxidative stress. These miRNAs are also responsible for the morphological and functional changes in cells and tissues induced by hypoxia [10, 11]. As a member of the miR family, the functional significance of miR-592 in the etiology of HIBD in the developing brain is limited. Prostaglandin D2 (PGD2) is an inflammatory regulator in peripheral tissues, synthesized by hematopoietic PGD synthase (HPGDS) and lipocalin-type PGDS (L-CPGDS) [12], as well as can invoke airway inflammation, inhibit platelet aggregation, and induce cerebral vasodilatation [12]. PGD2 is the most abundant prostaglandin in the brain and exerts its functions through the prostaglandin D2 receptor (PTGDR) and the chemokine receptor 2 (CXCR2) [13]. The PTGDR is encoded by the PTGDR gene located on the long arm of chromosome 14 (14q31) [14]. The PTGDR is one of seven G-protein coupled receptors with an intracellular carboxyl terminus and an extracellular amino terminus [15]. CXCR2, also referred to as CXCR1, has also been reported, but it does not exhibit significant homology with PTGDR. PTGDR has various functions and cellular distributions, and a portion of its signaling is considered to be independent of PGD2 levels [16]. Currently, the role of PTGDR in the neonatal brain remains elusive. Therefore, our study aims to investigate the underlying mechanism through which miR-592-5p may affect hippocampal neurons through the regulation of the PGD2/DP signaling pathway in HIBD.

Materials and Methods

Ethics Statement

All experiments were strictly in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Besides, mice pain was minimized as much as possible.

Study Subjects and Construction of the HIBD Model

Neonatal Kunming mice aged 7 days (n = 30), either male or female, were provided by the Laboratory Animal Center of Liaoning University of Traditional Chinese Medicine (Liaoning, China). The mean body weight of the mice was 5.01 ± 0.51 g, and they were fed with maternal breast milk. The mice were maintained under a natural light cycle at appropriate temperatures of 18 to 22 °C and a relative humidity of 40% to 70%, with noise maintained below 50 dB. The mice had free access to water and food under a 12-h

light-dark cycle. These mice were randomly assigned to a normal group and an HIBD group (each $n = 15$). The model was established using the modified Rice-Vannucci method [17, 18]. The mice in the HIBD group administrated by intraperitoneal injection with 0.3% pentobarbital sodium (30 mg/kg) for anesthesia, fixed on an operating table with medical tape in the supine position, and their necks were disinfected with alcohol. An approximate 0.5 cm incision was made in the neck under an anatomical microscope (bat number PXS9-T, purchased from Xi'an CeWei Photoelectric Technology Co., LTD, Xi'an, China). The anterior neck muscles and trachea were separated, and the carotid triangle was subsequently exposed by a glass needle. Carotid pulsation could be observed next to the right side of the trachea and the vagus nerve. After careful separation of the carotid artery, it was permanently ligated with double ligature to block blood flow. The operation lasted no more than 5 min to prevent any injury to the neonatal mice due to non-operative factors resulting from prolonged procedure duration. A No. 4 needle was used to suture the incision. The neonatal mice were placed back with the female mice for 1 to 2 h after the operation [17, 19]. After the mice were put into a sealed, 50-mL low-oxygen container at a constant temperature of 37°C, after they were fully awake. After 2 h of hypoxia, the mice were returned to their mothers for breastfeeding. The mice in the normal group underwent carotid artery separation only (no ligation or hypoxia). The mice appeared to move slowly, exhibited poor stability and showed difficulty in turning over; then the establishment of the model was considered to be successful.

Morris Water Maze (MWM) Test

The MWM involves a double transparent plexiglass box (80 × 50 × 40 cm) containing a destination platform (10-cm diameter, 0.5 cm under the water surface), a video camera above the pool and a personal computer connected to a camera. The pool was 12 cm high and the water temperature was kept constant at $24 \pm 1^\circ\text{C}$. All space signs around the maze remained in the same position throughout the test. The MWM test was carried out when the mice were 30 days old (23rd day after HIBD model establishment). The mice were placed on the destination platform for 30 s of adaptation and then placed in the water facing upward. Next, the time to find the platform was recorded and defined as escape latency. If the mice were unable to find the platform within 60 s, they were guided to the platform so that they could remember its location. The mice were trained for 4 d (6 times each day), and the mean escape latency of each group was used for assessments of mice's spatial learning and memory capacity [20].

Hematoxylin Eosin (HE) Staining

The mice were anesthetized with 1% pentobarbital sodium (30 mg/kg), fixed in the supine position, and perfused with normal saline through their left ventricles. The right atrial appendage was incised until outflow of transparent liquid was observed. After that step, the mice were injected with 4% paraformaldehyde until the body became rigid and the tail cocked. Next, mouse hippocampal tissues were removed and placed in 4% paraformaldehyde for 6 h of fixation. The tissues were subjected to gradient ethanol dehydration, xylene transparency, wax dipping, and paraffin embedding and were cut into 5 μm continuous coronal sections by a paraffin sectioning machine (SM2400, Leica, Germany). One section among every five sections was selected to be attached on a lysine-treated glass slide. Ten brain tissue sections was taken from each mouse. The sections were stained with HE. The operation procedures were as follows: (1) the sections were sequentially soaked in xylene I and II 3 times (5 min each); (2) they were washed with 100%, 95%, 90% and 80% ethanol for one or two min and then fully rinsed under running water; (3) they were soaked in hematoxylin for 5 min, and then removed and fully rinsed under running water; (4) 1% hydrochloric acid ethanol was used for differentiation for 15 s, and then the sections were washed with running water; (5) the sections were stained by eosin for 3 min, and then washed with running water; (6) they were washed with 80%, 90%, and 95% ethanol, respectively, for one or two min and soaked in 100% ethanol for 3 or 4 min; (7) the sections were soaked in xylene I and II, respectively, for 5 min and then removed; (8) they were sealed by neutral balata; (9) the sections were observed under an optical microscope (DR12y; Olympus Optical, Tokyo, Japan) and photos were taken.

Terminal Transferase-Mediated DNA End Labeling (TUNEL) Assay

A TUNEL assay was performed using a conventional process. The procedures were as follows: paraffin sections were dewaxed with xylene I and II respectively for 15 min. After gradient alcohol hydration, the sections were washed with Tris solution (pH: 7.4-8.0) twice (3 min each), and then digested with Protein K in

a water bath at 37 °C for 30 min. Next, the sections were washed with Tris solution twice for 3 min each time. Next, 0.3% H₂O₂ methanol was added to the sections at room temperature for 10 min, followed by washing with Tris solution twice for 3 min each time. The sections were reacted with TUNEL solution in a water bath at 37 °C for 60 min and then washed with Tris solution three times (3 min each). Subsequently, biotinylated anti digoxin antibody (1 : 100, MK1020, Wuhan Yide Biotechnology Co., Ltd, Wuhan, China) was added to the sections in the water bath at 37 °C for 60 min, and then the sections were washed with Tris solution three times (3 min each). sSABC was added to the sections in a water bath at 37 °C for 30 min. The sections were then washed with Tris solution five times (3 min each), stained again by hematoxylin for 15~30 s, washed with water, dehydrated by gradient ethanol, soaked in xylene I and II respectively for 15 min, and mounted by neutral balata. A digital pathology section scanner (6504523001, Roche Diagnostics Co. Ltd., Shanghai, China) was used to scan the sections 200 times. Five non-overlapping fields were randomly selected to calculate the percentage of positive cells in each image of the HIBD group. The average was taken as the apoptosis index (AI). The experiment was repeated three times for each group.

Hydroxylamine Method and Thiobarbituric Acid (TBA) Method

Brain tissues of HIBD mice and normal mice were collected and washed with normal saline. After the blood was removed, the tissues were dried using filter paper. The tissues were homogenized by homogenizer in an ice bath and were centrifuged at 4000 r/min for 15 min at 4 °C. The supernatant was collected in an eppendorf tubes and stored at -20 °C. Superoxide dismutase (SOD) activity was detected by a kit (ml001998, Shanghai Meilian Biotechnology Co. Ltd, Shanghai, China), and the malondialdehyde (MDA) level was detected by another kit (ml1531021, Shanghai Meilian Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's instructions. SOD activity was determined by the hydroxylamine method and the MDA level was evaluated indirectly by the absorbance of red products that were produced by the reaction of sodium thiosulfate and MDA. The absorbance was evaluated by the Multiskan Spectrum (51119200, Thermo Scientific™, Waltham, USA). The experiments were repeated three times for each group.

Immunohistochemistry (IHC)

The steps of the IHC procedure were as follows: paraffin sections were dewaxed in xylene I and II respectively for 15 min using a gradient ethanol (100%, 95%, 90%, 80%, 70%, 5 min each) and rinsed with phosphate-buffered saline (PBS) solution three times for 3 min each. The sections were poured into 0.01 mol/L citric acid buffer and heated, followed by cooling to room temperature. The sections were rinsed with PBS solution three times for 3 min each. Each section received the addition of normal goat serum (blocking solution) (CW0130S, Beijing ComWin Biotech Co., Ltd., Beijing, China) and was incubated at 20 °C for 10 min. Next, diluted primary antibody and rabbit anti-mouse antibody 1:1000 (1:500, GTX71677, GeneTex, Owen, California, USA) were added to the sections, which were then shaken overnight at 4 °C and washed with PBS three times (3 min each). Thoroughly washed sections were set flat at 20 °C for 15~20 min, and then washed with PBS three times (3 min each). Then, the sections were incubated on a shaking table with goat anti-rabbit secondary antibody (1:1000, CW0130S, Beijing ComWin Biotech Co., Ltd., Beijing, China) for 1 h. Next, the sections were incubated at 20 °C for 10 min and rinsed with PBS solution three times (3 min each). Streptavidin working solution was marked by horseradish peroxidase (0343-10000U, Imunbio technology Co., Ltd, Beijing, China). Subsequently, 50 µL of diaminobenzidine (DAB) was added to every section. The sections were observed under an optical microscope (DR12, Olympus, Tokyo, Japan) and rinsed with running water two times (10 min each). Next, the sections were stained with hematoxylin for 10 s, washed with running water, and dehydrated by gradient ethanol (70%, 80%, 90%, 95%, 100%, each for 5 min). The sections were dewaxed twice with xylene (15 min each). The negative control (NC) group underwent the same procedure, except that the primary antibody was replaced by 0.1 mol/L of PBS. Image-Pro Plus Analysis Software (Bio-Rad Laboratories, Inc. U.S) was used to collect images under an optical microscope, and then the number of positive cells was counted. Each group had three replicates.

Enzyme-Linked Immunosorbent Assay (ELISA)

Normal saline (0.9%) was used to prepare 10% tissue homogenate, which was then centrifuged at a low temperature (8000 rpm/min). The supernatant was collected, and the antigens were diluted to the proper concentration with coating diluted solution and stored at room temperature until further use. The

diluted samples were evaluated, and the blank control group and the positive control group were established. Standard control reagent or samples (both 100 μ L) were added to coating wells, which were then shaken and mixed, covered with a plate, and incubated at 37 °C for 3 h. The samples were rinsed with PBS 3 times (3 min each). The reaction solution in the well was discarded, and the well was dried using absorbent paper. Biotin-labeled antibody reaction solution (100 μ L) was added to each well, and then the samples were covered with new plate and incubated at 37 °C for 1 h. The antibody reaction solution was discarded, and the wells were dried and washed 3 times (3 min each). Peroxidase-labeled reaction solution (100 μ L) was added to each well, and then the samples were covered with another new plate. The reaction wells were incubated at 37 °C for 1 h. The reaction solution was discarded and the wells were dried using absorbent paper. The plates were washed three times (3 min each). The wells were visualized with 80 μ L of substrate solution, and then incubated at 37 °C for 20~30 min. The reaction was terminated with the addition of 50 μ L of stop solution. The optical density (OD) value of each well was measured with a microplate reader (51119200, Thermo Scientific™, Waltham, USA). Each group had three replicates.

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

A total RNA extraction kit (Axygen Biotechnology Co., Ltd., AP-MX-MS-RNA, Guangzhou, China) and reverse transcription and RT-qPCR reagents (AE101-03, AQ401-02, Beijing Gold-tide Biotechnology Co., Ltd., Beijing, China) were used in this experiment. The primers were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China) (Table 1). Total RNA was extracted using Trizol reagent (150-0256, Invitrogen, Car, Cal, USA). Diethyl pyrocarbonate (DEPC)-treated water was used to dissolve the RNA. The concentration and purity of RNA were determined by ultraviolet spectrophotometry. RNA integrity was detected by 1% agarose gel electrophoresis. A reverse transcription reaction was performed using the RT-MLV Reverse Transcriptase Kit (Invitrogen Inc., CA, USA). The reaction system was prepared and the procedures were carried out according to the manufacturer's instructions. The reaction system included 20 μ L of One Step SYBR RT-PCR Buffer III, 0.4 μ L of TaKaRa Ex Taq™ HS (5 U μ L⁻¹), 0.4 μ L of Prime Script™ RT Enzyme Mix I, 0.4 μ L of ROX Reference Dye or Dyel (50 $\times$), 0.3 μ L of PCR primer (10 μ mol/L), 0.3 μ L of PCR reverse primer (10 μ mol/L), 2.0 μ L of total RNA and 12 μ L of RNase Free dH₂O. U6 was the internal reference for miR-592-5p and glyceraldehyde phosphate dehydrogenase (GAPDH) was the internal reference for other substances. The relative transcription level of the target genes was analyzed using the 2^{- $\Delta\Delta$ Ct} method ($\Delta\Delta$ Ct = Δ Ct HIBD group - Δ Ct control group, Δ Ct = Ct target gene - Ct internal reference gene). The expression levels of miR-592-5p, PTGDR, DP2, Bcl-2, B-cell lymphoma 2 (Bcl-2), and Bcl-2-associated X protein (Bax) in tissue were calculated (This method can also be applied for the detection of RT-qPCR in cells). Each group had three replicates.

Western Blotting

Protein extraction tissues were selected to be weighed and cut into small pieces, which were then added to a reagent. The newly prepared protein extraction reagent consisted of a mixture of 1 mL of extraction reagent and 5 μ L of protease inhibitor and a mixture of 5 μ L of phenylmethylsulfonyl fluoride and 5 μ L of phosphatase inhibitor. The milliliter of pre-cooled protein extraction reagent was added to each 250-mg sample, and the tissue samples were immediately homogenized at high speed (30 s each time) and placed in ice for 1 min per homogenization until they were completely lysed. Next, the tissues were put into a pre-cooled 4 °C centrifuge and centrifuged at 14,000 rpm for 15 min. The supernatant was stored in a new centrifuge tube until further use. Protein expression levels were determined using the bicinchoninic acid (BCA) Protein Assay Kit (CW2011S, ComWin Biotech Co.,

Table 1. Primer sequences for RT-qPCR. Notes: miR, microRNA; PTGDR, prostaglandin D2 receptor; GAPDH; glyceraldehyde 3-phosphate dehydrogenase; DP2, prostaglandin D2 receptor 2, Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; RT-qPCR, reverse transcription polymerase chain reaction

Gene	Sequence
miR-592-5p	F: 5'-CCATGACATTGTGTCAATATGCCA-3' R: 5'-CGTCATGATGTTGCGTCACC-3'
PTGDR	F: 5'-CGGAATTCATGGTCCACAGCATTCCGCTG-3' R: 5'-CGGGATCCCTAATTGATCCCGCTGCTCA-3'
GAPDH	F: 5'-CATCACCATCTTCCAGGAGCG-3' R: 5'-TGACCTTGCCCACAGCCTTG-3'
DP2	F: 5'-CATGTGCTACTACAACCTTGC-3-3' R: 5'-GCAGACTGAAGATGTGGTAGG-3'
Bcl-2	F: 5'-CGACTTTGCAGAGATGTCCA-3' R: 5'-ATGCCGGTTCAGGTACTCAG-3'
Bax	F: 5'-CTGCAGAGGATGATTGCTGA-3' R: 5'-GATCAGCTCGGGCACTTTAG-3'
U6	F: 5'-AGAGCCTGGTGTCCGC-3' R: 5'-CATCTTCAAAGCACTTCCCT-3'

Ltd., Beijing, China). The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on separation gel. Protein samples were boiled at 100 °C for 5 min, and 20 µg of the samples was added to each well of new plate. Target proteins were separated in electrophoresis buffer and gel block containing the cut target protein. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 µm diameter) at 10 V using semi-dry electric transfer methods for 30 min. Next, the membranes were blocked with 5% bovine serum albumin (BSA) supplemented with Tris Buffer (pH 7.5) Saline with Tween (TBST) for 2 h. After that step, the membranes were incubated for 1 h at 37 °C with the following primary antibodies: rabbit antibody PTGDR (1: 500, ab99446), rabbit antibody DP2 (1: 100, ab80647), rabbit antibody Bcl-2 (1: 500, ab129775), rabbit antibody Bax (1: 1000, ab32593) and rabbit antibody GAPDH (1: 500, ab8245) (Abcam, Cambridge England). Next, the membranes were incubated with 3% BSA at 37 °C for 1 h, and then at 4 °C on a shaking table overnight. The membranes were washed with TBST 3 times (5 min each) and incubated at 37 °C for 1 h with secondary antibody (goat anti rabbit IgG 10000, Boster Biological Technology, Ltd., Hubei, China). Next, the membranes were washed with TBST (5 min each) and visualized with Electrochemiluminescence (ECL). The membranes were then scanned after exposure. The OD value was analyzed by ImageJ (National Institutes of Health, Bethesda, USA). The gray value between the target band and the internal reference band represents the relative expression of protein (This method can also be applied for the detection of Western blotting in 2017). Each group had three replicates.

Dual Luciferase Reporter Gene Assay

The target gene of miR-592-5p was analyzed by miRBase.org to verify whether PTGDR was a direct target gene. A luciferase reporter gene assay was performed to confirm that PTGDR was a direct target gene of miR-592-5p. An artificial synthetic PTGDR 3'UTR gene construct was introduced into the pMIR-reporter (Beijing Hua Yueyang Biotechnology Co., Ltd, Beijing, China) between restriction sites Spe I and Hind III. The mutation sites of the complementary sequence were introduced into wild-type PTGDR. After digestion of the restriction endonuclease, the target fragment was ligated into the pMIR-reporter plasmid using T4 DNA ligase. The wild-type (WT) and mutant (mutant) luciferase reporter plasmids, which had the correct sequences, were transfected into HEK-293T cells (Beijing Beino Biotechnology Co., Ltd, Beijing, China) with miR-592-5p. Forty-eight h after transfection, the cells were collected and lysed, and then a luciferase assay kit (K801-200, Biovision, San Francisco, USA) was used to detect luciferase activity individually.

Cell Culture

Mouse hippocampal tissues were cut into pieces of approximately 3 mm and washed by D-Hanks (HMK0011, Beijing Huamaili Biotechnology Co., Ltd, Beijing, China). The supernatant was discarded and dulbecco minimum essential medium (DMEM) containing 0.15% collagenase was added to the tissues (SH30021, Beijing Nantong Biotech Develop, Co. Ltd, Beijing, China). Subsequently, the tissues were transferred to sterile centrifuge tube, digested at 37 °C in an electromagnetic stirrer (1008011909, Tianjin Standard Testing Co., Ltd, Tianjing, China) for 30 min and centrifuged at 700 rpm/min for 5 min. The supernatant was removed, and the tissues were re-suspended with proper low-sugar DMEM containing 10% fetal bovine serum (FBS) at 700 rpm/min. Cell deposits were added to the medium to form a cell suspension. Next, the cell deposits were mixed thoroughly and transferred to a disposable medium such that they could distribute evenly in the medium. The suspension was washed by D-Hanks, combined with 0.25% trypsin (supplemented with 0.02% ethylene diamine tetraacetic acid [EDTA]), and then incubated in a 5% CO₂ incubator (XBQ-3H, Hualida Experiment Equipment Co., Ltd., Taicang, China) at 37 °C for 7~9 min. Next, the medium containing 20% FBS was added into the suspension to terminate digestion. The suspension was centrifuged at 1200 rpm/min for 5 min. The supernatant was discarded, and the cells were re-suspended using DMEM containing 20% FBS and later seeded in new medium. The cells were incubated in a 5% CO₂ incubator at 37 °C. The medium was refreshed every 2~3 d.

The establishment of an oxygen and glucose deprivation (OGD) model in mouse primary hippocampal neurons was conducted according to the following steps: after 5 d of culture, the primary neuronal cells with a rapid growth rate were interrupted. As the cells reached 90% confluence, the medium was replaced with Earle balanced salt solution (PH1509, HyClone, USA). Subsequently, neuronal cells were cultured in an incubator containing 5% O₂ and 95% N₂ at 37 °C for 6 h. Normal neuronal cells did not undergo OGD model induction and were used as the control group [21-23].

Cell Grouping and Transfection

The hippocampal primary neurons were divided into 7 groups: a control group (healthy neuronal cells), a blank group (neuronal cells of OGD models that did not undergo transfection), an NC group (neuronal cells of OGD models transfected with empty plasmid), an miR-592-5p mimics group (neuronal cells of OGD models transfected with miR-592-5p mimics), an miR-592-5p inhibitors group (neuronal cells of OGD models transfected with miR-592-5p inhibitors), an siRNA-PTGDR group (neuronal cells of OGD models transfected with siRNA-PTGDR) and an miR-592-5p inhibitors + siRNA-PTGDR group (neuronal cells of OGD models transfected with miR-592-5p inhibitors and siRNA-PTGDR). Inhibitors and mimics were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). When the cells density reached 80~90%, cells were transfected according to the instruction of Lipofectamin™ 2000 (11668030, Invitrogen, Carlsbad, USA). Plasmid was diluted in 250 µL of Opti-MEM I low-serum medium (or other serum-free medium). Likewise, Lipofectamine™ 2000 was gently shaken, and 5 mL was taken for dilution in Opti-MEM I low-serum medium. The mixture was placed at room temperature for 5 min. The dilutions of Lipofectamine™ 2000 and plasmid were percussed and mixed, and then placed at room temperature for 20 min. Then, cells were cultured in a CO₂ incubator at 37 °C for 18~48 h for further experiments. Each group had three replicates.

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-Diphenyl Tetrazolium Bromide Assay

When the cells density reached 80% after transfection for 24 h, the cells were adjusted to different concentrations with complete medium (RPMI 1640). Then, the cells were seeded into 96-well plate and each group had 8 replicates. The cells were cultured in a 5% CO₂ incubator at 37 °C. The OD value was measured at 490 nm by MTT assay at 24 h, 48 h and 72 h. The above experiments were repeated 3 times. Neuronal cell proliferation was analyzed and compared in each group.

Flow Cytometry

Propidium iodide (PI) single-staining was employed to detect the cell cycle. Neuronal cells were collected 48 h after transfection and washed with pre-cooled PBS three times. The cells were centrifuged to remove the supernatant, re-suspended in PBS and adjusted to a concentration of 1×10^5 cells/mL. Next, the cells were fixed with pre-cooled 70% ice-cold ethanol (20 °C) and stored at 4 °C overnight. The cells were centrifuged at 800 rpm/min at 4 °C and then the supernatant was removed and the cells were washed with PBS (containing 1% FBS). Two million cells were suspended with 400 µL of binding buffer with 50 µL of RNase A (R4875, Shanghai Weigene Biotechnology Co., Ltd., Shanghai, China) and incubated at 37 °C for 30 min. PI (50 µL) (50 mg/mL GK3691-50T, Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was added to the cells, which were then incubated at room temperature for 30 min. Flow cytometry was used to detect the cell cycle. Each group had three replicates.

Annexin-V-FITC double-staining were used to detect cell apoptosis: Forty-eight h after transfection, cells were digested with EDTA-free trypsin and collected in a flow tube. The cells were centrifuged to remove the supernatant, washed by pre-cooled PBS three times, and then centrifuged again to remove the residual supernatant. The cells received further additions of FITC (5 µL) and PI (5 µL) mixture to prepare Annexin-V-FITC/PI staining solution and were later incubated at room temperature for 15 min in the dark. The cells were seeded in a 6-well plate at a concentration of 2×10^5 cells per well. The blank, NC, and test groups were established and transfected with 100 nmol/L Annexin-V-FITC/PI staining solution according to the instruction of the cell apoptosis detection kit (Annexin V-APC Apoptosis Detection Kit-556547, Becton, Dickinson and Company, Los Angeles, USA). The cells were suspended in 500 µL of binding buffer. Flow cytometry was used to analyze cell apoptosis. Each group had three replicates.

Statistical Analysis

SPSS 21.0 (IBM corp. Armonk, NY, USA) was applied to analyze the data. Measurement data with a normal distribution were expressed as the mean value $\pm$ standard deviation. The escape latency in the Morris water maze test was analyzed by a repeated measures analysis of variance (ANOVA). Differences between two groups were compared by the independent-sample *t* test. Comparisons among multiple groups were conducted by a one-way ANOVA, and pair-comparisons were determined with the Student-Newman-Keuls test [20]. *P* < 0.05 was considered to be significant.

Results

Injury to hippocampal primary neurons occurred in HIBD model mice

Compared with the normal group, most of the mice in the HIBD group showed physical decline. For example, the HIBD model mice exhibited tonic convulsion, reduced flexibility, slow motion, poor stability, difficulty in turning over, and significant physical disabilities. Hypoxia-ischemia contributed to limb movement disorder, so the mice walked in the direction of the paralyzed side or fell due to limb weakness. These symptoms were not observed in the normal group. The behaviors mentioned above demonstrated successful establishment of the HIBD model.

The escape latencies of the neonatal mice in the HIBD and normal groups were not different on the first day ($P > 0.05$). From the second day on, the escape latency in the HIBD group was significantly higher than the normal group ($P < 0.05$).

These results suggest that HIBD impaired spatial learning and memory capacity in mice (Table 2).

Nuclei stained with hematoxylin were blue and cytoplasm stained with eosin was pink. The normal group showed well-structured hippocampal tissues, clear intercellular levels, and normal cellular morphology, no edema, and few apoptotic cells, reflecting the absence of pathological changes. However, the HIBD group exhibited disordered hippocampal cells, cytoplasmic edema, deeply stained nuclei, pyknosis, and visible apoptotic cells (Fig. 1).

HIBD exacerbated hippocampal neuronal apoptosis

Brown granules were evident in the nuclei of apoptotic cells by light microscopy. Apoptotic cells could have different shapes, such as round and oval, and dark brown apoptotic bodies were observed. In the normal group, apoptotic cells were rare, but a few normal, light-yellow apoptotic cells could be observed. Numerous apoptotic cells were observed in the HIBD group, significantly more than those observed in the normal group ($P < 0.05$) (Fig. 2). Each experiment was repeated three times.

HIBD increased MDA expression and suppressed SOD expression in hippocampal tissues

Compared with the normal group, the level of MDA in hippocampal tissues of the mice in the HIBD group was significantly increased, while SOD activity was significantly decreased

Table 2. MWM test of the normal and HIBD groups. Notes: MWM, Morris water maze; HIBD, hypoxic-ischemic brain damage; *, $P < 0.05$ compared with the normal group

group	number	the first day	escape latency (s)		
			the second day	the third day	the fourth day
HIBD	15	74.35 ± 4.78	64.25 ± 5.45*	59.22 ± 4.49*	46.34 ± 4.43*
normal	15	72.08 ± 3.35	52.04 ± 3.15	45.84 ± 7.91	32.17 ± 4.12

Fig. 1. HE staining of hippocampal tissue from the normal and HIBD groups (400 ×). Notes: MWM, Morris water maze; HE, hematoxylin eosin; HIBD, hypoxic-ischemic brain damage.

Fig. 2. Neuronal apoptosis in the normal and HIBD groups by TUNEL staining. Notes: A, cell apoptosis in both groups under microscopy (200 ×); B, apoptosis rates in the two groups; HIBD, hypoxic-ischemic brain damage; TUNEL, transferase-mediated deoxyuridine triphosphate-labeled nucleoside end labeling; *, $P < 0.05$ compared with the normal group; n = 3.

(both $P < 0.01$). The results suggested that the MDA level increased, but SOD activity decreased in HIBD (Table 3). Each experiment was repeated three times.

HIBD increased the expression levels of PTGDR and PGD2 in hippocampal tissues

IHC staining showed (Fig. 3) that in the normal group, PTGDR proteins were lightly stained cytoplasmic proteins, the expression of which was weakly positive in hippocampal tissues. In the HIBD group, PTGDR proteins were deeply stained, with highly positive expression compared with the normal group ($P < 0.05$) (Fig. 3A-B). The ELISA results revealed that PGD2 expression was higher in the HIBD group than that in the normal group ($P < 0.05$). Each experiment was repeated three times. The findings revealed that HIBD increased PGD2 expression in hippocampal tissues (Fig. 3C).

Expression levels of miR-592-5p and Bcl-2 decreased, and expression levels of PTGDR, DP2, and Bax increased in hippocampal tissues of HIBD mice

The RT-qPCR results showed that compared with the normal group, the HIBD group exhibited lower miR-592-5p expression and mRNA expression of Bcl-2, and higher mRNA expression levels of PTGDR, DP2 and Bax (all $P < 0.05$). The Western blotting results showed that, compared with the normal group, the protein expression levels of PTGDR, DP2 and

Table 3. SOD and MDA activity in the normal and HIBD groups. Notes: SOD, superoxide dismutase; MDA, malondialdehyde; HIBD, hypoxic-ischemic brain damage; **, $P < 0.01$ compared with the normal group, $N = 3$

group	MDA/nmol-mg-1	SOD/U-mg-1
HIBD	$9.41 \pm 1.01^{**}$	$48.34 \pm 5.96^{**}$
Normal	7.44 ± 1.02	64.22 ± 8.4

Fig. 3. Expression levels of PTGDR and PGD2 in hippocampal tissues of the normal and HIBD groups. Notes: A, PTGDR protein expression by IHC; B, histogram of PTGDR protein expression; C, histogram of PGD2 protein expression by ELISA assay; PTGDR, Prostaglandin D2 Receptor; PGD2, Prostaglandin D2; IHC, immunohistochemistry; HIBD, hypoxic-ischemic brain damage; *, $P < 0.05$ compared with the normal group.

Fig. 4. Expression levels of miR-592-5p, PTGDR, DPs, Bcl-2 and Bax in hippocampal tissues of the HIBD and normal groups. Notes: A, histogram of miR-592-5p expression and mRNA expression levels of PTGDR, DPs, Bcl-2 and Bax by RT-qPCR; B, histogram of the protein expression levels of PTGDR, DP2, Bcl-2 and Bax as well as protein bands by Western blotting; miR, microRNA; PTGDR, prostaglandin D2 receptor; DP2, prostaglandin D2 receptor 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HIBD, hypoxic-ischemic brain damage; *, $P < 0.05$ compared with the normal group.

Bax increased significantly, while the expression of Bcl-2 protein decreased significantly in the HIBD group (all $P < 0.05$) (Fig. 4). Each experiment was repeated three times.

PTGDR is a Direct Target Gene of miR-592-5p

The biological prediction website, microRNA.org, showed that miR-592-5p could target PTGDR. According to the results of the dual luciferase reporter gene assay, the luciferase activity of Wt-miR-592-5p/PTGDR in the miR-592-5p mimics group decreased compared with that of the NC group ($P < 0.05$). However, the luciferase activity of Mut-miR-592-5p/PTGDR showed no significant difference between the miR-592-5p mimics group and the NC group. Therefore, miR-592-5p can specifically bind to PTGDR (Fig. 5A-B).

miR-592-5p downregulated the mRNA expression levels of PTGDR, DP2 and Bcl-2, up-regulated the mRNA expression of Bax, and the expression of miR-592-5p.

Compared with the control group, miR-592-5p expression and mRNA expression of Bcl-2 were decreased, and the mRNA expression levels of PTGDR, DP2, and Bax were increased in the other six groups (all $P < 0.05$). Compared with the blank group and the NC group, the siRNA-PTGDR group exhibited significantly reduced mRNA expression levels of PTGDR, DP2 and Bax, significantly increased mRNA expression of Bcl-2 (all $P < 0.05$), and insignificantly altered miR-592-5p expression ($P > 0.05$). The miR-592-5p mimics group showed significantly lower mRNA expression levels of PTGDR, DP2 and Bax, and significantly higher miR-592-5p expression and mRNA expression of Bcl-2 (all $P < 0.05$). The miR-592-5p inhibitors group displayed decreased miR-592-5p expression and mRNA expression levels of PTGDR, DP2 and Bax (all $P < 0.05$). The siRNA-PTGDR + miR-592-5p inhibitors group showed no significant differences in the mRNA expression levels of PTGDR, DP2, Bax or Bcl-2

Fig. 5. Target relationship between the PTGDR gene and miR-592-5p. Note: A, sequence of PTGDR mRNA and miR-592-5p 3'-UTR binding region; B, luciferase activity in the NC and miR-592-5p groups; miR, microRNA; PTGDR, prostaglandin D2 receptor 2; *, compared with the NC group, $P < 0.05$; NC, negative control.

Fig. 6. Expression of miR-592-5p and mRNA expression levels of PTGDR, DP2, Bcl-2 and Bax in the control, blank, NC, miR-592-5p mimics, miR-592-5p inhibitors, siRNA-PTGDR, and miR-592-5p inhibitors + siRNA-PTGDR groups by RT-qPCR. Notes: miR, microRNA; PTGDR, prostaglandin D2 receptor; DP2, prostaglandin D2 receptor 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the NC and blank groups; NC, negative control.

Fig. 7. Protein expression levels of PTGDR, DP2, Bcl-2 and Bax in the control, blank, NC, miR-592-5p mimics, miR-592-5p inhibitors, siRNA-PTGDR, and miR-592-5p inhibitors + siRNA-PTGDR groups by Western blotting. Notes: miR, microRNA; PTGDR, prostaglandin D2 receptor; DP2, prostaglandin D2 receptor 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the NC and blank groups; NC, negative control.

(all $P > 0.05$), but significantly decreased miR-592-5p expression ($P < 0.05$) (Fig. 6).

miR-592-5p downregulated the protein expression levels of PTGDR, DP2 and Bax, upregulated the protein expression level of Bcl-2.

Compared with the control group, the protein expression levels of PTGDR, DP2 and Bax increased, while the protein expression level of Bcl-2 decreased in the other six groups (all $P < 0.05$). Compared with the NC and blank groups, the protein expression levels of PTGDR, DP2 and Bax increased, while the protein expression level of Bcl-2 decreased in the siRNA-PTGDR and miR-592-5p inhibitors groups (all $P < 0.05$). The protein expression levels of PTGDR, DP2, Bax and Bcl-2 were not significantly different between the siRNA-PTGDR + miR-592-5p inhibitors group, NC group and blank group (all $P > 0.05$). The protein expression levels of PTGDR, DP2 and Bax increased, while the protein expression level of Bcl-2 decreased in the miR-592-5p inhibitors group (all $P < 0.05$) (Fig. 7). Each experiment was repeated three times.

miR-592-5p increased hippocampal neuronal cell proliferation

After 24 h of transfection, the growth rate between the groups was not significantly different (all $P > 0.05$). Forty-eight h and 72 h after transfection, the relative growth rates of the other groups were decreased compared with those of the control group (all $P < 0.05$). Compared with the blank group and the NC group, the cell growth rate was not significantly different in the miR-592-5p inhibitors + siRNA-PTGDR group after 48 h or 72 h of transfection (all $P > 0.05$). The cell growth rate increased in the miR-592-5p mimics and siRNA-PTGDR groups and decreased in the miR-592-5p inhibitors group after 48 h or 72 h of transfection (all $P < 0.05$) (Fig. 8). Each experiment was repeated three times. The results indicated that miR-592-5p negatively regulates PTGDR and that PTGDR can decrease cell proliferation.

Fig. 8. Cell proliferation after transfection in the control, blank, NC, miR-592-5p mimics, miR-592-5p inhibitors, siRNA-PTGDR, and miR-592-5p inhibitors + siRNA-PTGDR groups by MTT assay. Note: miR, microRNA; PTGDR, prostaglandin D2 receptor; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the NC and blank groups; NC, negative control; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyl Tetrazolium Bromide.

Fig. 9. Distribution of the cell cycle after transfection in the control, blank, NC, miR-592-5p mimics, miR-592-5p inhibitors, siRNA-PTGDR, and miR-592-5p inhibitors + siRNA-PTGDR groups. Note: A, cell cycle diagram for each group; B, histogram of the cell cycle for each group. miR, microRNA; PTGDR, prostaglandin D2 receptor; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the NC and blank groups; NC, negative control.

Fig. 10. Cell apoptosis after transfection in the control, blank, NC, miR-592-5p mimics, miR-592-5p inhibitors, siRNA-PTGDR, and miR-592-5p inhibitors + siRNA-PTGDR groups. Note: A, cell apoptosis diagram for each group; B, histogram of cell apoptosis for each group. miR, microRNA; PTGDR, prostaglandin D2 receptor; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the NC and blank groups; NC, negative control.

miR-592-5p affected the hippocampal neuronal cell cycle

The flow cytometry results showed (Fig. 9) that compared with the control group, the cells in the S phase were decreased, and the cells in the G1 phase were increased, in the other six groups (all $P < 0.05$). Compared with the NC group and the blank group, the cells in the S phase increased and the cells in the G1 phase decreased in the siRNA-PTGDR and miR-592-5p mimics groups. The cells in the S phase decreased and the cells in the G1 phase increased in the miR-592-5p inhibitors group ($P < 0.05$). No significant difference was found in the siRNA-PTGDR + miR-592-5p inhibitors group ($P > 0.05$). The number of cells in the G2 phase was not significantly different between the groups (all $P > 0.05$). The results indicated

that miR-592-5p expression reduced cell proliferation by inhibiting the PTGDR signaling pathway.

miR-592-5p reduced hippocampal neuronal cell apoptosis

Compared with the control group ($2.02 \pm 0.07\%$), the apoptosis rates of the other si groups were significantly increased (all $P < 0.05$). Compared with the blank group ($6.5 \pm 0.15\%$) and the NC group ($6.23 \pm 0.79\%$), cell apoptosis increased in the miR-592-5p inhibitors group ($10.77 \pm 1.01\%$) ($P < 0.05$), and the apoptosis rate was not significantly different in the miR-592-5p inhibitors + siRNA-PTGDR group ($6.51 \pm 0.79\%$) ($P > 0.05$). Cell apoptosis decreased significantly in the miR-592-5p mimics group ($4.3 \pm 0.23\%$) and the siRNA-PTGDR group ($4.28 \pm 0.33\%$) ($P < 0.05$) (Fig. 10). Each experiment was repeated three times. The results indicated that downregulated miR-592-5p expression can activate the PTGDR signaling pathway and aggravate cell apoptosis.

Discussion

HIBD is caused by many factors related to neonatal hypoxia and ischemia during the perinatal period [1]. The hippocampus is an important part of the brain in higher animals and is involved in neurological such activities as recognition and learning [23]. The hippocampus is also vulnerable to ischemic and hypoxic damage, resulting in various mental illnesses [24]. Currently, the effects of PTGDR on neonatal HIBD remain elusive. Therefore, the present study was designed to investigate the underlying role of which miR-592-5p may affect hippocampal neurons through regulation of the PGD2/DP signaling pathway in HIBD. Our findings revealed that miR-592-5p was downregulated and PTGDR was increased in HIBD neurons. Upregulation of miR-592-5p and suppression of the PGD2/DP signaling pathway may protect neurons from HIBD in neonates.

First, our data indicated that the expression levels of miR-592-5p and Bcl-2 were reduced and the expression levels of PTGDR, DP2, and DP1 were increased in the HIBD group. Additionally, MDA expression increased and SOD expression decreased in the HIBD group. Studies have shown that neuronal apoptosis in an ischemia-reperfusion injury model may be associated with Bcl-2 upregulation and Bcl-2 downregulation [25]. Increased Bcl-2 expression and decreased Bax expression have been shown to be involved in the inhibition of HIBD in the subventricular zone of rats [26]. One study also showed that an increased ratio of Bcl-2 expression to Bax expression was positively correlated to decreased neuronal damage in the brain ischemic focus [27]. Intracerebroventricular prostaglandin administration has been reported to increase neural damage from global hemispheric hypoxic ischemia [28]. The study showed that upregulated prostaglandin release increases hypoxic ischemic damage [29]. Prostaglandins such as PGD2 and PGE2 have been revealed to exacerbate hypoxic injury in neurons [30]. Moreover, several miRs have recently been reported to be associated with hypoxia and ischemia [31, 32] and to have protective effects on neuronal damage, such as miR-34b-5p expression in astrocytes [33] and miR-210 downregulation of glucocorticoid receptor protein, and in the neonatal brain in response to HIBD [9]. Importantly, the miR-592 level has also been reported to decrease after cerebral ischemia, and miR-592 overexpression decreases ischemic injuries in neurons [34]. In central inflammatory pathologies, MDA epitopes function as biomarkers of oxidative stress [35], while SOD, a primary protective agent, can release oxidative stress mediators and activate inflammatory mediators [36]. Global hypoxia-ischemia results in extensive cerebral inflammation [37].

Furthermore, the expression levels of PTGDR, DP2 and Bax decreased, and the expression levels of miR-592-5p and Bcl-2 increased in cells transfected with miR-592-5p and siRNA-PTGDR. Bcl-2 has a specific oncogenic role in extending cell survival by inhibiting a broad range of apoptotic pathways. Bax heterodimerizes with Bcl-2, and its overexpression counteracts Bcl-2 [38]. Therefore, Bcl-2 can inhibit apoptosis, and Bax can promote apoptosis [39]. PGD2 is synthesized in many organs and may function as a signaling molecule in the modulation of

multiple biological processes, such as broncho-constriction, platelet aggregation, seizures, sleep and wakefulness, and hypoxia [40]. As the most abundant prostaglandin in the brain, PGD2 exerts its functions primarily through PTGDR, which is also referred to as DP [13]. PGD2 may regulate neuronal cell death by activating DP receptors or their metabolism, inducing cell apoptosis independently of prostaglandin receptors [41]. Studies have revealed that PGD2 induces apoptosis in human leukemia cells [42] and in human osteoclasts by activating the CRTH2 receptor (PTGDR) [43]. Combined with the finding that siRNA-PTGDR exerts no effect on miR-592-5p expression, our data indicated that miR-592-5p inhibited the PGD2/DP signaling pathway in hippocampal cells suffering from HIBD.

Lastly, the S phase of the cell cycle was prolonged, the G1 phase was shortened, and cell proliferation was increased in cells transfected with miR-592-5p or siRNA-PTGDR. MiR-592 has been reported to promote the progression, metastasis, and proliferation of human colorectal cancer cells [44] and the proliferation of prostate cancer cells [45]. Studies have also shown that the expression levels of PGD2 and PTGDR can influence T cell proliferation in allergic inflammation [46], and that Prostaglandin D synthase and PGD2 can increase gastric cancer cell proliferation [47]. Therefore, the results regarding the positive role of miR-592-5p in enhancing the proliferation of hippocampal neurons are consistent with those of previous reports.

Conclusion

Our experiment revealed novel findings that miR-592-5p upregulation and suppression of the PGD2/DP signaling pathway could protect hippocampal neurons from HIBD in neonates. Our results indicate a new target for the management and treatment of HIBD. Considering the complex molecular mechanism, further research is required to accurately determine the functions of miR-592-5p and PTGDR in HIBD.

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Disclosure Statement

The authors have declared that no competing interests exist.

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