Article

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The Effect of Riboflavin on Neurological Rehabilitation after Traumatic Brain Injury in Children

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Background: Traumatic brain injury (TBI), which is the brain impairment and lesion caused by the external force injuring the head and the underlying brain, can cause pediatric death, disability, neurological disorders, and even lifelong disability. This study was to explore the effect of riboflavin (RF) on neurological rehabilitation and functional recovery after TBI.
Methods: The rat models of TBI were constructed by treating rats with controlled cortical impact (CCI). By treating TBI rats with RF, we investigated whether the administration of RF would affect the sensorimotor function and cognitive ability recovery through adhesive removal test, modified neurological severity score (mNSS), corner test, wire-grip test and the Morris water maze. The effects of RF on lesion volume and water content were investigated using hematoxylin and eosin (H&E) staining and wet-dry method. The Nissl staining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were used to demonstrate the effect of RF on neural apoptosis. Inflammation-related cytokines of interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1 were measured by enzyme-linked immunosorbent assay (ELISA) to evaluate the effect of RF on neuroinflammation. The impact of RF on oxidative stress was assessed by measuring malondialdehyde (MDA) content and superoxide dismutase (SOD) activity, and the platelet endothelial cell adhesion molecule-1 (CD31) staining for observing vessel density, the reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) for measuring vascular endothelial growth factor (*VEGF*) mRNA expression and western blot for VEGF protein expression were used for evaluated angiogenesis.
Results: The administration of RF could facilitate the recovery of neurological function by promoting the recovery of sensorimotor function and cognitive ability ($p < 0.05$). Furthermore, RF could reduce the lesion volume and water content after TBI and ameliorate neural apoptosis, neuroinflammation, and oxidative stress ($p < 0.05$). Finally, RF increased vessel density ($p < 0.01$) and *VEGF* levels ($p < 0.01$) in brain tissues after TBI, promoting angiogenesis.
Conclusion: RF benefits neurological rehabilitation after TBI by promoting neurological function recovery, ameliorating the pathogenesis after TBI, and facilitating brain vascular remodeling. These findings provide a novel mechanism for RF treating pediatric TBI.

Keywords: traumatic brain injury; TBI; riboflavin; neurological rehabilitation

Introduction

Traumatic brain injury (TBI) is the lesion and impairment of brain functions caused by the external force injuring the head and the underlying brain [1]. As the leading cause of pediatric death and severe disability, TBI is also the leading cause of children admitted to pediatric emergency departments [2,3]. The previous data have demonstrated that there were about 10 million children that came down with TBI globally in an individual year, resulting in significant impacts on the mortality and morbidity of people in pediatric age [4,5]. It has also been reported that in 2017, the number of people who were hospitalized due to TBI was approximately 224,000, of which 7.8% were children between the ages of 0–17. Among these patients with TBI, there were 61,000 deaths resulting from TBI, of which 4.5% were children [6]. For survivors, TBI, as one of the top 10 causes of disability and neurological disorders, would result in lifelong disability of infants, children, and adolescents [7]. Therefore, effective therapies for alleviating and treating TBI urgently need to be explored.

Riboflavin (RF), the essential vitamin B_2, was first found in milk and considered a yellow pigment by Blythto in 1879 [8]. RF is soluble in water and stable under heat, and oral supplementation of RF is required due to humans' limited absorption of RF [9,10]. RF is the cofactor that plays critical roles in numerous enzymatic reactions and facilitates vital metabolic functions, maintaining skin integrity, eyes, mucous membranes, and the nervous system [11]. At the same time, a deficiency of RF may induce various biological abnormalities such as renal damage, skin lesions, anemia, growth retardation, and nervous system degradation [12]. RF-deficient rat models have been used to prove that RF was significant for the early post-

natal development of the brain [13], and a previous study has reported that the supplementation of RF could combat childhood neuropathy, including the development of neuro-degradation [14]. It is noted that an earlier study has verified the ability of RF to decrease the area of brain infarct induced by the occlusion of the middle cerebral artery [15]. Moreover, it was claimed that the RF could suppress stroke development, prevent brain ischemic injury, and inhibit apoptosis and death of cultured neurons [16]. As described above, RF can protect the brain and nervous system; however, whether RF plays a role in ameliorating neural disorders and promoting neurological rehabilitation after TBI remains unclear.

In the present study, we explored the functions of RF for neurological rehabilitation after TBI in rats by investigating the effect of RF on the brain and nervous system of established TBI rat models, providing a novel therapeutic strategy of RF supplementation for pediatric TBI.

Materials and Methods

Preparation of Animals

A total of thirty male Sprague-Dawley (SD) rats at 12 weeks old were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained in the pathogen-free environment for seven days with sufficient food and under the condition of standard 12-h light/dark cycles and 26–28 °C. As for the supplied water, rats were randomly divided into three groups: Group 1 (n = 10) received normal saline, Group 2 (n = 10) received normal saline, and Group 3 (n = 10) received the solution of RF (HY-B0456, MedChemExpress, Monmouth Junction, NJ, USA) dissolved in deionized water (40 mg/L) [17]. Animal experiments were complied with the guidelines of the Tianjin Medical Experimental Animal Care, and animal protocols were approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Co., Ltd. (protocol number YSY-DWLL-2023305).

Rat-Controlled Cortical Impact (CCI) Model of Brain Injury

The Rat-Controlled Cortical Impact (CCI) model was achieved based on the previous reports [18,19]. Rats were anesthetized using a vaporizer with 4% isoflurane in 70% N_2O and 30% O_2. Then, the isoflurane concentration was adjusted to 2% to maintain the anesthesia during the surgery. All rats were shaved head and positioned in a stereotaxic apparatus, and a midline scalp longitudinal incision was made in the skin and underlying fascia, exposing the skull. For rats in Group 2 and all rats from Group 3, a 6.0-mm circular craniotomy was performed laterally using a specially designed portable drill to prevent damage to the cortex and meninges. The craniotomy was in the left parietal bone between the bregma and lambda, and the distance between the medial edge of the craniotomy and the sagittal suture was 1 mm. A controlled impactor constructed by attaching the sterile and stainless-steel flat impactor tip to a piston and controlled by condensed air was used for performing cortex injury. The cortex injury was made by impacting the cortex at 6.0 m/s velocity, 0.5 mm depth, and 0.15 s contact between the impactor tip and cortex, resulting in a compression of the cortex. The bleeding was controlled for all rats with a cold saline-soaked sterile sponge, the scalp was sutured closed with nylon material, and the wound was treated with antibiotic ointment. Then, rats were returned to their cages, maintained under 26–28 °C for recovery.

Drug Treatment

Based on the previous report [20], rats were treated as follows:

(1) The group of negative control (NC): rats in Group 1, which were daily supplied with normal saline (ST341, Beyotime, Shanghai, China) and received no CCI. Rats received saline (0.9%, 1.0 mL/kg, intraperitoneal injection) 15 min and 24 h after surgery. Daily supplied water was sterile saline.

(2) The group of CCI model with no RF treatment (CCI): Rats in Group 2 were supplied daily with normal saline and received CCI. Rats received saline (0.9%, 1.0 mL/kg, intraperitoneal injection) 15 min and 24 h after surgery. Daily supplied water was sterile saline.

(3) The group of CCI model with RF treatment (CCI+RF): Rats in Group 3 were supplied daily with aqueous solutions of RF and received CCI. Rats received RF (7.5 mg/kg, intraperitoneal injection) 15 min and 24 h after surgery. Daily supplied water was sterile aqueous solutions of RF (0.9%).

Sensorimotor Assessment

Behavioral tests were performed on rats, including an adhesive removal test (ART) and the modified neurological severity score (mNSS). Based on the report, tests were performed after the injury and on the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th days [21]. The rats were first removed from their resting cages, and two small adhesive bands of equal size, acting as tactile stimuli, were attached to the wrist of each forelimb onto the distal-radial region. Then, rats were returned to their cages, and normally, they would remove each adhesive band using their teeth at the first time of teeth contacting the band. Each rat would be tested repeatedly five times per day, and the time of the rat removing both bands was recorded. Before the CCI surgery, all rats were trained five times/day for three days, familiarizing them with this test and enabling them to remove both bands within 10 s. The rats would get points if they failed to fulfill tasks, and the motor, sensory, balance, and reflex measures were used to calculate a score ranging from 1 to 18, with the higher score implying greater neurological injury.

According to the previous study [22], the corner test was performed on the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th

days after the injury. In the test, two boards that were vertical to the ground were attached at an angle of 30° with an opening along the joint. The rat was placed between these two boards facing the corner and encouraged to enter the corner. When the rat entered deeply into the corner, and the vibrissae were stimulated, the rat would turn back and face the opening. Rates with no brain injury would turn left or right, while rates with brain injury would be inclined to turn toward the side with brain injury. Each rat would be tested for 10 times, and the turning direction was recorded.

The standard wire-grip test was performed on the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th days after the injury to test the vestibulocochlear function, according to the previous report [23]. In the trial, the rat could traverse a metal wire about 50 cm long and be suspended 50 cm above a pad. The score of the individual wire-grip test was quantified according to the latency time of the rat staying at the wire: score 0 represented the rat fell off the wire within 30 s, score 1 represented the rat held on the wire for 30 s, score 2 represented the rat held on the wire with four paws for no less than 5 s, score 3 represented the rate held on the wire with four paws and placed the tail on wire for no less than 5 s, score 4 represented the rat held on the wire with four paws and move to the end of the wire and score 5 represented the rat moved to the end of wire and went down to the ground. Each rat would be tested for five times.

Cognitive Assessment

The Morris water maze (MWM) was used to determine cognitive functions, including learning and spatial memory [24,25]. In this test, a glass tank of 1.5 m in diameter and 50 cm in height was filled with water (23–25 °C) to a depth of 35 cm. The water was made opaque by adding nontoxic white paint, and a platform of 14 cm in diameter was submerged 1.0 cm below the water's surface, leading to an invisible escape platform.

The reference memory test started 22 days after the injury and lasted five days. The hidden platform was located in the same quadrant for all the time and all rats. When being tested, the rat would be inserted into the water, and the time of the rat reaching the platform within 90 s would be recorded. If the rat failed to find the platform within 90 s, it would be gently pushed, maneuvered toward it, and allowed to remain on the platform for 10 s. Each rat was tested four times daily with different randomly chosen points of the rat being released into the water for every trial and with the intertrial intervals (ITI) of 5 min. During the ITI, the rat would return to the resting cage and be kept warm using a heating pad.

Another test for working memory started 27 days after the injury and lasted for three days. During the test, the hidden platform was located in the different randomly chosen locations in quadrants 2, 3, or 4 for each day, while the platform's location was the same for all rats being tested. Each rat was tested repeatedly four times per day with the intertrial intervals (ITI) of 5 min, and the time of the rat reaching the platform would be recorded. Among the four trials for each rat, the first trial was considered an information trial and not used to assess working memory.

Lesion Volume and Brain Content Measurement

At 28 days after the injury, all rats were anesthetized with tribromoethanol (350 mg/kg) through intraperitoneal injection and were firstly trans-cardially perfused with phosphate-buffered saline (0.9%; ST447, Beyotime, Shanghai, China) and secondly with phosphate-buffered formalin (10%; SF100, Thermo Fisher Scientific Inc., Waltham, MA, USA). The brain was removed and first fixed in formalin, then cryopreserved in sucrose (30%; HY-B1779, MedChemExpress, Monmouth Junction, NJ, USA) for three days before the brain was sectioned.

For determining the lesion volume, the brain was sectioned into slices of 20 µm, and every 20th section was stained with hematoxylin and eosin (H&E; C0105, Beyotime, Shanghai, China). The lesion area for all stained sections was quantified using ImageJ software (1.48, National Institutes of Health, Rockville, MD, USA). It was finally integrated to get the total volume of the lesion.

The wet-dry method was used for measuring brain water content. Briefly, the brain was removed from the rat, and the wet weight was obtained before the fresh wet brain was dried and the dry weight was obtained. The brain water content (%) was calculated using the formula (wet weight – dry weight)/wet weight × 100%.

Nissl Staining and Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

The Nissl staining was performed as previously described [26]. Brain sections were dewaxed and immersed in 100% ethanol for 5 min, 95% ethanol for 30 s, and 70% ethanol for 30 s. After being washed with ultrapure water, sections were stained by immersion in 1% toluidine blue (E670105, Sangon Biotech Co., Ltd., Shanghai, China) for one hour, followed by being washed with ultrapure water. Subsequently, sections were immersed in 70% ethanol (60 s), 95% ethanol (60 s), 100% ethanol (60 s), 100% chloroform (5 s), a differentiation agent (15 s), 95% ethanol (30 s), 100% ethanol (60 s) and xylene (2 h), in sequence. After being sealed, stained brain sections were observed (Olympus CK31, Olympus, Tokyo, Japan).

For terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining, brain sections were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 30 min. At room temperature, cells were incubated with 0.3% Triton X-100 (T8200, Solarbio, Beijing, China) for 5 min and then treated with TUNEL Assay Kit (C1088, Beyotime, Shanghai, China) following the manufacturer's instruction. After washed with PBS twice, cells were stained with DAPI (C0060, So-

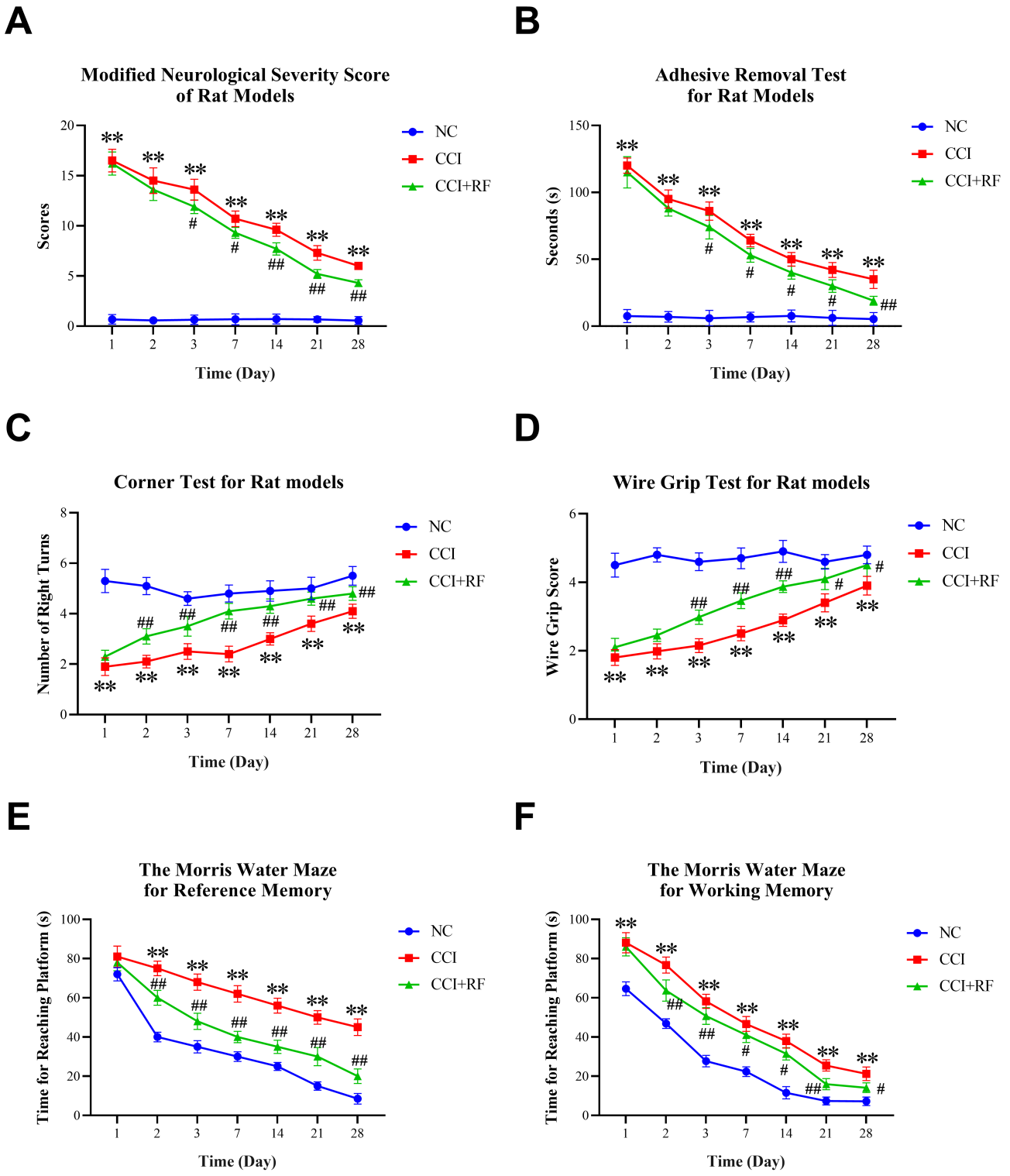

Fig. 1. Riboflavin (RF) supplementation improves the recovery of sensorimotor function and cognitive ability in rats after traumatic brain injury (TBI). For rats among different groups on different days after treatment, (A) the results of modified neurological severity score (mNSS), (B) the results of adhesive removal test, (C) the results of corner test, (D) the results of wire grip test, (E) the results of Morris water maze test for reference memory, and (F) the results of Morris water maze test for working memory. N = 10. NC: the group of negative control, CCI: the group of controlled cortical impact (CCI) treatment, CCI+RF: the group of CCI treatment with RF supplementation. $^{**}p < 0.01$ compared with the NC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with CCI group.

larbio, Beijing, China). Then, the stained sections were visualized under a fluorescence microscope (CKX53, Olympus, Tokyo, Japan) for quantifying the apoptotic cells, and three microscopic regions were selected for counting the mean of TUNEL-positive cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) analyses were used to measure the levels of interleukin (IL)-6 (ab234570, Abcam, Cambridge, MA, USA), IL-1β (ab255730, Abcam, Cambridge, MA, USA), tumor necrosis factor (TNF)-α (ab236712, Abcam, Cambridge, MA, USA) and transforming growth factor (TGF)-β1 (PT878, Beyotime, Shanghai, China) in brain tissues from different groups, following the manufacturer's instruction. The results were shown as pictograms per milligram (pg/mg).

Oxidative Stress Biomarker Assay

According to the manufacturer's instructions, the Lipid Peroxidation malondialdehyde (MDA) Assay Kit (ab233471, Abcam, Cambridge, MA, USA) was used to measure MDA content, and the superoxide dismutase (SOD) Assay Kit (ab65354, Abcam, Cambridge, MA, USA) was used to measure SOD activity.

Brain Vessel Density

Coronal sections of 12 µm were acquired and blocked with 3% bovine serum albumin (HY-CP002, MedChemExpress, Monmouth Junction, NJ, USA) for 1 h to determine brain vessel density. Then, sections were incubated with rat anti-cell adhesion molecule-1 (CD31) antibody (14-0311-82, Invitrogen, Carlsbad, CA, USA) at 4 °C overnight and then incubated with the Goat Anti-Rat IgG2a secondary antibody (PA184755, Invitrogen, Carlsbad, CA, USA) for 1 h after being washed. The fluorescence microscope (CKX53, Olympus, Tokyo, Japan) was used to examine the coronal sections, and the vessel density of individual sections was represented by the number of CD31-positive vessels in 3 fields of the section and analyzed by ImageJ software (1.48, National Institutes of Health, Rockville, MD, USA).

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

Extracting total RNAs from brain tissues by TRIzol® (DP424, Tiangen, Beijing, China) and evaluating RNA purity by a full-wavelength spectrophotometer (OSE-260, Tiangen, Beijing, China) as well as by agarose gel electrophoresis. According to the manufacturers' instructions, reversely transcribing RNAs to cDNAs using a reverse transcription kit (KR116, Tiangen, Beijing, China). The fluorescence quantitative instrument for PCR (LightCycle96, Roche, Shanghai, China) was utilized to perform qPCR following a thermal cycling program. The primer sequences used in this study were for vascular endothelial growth factor (*VEGF*) (forward 5′-CCAGGAGTACCCCGATGAGA-3′, reverse 5′-CTTCATCATTGCAGCAGCCC-3′) and β-*Actin* (forward 5′-AACACAGTGCTGTCTGGTGG-3′, reverse 5′-GAGCCAGGGCAGTAATCTCC-3′). Relative expression levels were normalized by the expression of the housekeeping gene β-Actin, and the method of $2^{-\Delta\Delta Cq}$ was used for processing the data.

Western Blot

Extracting proteins from brain tissues and the total protein concentration was measured by the BCA Protein Quantitation Kit (P0012, Beyotime, Shanghai, China). Adding the loading buffer at 1/4 of the total protein solution volume, cell lysis was denatured at 100 °C, spoiling water for 15 min for the subsequent SDS-PAGE electrophoresis. Subsequently, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore Sigma, Billerica, MA, USA) at a current of 250 mA for 90 min. Then, they were blocked with 5% skim milk (dissolved in 1 $\times$ TBST). Then, proteins were incubated with primary antibodies (VEGF: 1:1000 dilution, Cat. No. A12303, Abclonal; β-Actin: 1:1000 dilution, Cat. No. TA-09, ZSGB-Bio, Beijing, China) at 4 °C overnight. Washing the membranes with 1 $\times$ PBST 3 times and incubating membranes with the secondary antibody (ZB-2305, ZSGB-Bio, Beijing, China). The antibody-reactive bands were revealed (Tanon-5200, Shanghai Tanon Life Science Co. Ltd., Shanghai, China). Here, Actin was used as the internal reference.

Statistical Analysis

Experimental data from repeated experiments were presented as the mean $\pm$ standard deviation. The data and images of all experiments were analyzed through GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) pairwise, and statistical analyses between two groups or multi-group comparisons were performed using the t-test or analysis of variance (ANOVA) with Tukey's post hoc test. The threshold of $p < 0.05$ was considered statistically significant.

Results

RF Supplementation Improves the Recovery of Sensorimotor Function and Cognitive Ability in Rats after TBI

Several assessments were performed 1, 2, 3, 7, 14, 21, and 28 days after the modeling and treatment to explore whether RF could improve the recovery of neurological function. As shown in Fig. 1A, the mNSS was increased significantly after CCI ($p < 0.01$) compared with that in the NC group and decreased in the following days by neural recovery. While the mNSS of the CCI+RF group was consistently lower than that of the CCI group ($p < 0.05$) at individual time points indicated, indicating that RF could improve

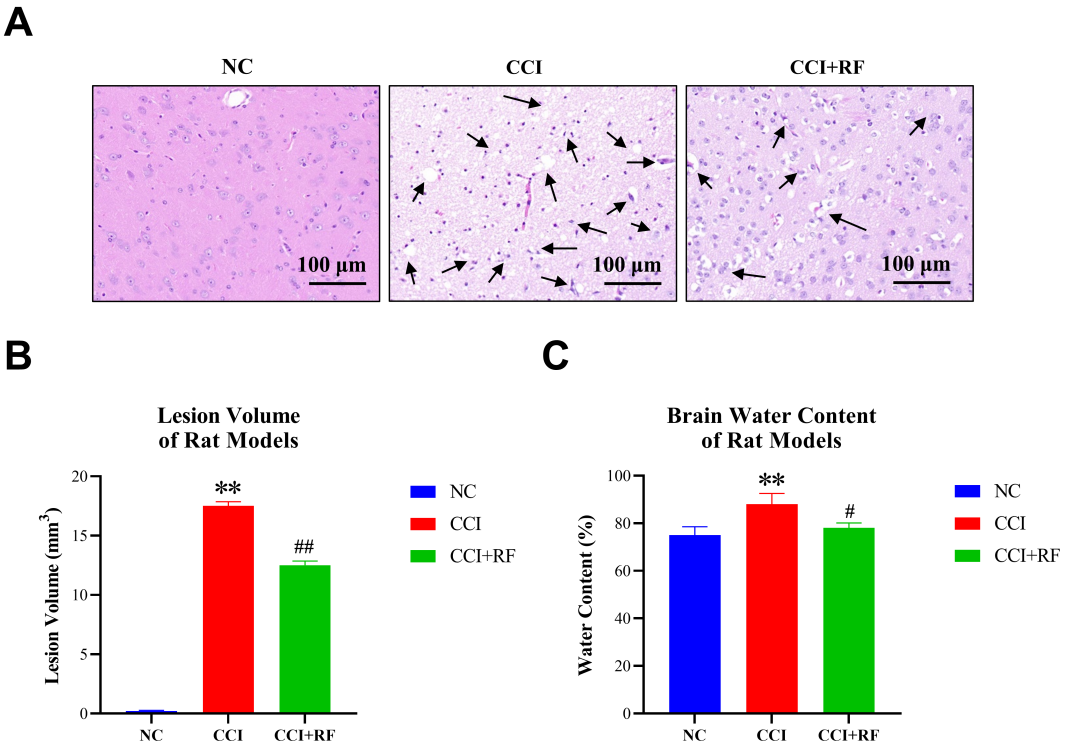

Fig. 2. RF reduced brain lesion volume and brain water content in rats after TBI. (A) The representative images of neurons in brain sections were observed by the hematoxylin and eosin (H&E) staining (arrows indicated the damaged neurons; 40× magnification). (B) The quantification of brain lesion volume (n = 5). (C) The quantification of brain water content (n = 5). $^{**}p < 0.01$ compared with the NC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with CCI group.

the recovery of neurological function after the TBI in the model. The results of the adhesive removal test are shown in Fig. 1B,D; the rats in the CCI group spent significantly increased time on removal ($p < 0.01$) and got significantly lower wire grip scores ($p < 0.01$) compared with rats in the NC group, demonstrating a significant behavioral function deficit. On the other hand, compared with rats in CCI, rats in CCI+RF performed better on both adhesive removal and wire grip at individual time points indicated after CCI ($p < 0.05$). In Fig. 1C, rats in the CCI group performed significantly decreased right turns ($p < 0.01$) due to the injury on the left brain compared with rats in the NC group. At the same time, the RF treatment increased the number of right turns ($p < 0.01$), indicating the ameliorated injury on the brain and the improved neurological recovery. All these results indicated that RF improved the recovery of sensorimotor function in rats after TBI.

The results of Morris water maze tests shown in Fig. 1E,F demonstrated that after the successive trials, the efficiency of reaching and finding the hidden platform was improved for all rats. However, CCI rats demonstrated severe reference and working memory deficits compared with NC rats. They presented more time ($p < 0.01$) needed for finding and reaching the hidden platform. The severity of cognitive deficits was significantly lowered ($p < 0.05$) by the treatment of RF, presenting less time needed for finding and reaching the hidden platform ($p < 0.05$) compared with rats in the CCI group. These findings indicated the effect of RF on improving the recovery of cognitive ability.

RF Reduced Brain Lesion Volume and Brain Water Content in Rats after TBI

Fig. 2A presents the representative images showing neurons in brain sections. It could be found that neurons in the NC group were intact and with normal morphology, and the nucleoli were observed. As indicated by the arrows in Fig. 2A, neurons in the CCI group were incomplete and had irregular cell contours. There were also blebbing membranes, and the nucleus was concentrated. RF alleviated all these symptoms of brain lesions in the CCI+RF group, in which most cells remained regular and complete despite irregular cell contours and membrane blebbing. Moreover, the quantification of the H&E staining (Fig. 2B) demonstrated that the lesion volume was significantly increased after CCI treatment compared with the NC group ($p < 0.01$). In contrast, the increase in lesion volume was reversed through the treatment of RF ($p < 0.01$). Moreover, the brain water content (Fig. 2C) was significantly increased after CCI treatment compared with the NC group ($p < 0.01$), and the increase of brain water content was reversed through the treatment of RF ($p < 0.05$).

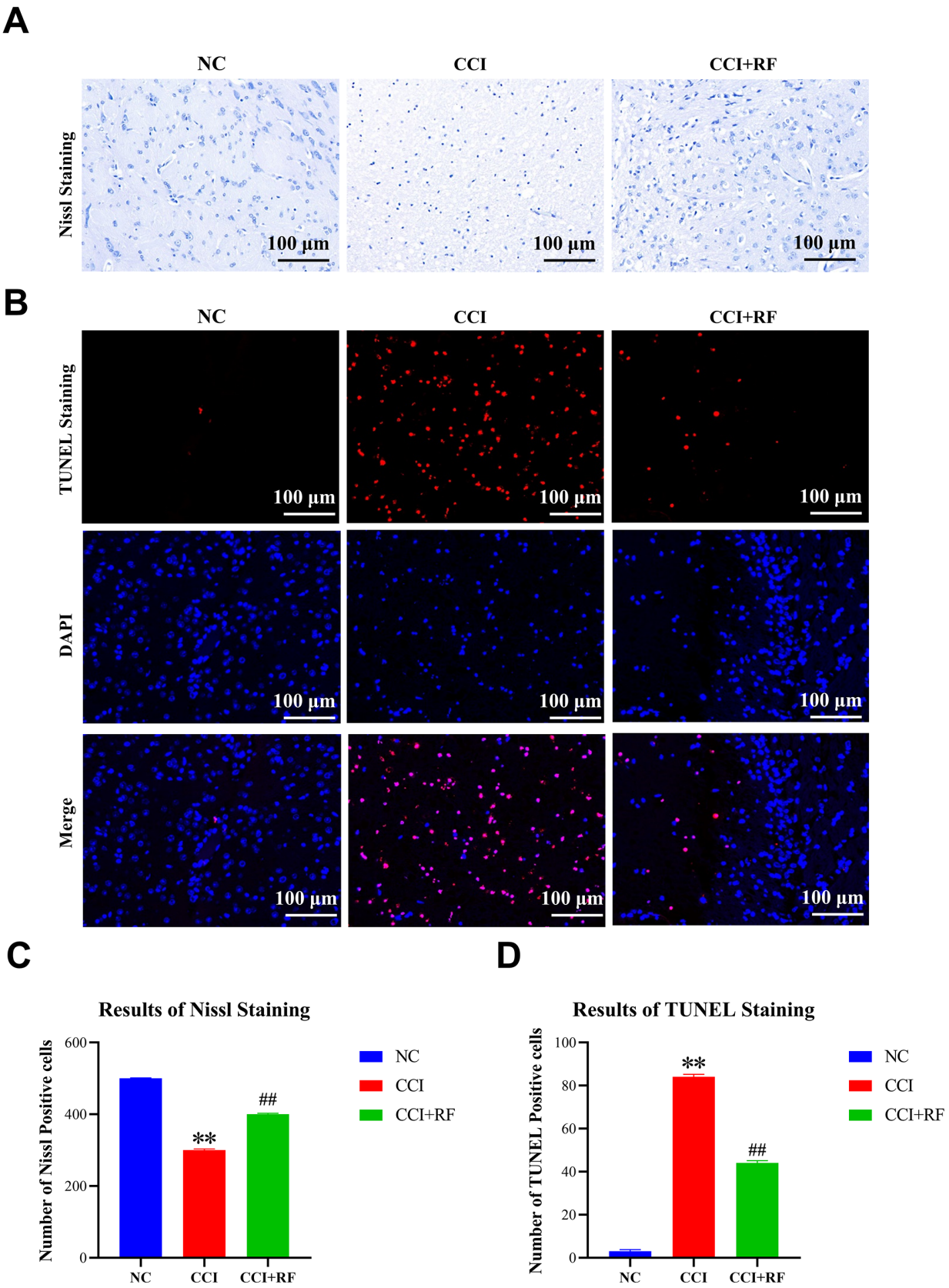

Fig. 3. RF alleviates apoptotic neural death in rats after TBI. Representative micrographs of (A) Nissl staining and (B) terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining. Scale bar = 100 μm for Nissl staining. Scale bar = 50 μm for TUNEL staining. (C) The quantification of Nissl-positive neurons (n = 5). (D) The quantification of TUNEL-positive neurons (n = 5). $^{**}p < 0.01$ compared with the NC group. $^{\#\#}p < 0.01$ compared with the CCI group.

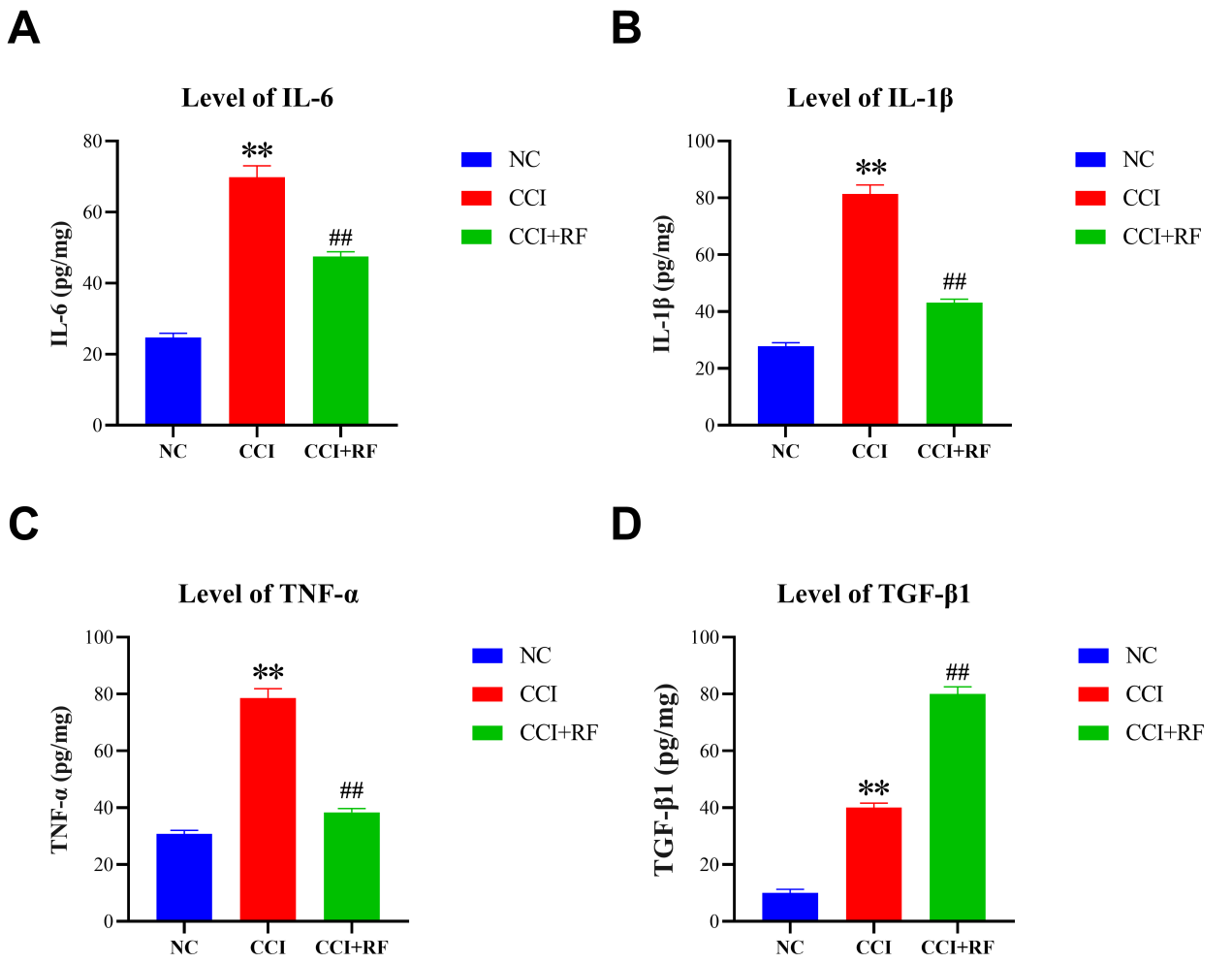

Fig. 4. RF ameliorates neuroinflammation in rats after TBI. The levels of (A) Interleukin (IL)-6, (B) IL-1β, (C) Tumor necrosis factor (TNF)-α, and (D) Transforming growth factor (TGF)-β1 in brain tissues obtained by enzyme-linked immunosorbent assay (ELISA). N = 5. **$p < 0.01$ compared with the NC group. $^{\#\#}p < 0.01$ compared with the CCI group.

RF Alleviates Apoptotic Neural Death in Rats after TBI

The Nissl staining was used to evaluate the number of viable cortical neurons. As shown by the histological analysis (Fig. 3A) and the quantification (Fig. 3C), markedly fewer Nissl-positive neurons, which represented the viable neurons, were observed in the CCI group compared with the NC group ($p < 0.01$). The treatment of RF after CCI improved the number of Nissl-positive neurons compared with the CCI group ($p < 0.01$). Moreover, the neuronal apoptosis was measured by TUNEL staining in which the TUNEL-positive neurons represented the apoptotic death of neurons. As shown in Fig. 3B,D, the CCI treatment significantly improved the apoptotic death of neurons compared with the NC group ($p < 0.05$). At the same time, the RF supplementation reversed the increase of apoptosis in the CCI group and led to fewer neuron apoptosis than in the CCI group ($p < 0.01$).

RF Ameliorates Neuroinflammation in Rats after TBI

As shown in Fig. 4A–C, the levels of IL-6, IL-1β, and TNF-α, these three proinflammatory cytokines, were increased significantly ($p < 0.01$) by CCI treatment ($p < 0.01$), and the increased expressions were reversed via RF treatment ($p < 0.01$). At the same time, CCI caused an increased TGF-β1 expression (Fig. 4D), which was further increased by the treatment of RF and with significance ($p < 0.01$).

RF Ameliorates Oxidative Stress Damage in Rats after TBI

The levels of oxidative stress induced by neuronal damage were measured. As shown in Fig. 5A, the activity of SOD was decreased after CCI treatment ($p < 0.01$), while this decrease was reversed by the supplementation of RF ($p < 0.01$). Furthermore, the content of MDA (Fig. 5B) was significantly increased after the CCI treatment ($p < 0.01$), while this increase was reversed by the RF treatment ($p < 0.01$).

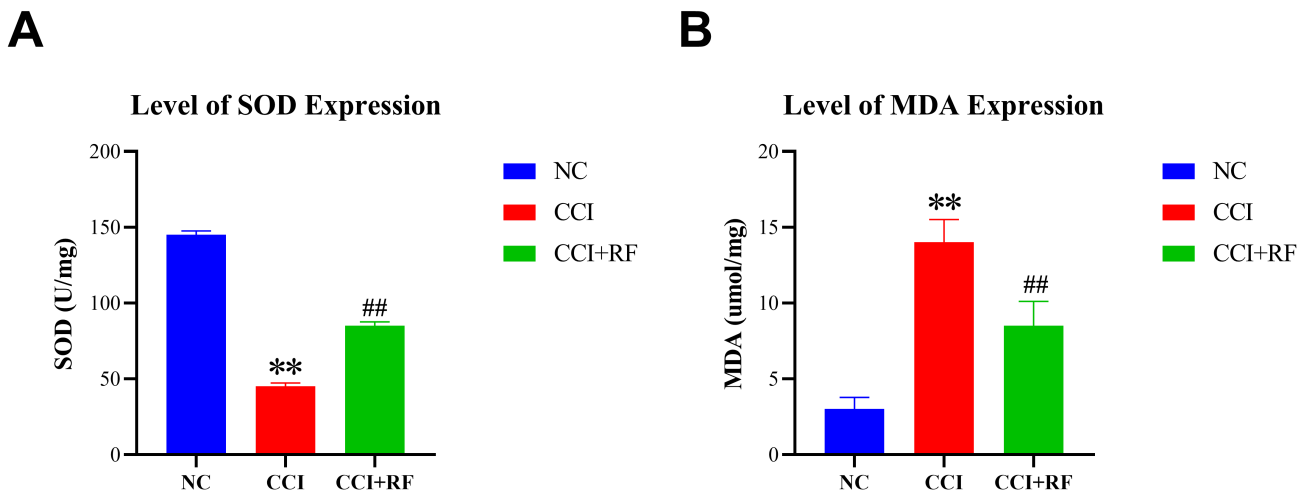

Fig. 5. RF ameliorates oxidative stress damage in rats after TBI. (A) The results of superoxide dismutase (SOD) activity (n = 5). (B) The results of malondialdehyde (MDA) content (n = 5). $^{**}p < 0.01$ compared with the NC group. $^{\#\#}p < 0.01$ compared with the CCI group.

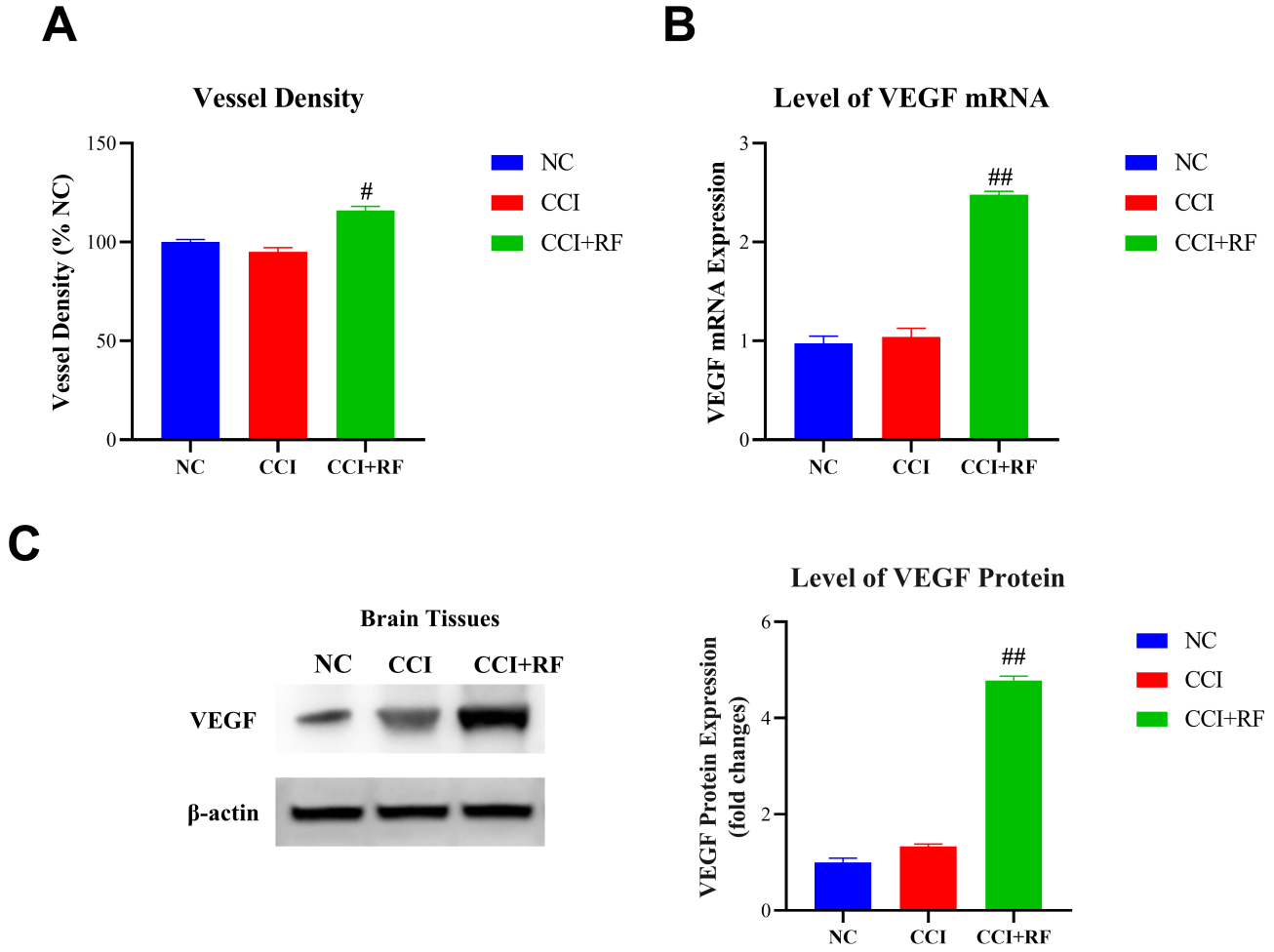

Fig. 6. RF increases brain vessel density in rats after TBI. (A) The quantification of vessel density (n = 5). (B) The level of vascular endothelial growth factor (*VEGF*) mRNA expression obtained by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) (n = 5). (C) Representative images of VEGF western blot and the quantification of levels of VEGF protein (n = 5). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with CCI group.

RF Increases Brain Vessel Density in Rats after TBI

Angiogenesis, the long-lasting process of recovering functions after TBI, was measured in the present study. According to the endothelial biomarker of CD31 and corresponding quantification (Fig. 6A), there were no significant changes in vessel density between rats with or without CCI treatment. In contrast, the brain vessel density was significantly increased after rats were treated with RF ($p < 0.05$), exhibiting the effect of RF on promoting vascular remodeling. In addition, as shown in Fig. 6B,C, the levels of *VEGF* mRNA and VEGF protein were significantly increased by the RF supplementation ($p < 0.01$), indicating that the RF promoted vascular remodeling through, at least partially, up-regulating the expression of *VEGF*.

Discussion

In this study, we verified our hypothesis that RF possessed a promotive effect on neural rehabilitation after the TBI in rats. We established the rat models of TBI by performing CCI on rats and found that, for rats with TBI, RF could promote sensorimotor function and cognitive ability recovery, reduce brain lesions and neuronal apoptosis, ameliorate neuroinflammation and oxidative stress, and promote angiogenesis. These results suggest that RF may play significant roles in neurological rehabilitation after TBI and could act as the therapeutic target for treating TBI in rats, providing more understanding about RF in TBI treatment.

In this study, it has been shown that the administration of RF could significantly improve the recovery of the sensorimotor function and cognitive ability of rats after brain TBI. Rats with RF could recover more from behavioral deficits and were more facilitated on the test of reference and working memory. Moreover, the mNSS is the multifunctional scale of evaluation used for determining functions of neurology [27], and in this study demonstrated the beneficial effects of RF on neuronal rehabilitation. Additionally, RF reduced the lesion volume and the water content in the brains of TBI rat models. Since approximately 80% of cases of TBI suffer from cognitive, motor, and somatosensory symptoms such as dizziness, headache, and attention loss [28,29], these findings suggest that RF could alleviate symptoms and brain damage induced by TBI and facilitate the recovery from TBI structurally and functionally.

It has been proved that brain injury and remodeling after TBI were associated with the inflammatory cells and response in the brain and peripheral organs [30,31]. Thus, proinflammatory and anti-inflammatory cytokines that trigger the inflammatory response are associated with the pathogenesis of brain injury. The TGF-β1 has been reported as an anti-inflammatory cytokine and neuroprotective [26]. While the IL-6, IL-1β, and TNF-α, which are proinflammatory cytokines and could be detected in brain and cerebrospinal fluid, would trigger an inflammatory response and further exacerbate brain injury, have been proven to be related to the increased brain lesion volume and neuronal damage [32,33]. In this study, we found that the RF treatment after brain injury could decrease the level of IL-6, IL-1β, and TNF-α and increase the level of TGF-β1, suggesting that RF's effect on ameliorating inflammatory response in the brain after TBI. Furthermore, oxidative stress was treated as the most relevant factor among all the factors related to brain injury due to the damage to tissue structure and function caused by the excessive radicals that are produced by unbalanced oxidation and antioxidation [34]. Moreover, it has been reported that the oxidative stress induced by brain injury and the mitochondrial dysfunction induced by neuronal damage would eventually result in the cascade of apoptosis [35,36]. The previous study has claimed that suppressing oxidative stress and apoptosis during brain injury would lead to neuroprotective effects and effective treatment for brain injury [35]. In the present study, we measured the MDA content and SOD activity, which were two enzymes often used for evaluating the level of oxidative stress and the degree of oxidative damage [37]. In this study, the increased SOD activity and the decreased MDA content induced by the RF treatment in the injured brain indicated that the RF could reduce the oxidative stress in the brain after TBI. Correspondingly with the reduced oxidative stress by RF, the apoptosis of neurons in the injured brain was also lowered after RF treatment. These findings of RF's effects on ameliorating inflammation, oxidative stress, and neural apoptosis in the brain after TBI were consistent with the previous report in which RF was claimed to have preventive effects on migraines through reducing neuroinflammation, oxidative stress, and mitochondrial dysfunction [38].

A study has proved that the angiogenesis and the following neurogenesis play essential roles in regulating the functional recovery after TBI [39], and agents that are neuroprotective and improve functional recovery after TBI would increase the angiogenesis and neurogenesis [40]. A review summaries the relationship between angiogenesis and the recovery of neurological function after TBI and the critical neurovascular niches provided by brain angiogenesis for neuronal remodeling [41]. In our study, RF was proven to improve the angiogenesis in the brains of TBI rat models, suggesting the effect of RF on promoting recovery from TBI.

Conclusion

RF treatment significantly improves the recovery of neurological functions by inhibiting the brain inflammatory reactions, reducing neuronal apoptosis, brain oxidative stress, and lesion volume, and enhancing brain vascular remodeling. These findings provide a novel mechanism for RF treating pediatric TBI.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

QC and YK performed the research and provided help and advice on the experiments. BZ contributed to the analysis and interpretation of the data. YK wrote the manuscript. All authors contributed to significant editorial changes in the manuscript. All authors read and approved the final manuscript. 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.

Ethics Approval and Consent to Participate

Animal experiments were complied with the guidelines of the Tianjin Medical Experimental Animal Care, and animal protocols were approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Co., Ltd. (protocol number YSY-DWLL-2023305).

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Conflict of Interest

The authors declare no conflict of interest.

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