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Original Article

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Cannabidiol alleviates the inflammatory response in rats with traumatic brain injury through the PGE_2-EP2-cAMP-PKA signaling pathway

Yan Cao[1,†], Hengxi Li[1,†], Jiali Li[1,†], Tenghan Ling[1,†], Aiping Yin[1], Xinyuan Luo[1], Ying Zhou[2], Jinghui Li[3], Hongyan Jiang[1], Huawei Wang[4], Li Yang[5], Haiying Wu[5,*], and Ping Li[1,*]

[1]Department of Anatomy and Histology/Embryology, Faculty of Basic Medical Sciences, Kunming Medical University, Kunming 650500, China, [2]Department of Electron Microscope Laboratory, Kunming Medical University, Kunming 650500, China, [3]Second Department of Neurosurgery, the First Affiliated Hospital of Kunming Medical University, Kunming 650032, China, [4]Department of Reproduction and Genetics, the First Affiliated Hospital of Kunming Medical University, Kunming 650032, China, and [5]Department of Emergency and Intensive Care Unit, the First Affiliated Hospital of Kunming Medical University, Kunming 650032, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-13608881671; E-mail: liping@kmmu.edu.cn (P.L.) / E-mail: why0209@hotmail.com (H.W.)

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Abstract

Traumatic brain injury (TBI) is a recognized global public health problem. However, there are still limitations in the available therapeutic approaches and a lack of clinically effective drugs. Therefore, an in-depth exploration of the secondary pathological mechanism of TBI and the identification of new effective drugs are urgently needed. Cannabidiol (CBD), a component derived from the cannabis plant, has potential therapeutic effects on neurological diseases and has received increasing attention. However, few reports on CBD intervention in TBI patients exist. Here, we use the Feeney free-fall method to establish a rat TBI model. CBD significantly improves neurological deficit scores, neuronal damage and blood-brain barrier permeability in rats and significantly inhibits the expressions of the brain injury markers S-100β and NSE. Mechanistically, CBD attenuates TBI-induced astrocyte activation, reduces inflammation, and attenuates the expressions of inflammatory prostaglandin system indicators. The use of TG6-10-1 (EP2 inhibitor) and H-89 (PKA inhibitor) indicates that CBD attenuates TBI-induced neurological damage via the PGE_2-EP2-cAMP-PKA signaling pathway. Overall, this research provides a novel drug candidate for the treatment of clinical brain trauma.

Key words cannabidiol, traumatic brain injury, astrocytes, neuroprotection, prostaglandin system

Introduction

Traumatic brain injury (TBI) caused by external mechanical force severely impacts brain function and instigates pathological alterations in neural tissue [1]. Classified as a central nervous system disorder, TBI is characterized by high incidence, disability, and mortality rates, with an estimated 69 million new cases reported globally each year [2], and is a common cause of death among adult males [3].

The pathophysiology of TBI includes the primary destruction of brain tissues and secondary cascading reactions. As primary brain injury stemming from direct mechanical force is typically irreversible, current therapeutic interventions primarily aim to mitigate the severity of subsequent secondary brain injuries. The neuroinflammatory response, a crucial element exacerbating secondary injury, involves the activation of microglia and astrocytes, the release of inflammatory factors, and the infiltration of peripheral immune cells into the brain parenchyma via a compromised blood-brain barrier (BBB) [4]. Astrocytes constitute approximately 30%–40% of the cells in the central nervous system and are integral to the structural and functional integrity of the BBB. These cells also engage in extensive interactions with other central nervous system cells, including neurons. After brain injury, astrocytes become overactivated, leading to the release of toxins that mediate brain edema and inflammatory reactions, further exacerbating brain

injury [5]. Consequently, targeting the suppression of excessive inflammation may be a pivotal strategy for alleviating astrocyte hyperactivation.

Prostaglandin E_2 (PGE_2) is a common inflammatory mediator synthesized from arachidonic acid (AA) through a series of enzymatic reactions. The enzyme cyclooxygenase (COX) catalyzes the conversion of AA to prostaglandin H_2 (PGH_2), a relatively unstable intermediate subsequently converted into PGE_2 by prostaglandin E synthase (PGES). Two isoforms of COX exist, including constitutive COX-1 and inducible COX-2. While COX-1 is ubiquitously expressed in many tissues and is primarily responsible for prostaglandin synthesis, COX-2 participates in the inflammatory response in various tissues and organs and is mainly induced by inflammatory stimuli [6]. PGES, another key enzyme in this enzymatic cascade, exists in three isozymic forms, including membrane-associated prostaglandin E synthase-1 (mPGES-1), mPGES-2, and cytosolic PGES (cPGES). Among these, mPGES-2 and cPGES are constitutive enzymes, whereas mPGES-1 acts primarily as an inducible isomerase stimulated by proinflammatory factors. mPGES-1-mediated synthesis of PGE_2 plays an important role in the posttraumatic inflammatory cascade and secondary injury and is implicated in the pathophysiological processes of many diseases [7,8]. PGE_2 exerts its effects by binding to four E-prostanoid (EP) receptors (EP1–4) on the cell membrane surface. EP2 receptors are abundantly expressed in cerebral regions, including the cortex, striatum, and hippocampus, and are closely associated with neuroinflammatory and neurodegenerative diseases [9]. The binding of PGE_2 to EP2 receptors leads to increased adenylate cyclase (AC) expression, which, in turn, promotes cytoplasmic cyclic adenosine monophosphate (cAMP) production and a series of downstream responses through the activation of effector protein kinase A (PKA).

Cannabidiol (CBD) is a nonpsychoactive phytocannabinoid that possesses a range of therapeutic properties, including analgesic, anti-inflammatory, antioxidant, antianxiety, and antitumor effects, as well as potential neuroprotective capabilities in neurological disorders such as Parkinson's disease, epilepsy, and Alzheimer's disease [10,11]. We previously demonstrated the therapeutic potential of CBD in TBI, which was effective in reducing brain edema, enhancing tight junction proteins, improving BBB permeability, and improving neurological function [12]. Despite these findings, the specific anti-inflammatory mechanisms of CBD in the context of TBI-induced secondary brain injury remain elusive. In addition, researchers have yet to determine whether CBD modulates neuroinflammation and attenuates the hyperactivation of astrocytes by interfering with the prostaglandin system, thereby conferring protection against secondary brain injury.

In the present study, we assessed the potential of CBD for reducing the inflammatory response and preserving the integrity of the BBB in the early stages of injury in a rat model of TBI.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (260–300 g) were obtained from the Animal Experimental Center of Kunming Medical University (Kunming, China). The rats were housed in a room under a stable temperature and a 12-h light/dark cycle and were provided with a standard diet and water *ad libitum*. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and the study design was approved by the Animal Ethics Committee of Kunming Medical University (Approval No. KMMU2020054).

TBI model

The TBI rat model was established as described previously [12]. In brief, the rats were anesthetized with pentobarbital sodium (40 mg/kg) and fixed in a stereotaxic frame (RWD Life Science, Shenzhen, China). After the surgical area was disinfected, a midline incision was made on the head to expose the skull. A 6-mm diameter bone window was created in the right parietal bone (2 mm posterior to the coronal suture, 1.5 mm lateral to the sagittal suture) with a dental drill. Cortical contusion was induced by freely falling 40 g of weight from a height of 15 cm and striking a cylinder placed above the dura mater (Figure 1A–C). After surgery, the bone window was closed with medical bone wax, and the scalp was sutured. Sham-operated rats underwent the same surgical procedure but without cortical strike injury.

Experimental design

To assess brain injury at different time points following TBI, the rats were randomly divided into seven groups: Sham, TBI 8h, TBI 1d, TBI 2d, TBI 3d, TBI 5d, and TBI 7d.

To investigate the impact of CBD on the acute inflammatory response following TBI, the rats were randomly divided into three groups: Sham-operated (Sham), TBI + vehicle (TBI), and TBI + CBD (CBD) groups. Changes in prostaglandin-related protein expression, astrocyte activation, neurological deficits, and BBB integrity were assessed.

To elucidate the specific mechanism underlying the regulatory effects of CBD on inflammation following TBI, the rats were randomly divided into five groups: Sham-operated (Sham), TBI + vehicle (TBI), TBI + CBD (CBD), TBI + CBD + EP2 inhibitor (TG6-10-1), and TBI + CBD + PKA inhibitor (H-89) groups. Changes in prostaglandin-related protein expression and astrocyte activation were detected. The detailed experimental design is shown in Figure 1D.

In accordance with our previous report [12], CBD (10 mg/kg; Hansu Biotechnology, Yunnan, China) was injected intraperitoneally 30 min before the TBI operation and then at 6 h and 24 h after the operation. EP2 inhibitors (TG6-10-1, 5 mg/kg; MedChemExpress, Monmouth Junction, USA) and PKA inhibitors (H-89, 5 mg/kg; MedChemExpress) were injected intraperitoneally 30 min before each CBD injection [13,14].

Modified neurological severity score (mNSS)

Neurological function was assessed via the mNSS [15]. The assessments included motor test (6 points), sensory test (2 points), beam balance test (6 points), and reflex absence and abnormal movement (4 points) (Table 1), with 0 indicating a normal score and 18 indicating the maximum deficit score. The higher the total score is, the more severe the neurological dysfunction. All behavioral tests were evaluated by investigators who were blinded to the groupings.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were drawn via cardiac puncture after the rats were anesthetized. The blood was left at room temperature for 1 h and then centrifuged at 4 °C and 1000 *g* for 10 min. The resulting supernatant was collected and stored at −80 °C for further analysis.

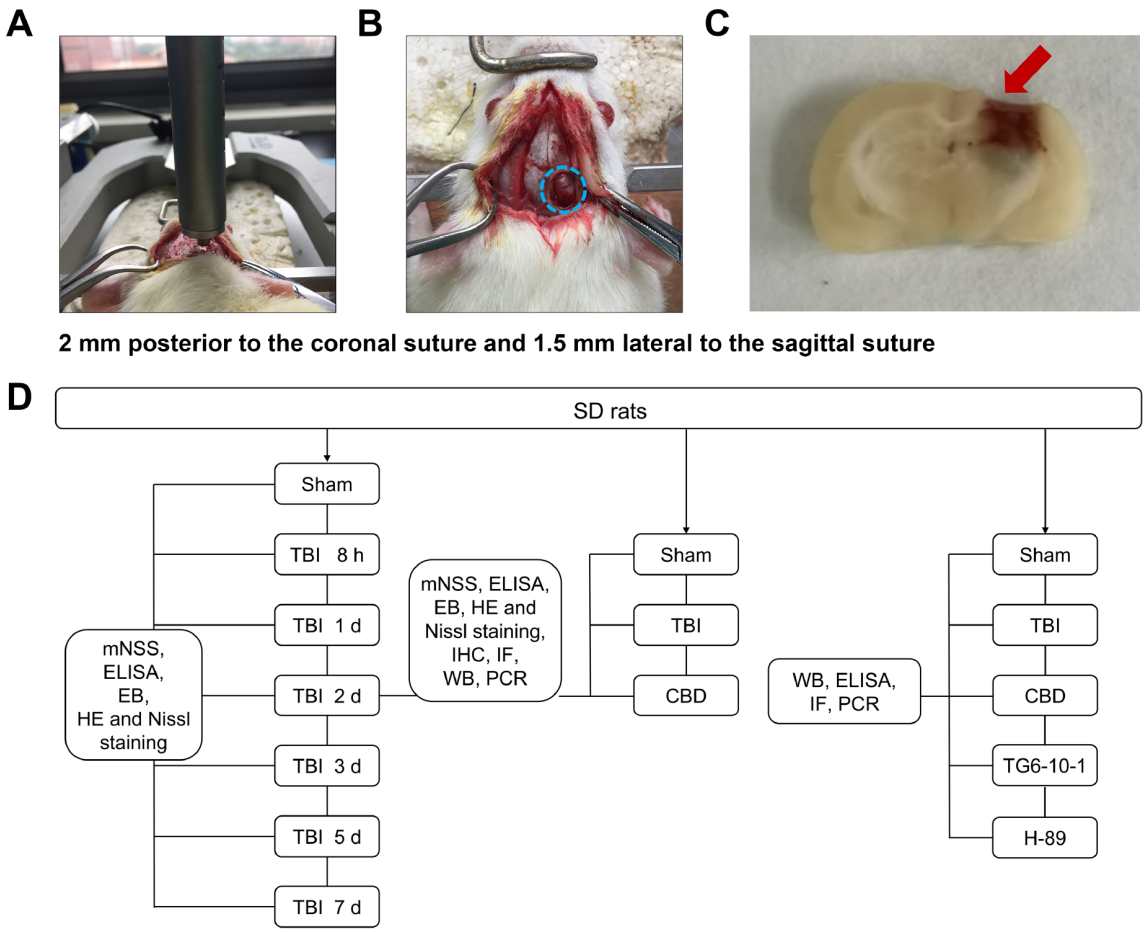

Figure 1. Schematic of the TBI rat model and experimental design (A) Based on the weight-drop method, 40 g of free-falling weight was dropped through the guiding tube to strike an exposed brain area in each rat. (B) Blood clots and tissue damage were observed in and around the injured brain region after traumatic brain injury (TBI). (C) Schematic of coronal sections of rat brain tissue showing the impact of weight loss on exposed tissue. (D) Experimental design.

ELISA kits of S-100β (Jianglaibio, Shanghai, China), neuron-specific enolase (NSE; Jianglaibio), PGE_2 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and cAMP (Nanjing Jiancheng Bioengineering Institute) were used to measure the levels of S-100β, NSE, PGE_2, and cAMP in the serum following the manufacturer's instructions.

BBB permeability assay

BBB permeability was determined by Evans blue (EB) dye extravasation. After the rats were anesthetized, 2% EB solution (4 mL/kg; Jinming Biotechnology, Beijing, China) was injected into the femoral vein and allowed to circulate for 2 h *in vivo*. The hearts were then perfused with saline until the fluid was colorless. The injured brain tissues were collected and placed in formamide (100 mg/mL; Mei5 Biotechnology, Beijing, China) for 48 h of incubation at 37°C. After centrifugation at 12,000 *g* for 20 min at 4°C, the supernatant was collected, and the absorbance was determined at 620 nm. The EB content was calculated from the standard curve.

Tissue preparation

The rats were anesthetized with pentobarbital sodium (40 mg/kg), and brain tissue samples were collected. For biochemical analyses, the rats were perfused transcardially with precooled saline, and the brain tissue around the cortical contusions was collected on ice. The obtained tissue samples were rapidly frozen and stored at −80 °C until further use. For histological analysis, the rats were perfused transcardially with precooled saline followed by 4% paraformaldehyde solution for 10 min, and the brain tissue was removed. The samples were fixed in 4% paraformaldehyde solution for 2 d, dehydrated in gradient ethanol, cleared in xylene, embedded in paraffin, and cut into 5-μm-thick coronal sections.

Hematoxylin-eosin (H&E) and Nissl staining

To evaluate neuronal damage, H&E staining (Solarbio, Beijing, China) and Nissl staining (Beyotime, Shanghai, China) were performed according to the manufacturer's instructions. The stained sections were observed with a light microscope (Leica, Wetzlar, Germany).

Immunofluorescence staining

The paraffin-embedded sections were dewaxed with xylene, dehydrated with gradient ethanol, subjected to antigen retrieval, and then subjected to high-temperature and high-pressure methods.

Table 1. Modified neurological severity score (mNSS) tests and scoring values

Test contents	Points
Motor test (normal = 0; maximum = 6)	
Flexion of forelimb after raising rat by the tail	1
Flexion of hindlimb after raising rat by the tail	1
Head moved > 10° to vertical axis within 30 s after raising rat by the tail	1
Normal walk placing rat on the floor	0
Inability to walk straight placing rat on the floor	1
Circling toward paretic side placing rat on the floor	2
Falls down to paretic side placing rat on the floor	3
Sensory test (normal = 0; maximum = 2)	
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance test (normal = 0; maximum = 6)	
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (> 60 s)	3
Attempts to balance on beam but falls off (> 40 s)	4
Attempts to balance on beam but falls off (> 20 s)	5
Falls off; no attempt to balance or hang on to beam (< 20 s)	6
Reflex absence and abnormal movement (normal = 0; maximum = 4)	
Pinna reflex (head shake when auditory meatus is touched with cotton)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a lipboard paper)	1
Seizures, myoclonus, myodystony	1
Total: normal = 0; maximum = 18	

The samples were blocked with 10% normal goat serum for 1 h and incubated overnight at 4°C with the following primary antibodies: rabbit anti-COX-1 (ab109025, 1:200; Abcam, Cambridge, UK) and rabbit anti-COX-2 (ab15191, 1:200; Abcam). The next day, the sections were incubated with a Cy3-labeled fluorescent secondary antibody (C2306, 1:200; Sigma, St Louis, USA) for 1 h at room temperature. Additionally, a mouse monoclonal CoraLite®488-conjugated GFAP antibody (CL488-60190, 1:200; Proteintech, Wuhan, China) was used to label the astrocytes, and a mouse monoclonal CoraLite®488-conjugated NeuN antibody (CL488-26975, 1:200; Proteintech) was used to label the neurons. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (F6057; Sigma). Images were observed and acquired via a fluorescence inverted microscope (Zeiss, Oberkochen, Germany).

Western blot analysis

Injured ipsilateral cerebral cortex tissue was collected and homogenized in RIPA buffer (Beyotime) to extract total protein. Protein concentrations were determined via a bicinchoninic acid (BCA) protein assay kit (Beyotime). Total protein (20 μg) was separated via 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA). After being blocked with 5% nonfat milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-GFAP (ab33922, 1:5000; Abcam), rabbit anti-COX-1 (ab109025, 1:5000; Abcam), rabbit anti-COX-2 (ab15191, 1:5000; Abcam), rabbit anti-mPGES-1 (160140, 1:2000; Cayman, Ann Arbor, USA), rabbit anti-EP2 (101750, 1:2000; Cayman), and rabbit anti-β-actin (20536-1-AP, 1:5000; Proteintech). After incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (SA00001-2, 1:5000; Proteintech) for 1 h at room temperature, the protein bands were visualized via enhanced chemiluminescence (ECL; Biosharp, Beijing, China). Subsequent detection and quantification were carried out with a gel imaging system (Bio-Rad, Hercules, USA) and ImageJ software, respectively.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from injured ipsilateral cortical tissue via an RNAiso Plus kit (Takara, Kyoto, Japan). cDNA was synthetized via a PrimeScript™ RT Reagent Kit (Takara). RT-PCR analysis was performed via the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, USA) with TB Green Premix Ex Taq (Takara) and corresponding primers. The amplification conditions were as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. The data were normalized and calculated via the $2^{-\Delta\Delta Ct}$ method. The primers were synthesized by Tsingke Biotechnology (Shanghai, China), and their sequences are shown in Table 2.

Statistical analysis

All the data were analyzed with GraphPad Prism v6 (GraphPad

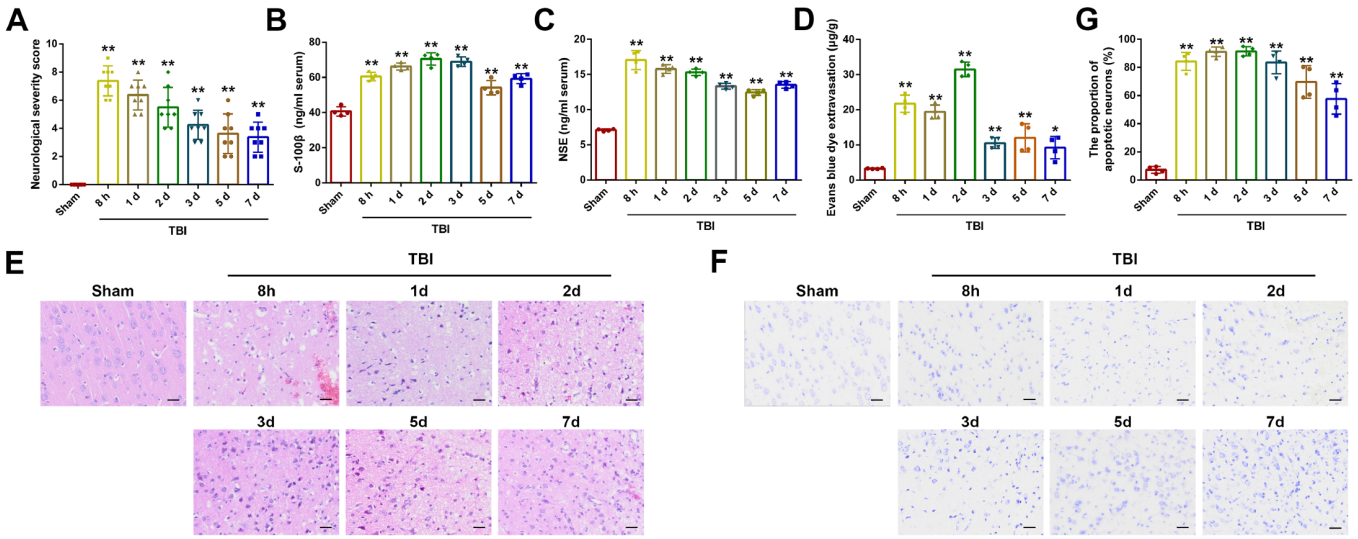

Figure 2. Brain injury at different time points after TBI (A) Neurological function was assessed via the modified neurological severity score (mNSS). (B) Serum level of S-100β. (C) Serum level of neuron-specific enolase (NSE). (D) EB dye extravasation. (E) H&E staining. (F) Nissl staining. Scale bar: 25 μm. (G) Quantitative analysis of Nissl staining. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Sham.

Table 2. Sequences of the primers used for RT-qPCR

Gene	Primer sequence (5′→3′)
GFAP	F: TAAGCTAGCCCTGGACATCG
	R: TACAGGAATGGTGATGCGGT
COX-1	F: ATTGCTGCTGAGAAGGGAGT
	R: CTGGTGGGTGAAGTGTTGTG
COX-2	F: CCAACCCATGTCAAAACCGT
	R: TCACCGTAGAATCCAGTCCG
mPGES-1	F: CAAGTCAGGCTGCGGAAGAA
	R: TGAGGACCACGAGGAAATGTA
EP2	F: ACTAATGCGCTCAGTCCTCT
	R: TGGGTCGAACAGGAAGCTC
IL-1β	F: ATCCCAAACAATACCCAAAGAAG
	R: GAACTGTGCAGACTCAAACTCCA
TNF-α	F: CCACCACGCTCTTCTGTCTA
	R: TGGAACTGATGAGAGGGAGC
iNOS	F: TCTTGGAGCGAGTTGTGGAT
	R: AGCCTCTTGTCTTTGACCCA
GAPDH	F: GACATGCCGCCTGGAGAAAC
	R: AGCCCAGGATGCCCTTTAGT

Software, La Jolla, USA). Data are expressed as the mean ± standard deviation (SD). Data were compared via unpaired *t*-test for two groups and one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple groups. Differences were considered statistically significant at $P < 0.05$.

Results

Changes in the time course of brain tissue damage post-TBI

Assessment of brain injury was conducted at different time intervals (8 h, 1 d, 2 d, 3 d, 5 d, and 7 d) following TBI. As shown in Figure 2, the mNSS values, serum levels of S-100β and NSE, EB exudation in brain tissues, and the number of necrotic neurons were elevated in the rats following TBI. Specifically, the mNSS (Figure 2A) and NSE levels (Figure 2C) peaked at 8 h, whereas the S-100β level (Figure 2B), EB exudation (Figure 2D) and the number of necrotic neurons (Figure 2F,G) peaked at 2 d. After reaching peak levels, all indicators declined but remained high on day 7 relative to those of the sham-operated rats. In addition, H&E staining and Nissl staining revealed significant pathological changes in neurons around the damaged cortex after TBI, including disordered arrangement of nerve cells, loose interstitial edema, destruction of the neuronal structure, and massive dissolution and necrosis (Figure 2E–G). These data indicated that brain injury in rats led to neurofunctional impairment, BBB disruption, and cellular necrosis, maintaining a relatively high level of injury on the 2nd day post-injury. Accordingly, the 2 d post-TBI time point was selected for subsequent studies.

CBD alleviates neurological function deficits and brain injury in TBI rats

The neuroprotective effects of CBD *in vivo* were examined in a rat model of TBI. As shown in Figure 3A, CBD ameliorated neurological deficit scores in TBI rats. The serum markers commonly associated with brain injury, S-100β and NSE, were elevated in TBI rats but significantly decreased following CBD treatment (Figure 3B,C). EB dye extravasation, which is commonly used to assess BBB permeability [16], was markedly greater in the injured ipsilateral cerebral hemisphere of the TBI group than in the sham-operated control group (Figure 3D), and this increase was attenuated by CBD intervention. Histological alterations were examined via H&E staining and Nissl staining. As shown in Figure 3E,F, cortical cells in the Sham-operated rats displayed an orderly arrangement and normal morphology. In contrast, the TBI group exhibited disrupted cellular arrangement, nuclear pyknosis and fragmentation, cell necrosis and dissolution, and altered cytoplasmic vacuolization. Notably, this neuronal damage was ameliorated in the CBD group.

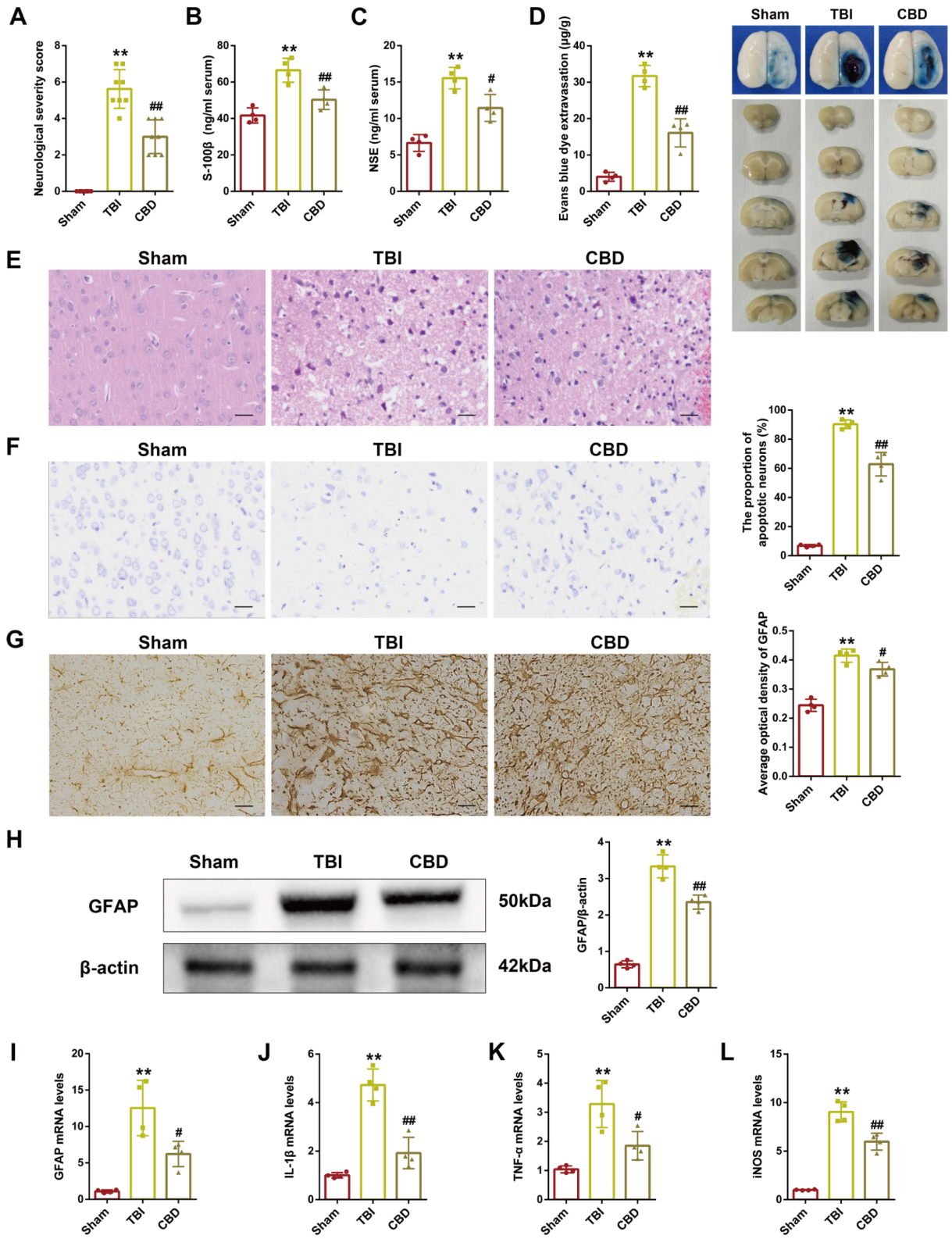

Figure 3. CBD improved brain injury in TBI-induced rats (A) Neurological function was assessed via the mNSS. (B,C) Serum levels of S-100β and NSE. (D) EB dye extravasation. (E) H&E staining. (F) Nissl staining. (G) Immunohistochemical staining of glial fibrillary acidic protein (GFAP) and average optical density (AOD) of GFAP-positive expression. Scale bar: 25 μm. (H) Representative western blot images of GFAP and relative protein levels of GFAP. (I–L) Relative mRNA levels of GFAP, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS). $^{**}P < 0.01$ vs Sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TBI.

These data indicated that CBD alleviated neurofunctional deficits and secondary injuries in TBI rats, exerting significant neuroprotective effects.

CBD alleviates TBI-induced astrocyte activation and inflammation

Glial fibrillary acidic protein (GFAP) was used as a marker for astrocyte analysis [17], and immunohistochemical staining was performed to analyze the morphology and average optical density (AOD) of the astrocytes in each group. As shown in Figure 3G, in the Sham-operated group, GFAP-labeled astrocytes were detected with small cytosomes and finely branched protrusions. Conversely, the TBI group presented elevated GFAP expression, astrocyte hyperplasia, enlarged cell bodies, and thickened protrusions, which are indicative of astrocyte activation. Compared with TBI, CBD treatment significantly reduced GFAP expression, suggesting attenuation of astrocyte activation. These findings were corroborated by western blot and RT-PCR analyses, revealing CBD-mediated inhibition of TBI-induced astrocyte expression (Figure 3H,I). In addition, CBD intervention also reduced the mRNA expressions of the inflammatory indicators IL-1β, TNF-α, and iNOS, thus alleviating inflammatory reactions (Figure 3J–L). These data indicated that the therapeutic effects of CBD may be associated with the attenuation of astrocyte activation and inflammatory responses.

COX-1 and COX-2 are not expressed in astrocytes

Astrocytes were labeled with GFAP via double immunofluorescence staining. Notably, our experiments revealed that COX-1 and COX-2 were not coexpressed with GFAP-labeled astrocytes, indicating that these enzymes were not present in these cells (Figure 4A,B). As shown in Figure 4C, quantification of the mean fluorescence intensity of GFAP confirmed that astrocytes were activated after TBI, an effect that was suppressed by CBD treatment. Similarly, the TBI group presented significantly greater levels of fluorescence labeling for both COX-1 and COX-2 than the Sham-operated control group did, and this increase was suppressed by CBD intervention

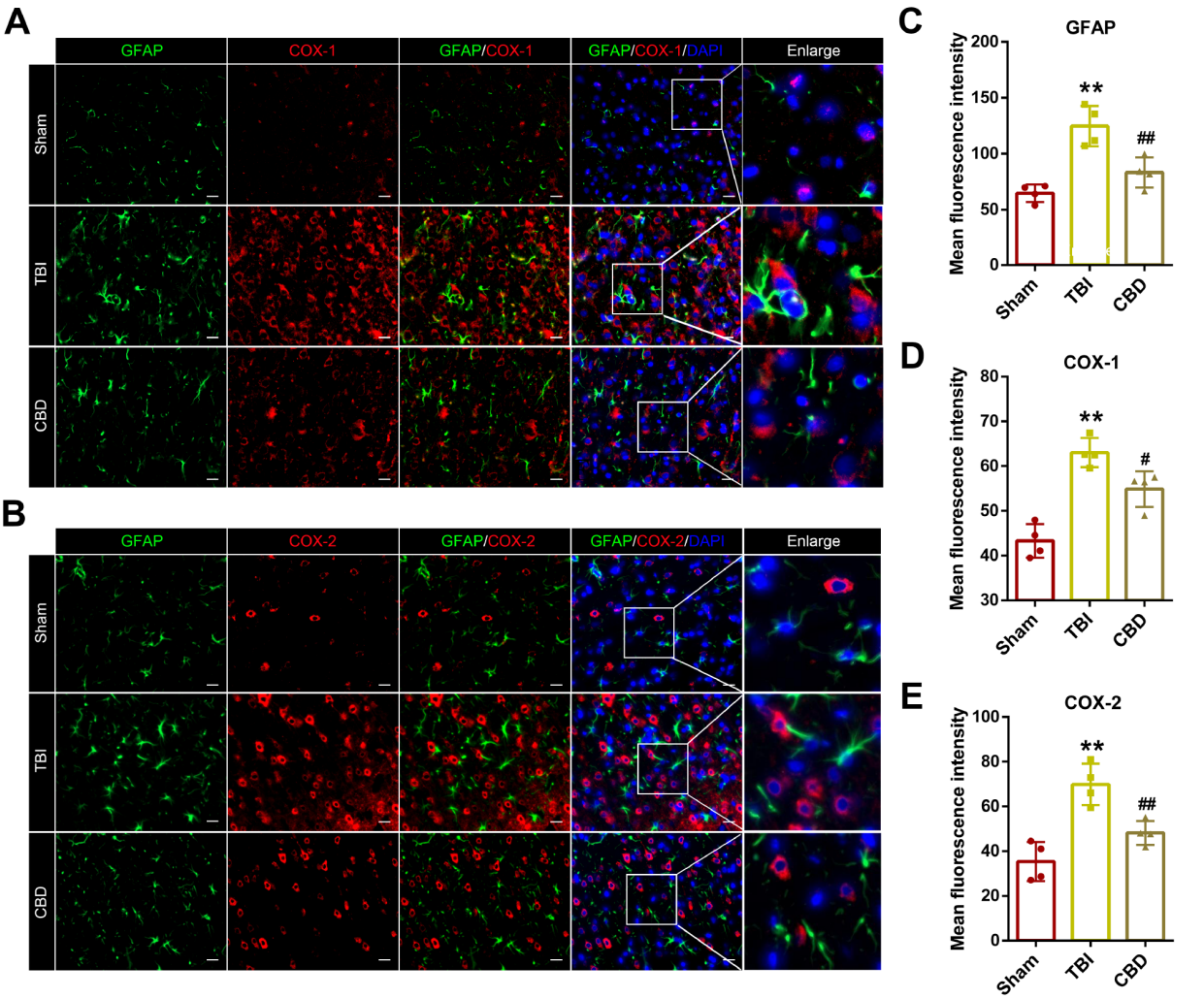

Figure 4. Double immunofluorescence staining of COX-1, COX-2, and GFAP in the injured ipsilateral cerebral cortex (A) Double immunofluorescence staining of GFAP (green)-positive astrocytes and cyclooxygenase-1 (COX-1) (red). (B) Double immunofluorescence staining of GFAP (green)-positive astrocytes and COX-2 (red)-positive astrocytes. Scale bar: 20 μm. (C) Mean fluorescence intensity of GFAP-positive cells. (D) Mean fluorescence intensity of COX-1-positive cells. (E) Mean fluorescence intensity of COX-2-positive cells. $^{**}P < 0.01$ vs Sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TBI.

(Figure 4D,E). These data indicated that CBD might indirectly inhibit astrocyte activation by decreasing COX-1 and COX-2 levels.

COX-1 and COX-2 are expressed in neurons

COX-1 and COX-2 were coexpressed with NeuN-labeled neurons, indicating that these enzymes were present in these cells (Figure 5A,B). Quantitative analysis of the density of colocalized cells revealed that, compared with the Sham-operated group, the TBI group presented an increased density of coincident cells, which was reduced following CBD intervention (Figure 5C,D). These data indicated that the protective effects of CBD may be correlated with the levels of COX-1 and COX-2 expressed in neurons.

CBD decreases COX-1, COX-2, mPGES-1, and EP2 expressions

As indicated in Figure 6A–E, western blot analysis revealed that the protein expression levels of COX-1, COX-2, mPGES-1, and EP2 were elevated in the TBI group compared with those in the Sham-operated group and were markedly attenuated following CBD intervention. The RT-PCR results revealed a similar trend (Figure 6F–I). These data indicated that CBD could regulate the prostaglandin metabolic system, resulting in decreased expressions of various indicators.

EP2 and PKA inhibitors attenuate the neuroprotective effects of CBD

To clarify the role of the prostaglandin system in the neuroprotective effects of CBD, rats were pretreated with the EP2 inhibitor TG6-10-1 and the PKA inhibitor H-89. As illustrated in Figure 7A–E, these interventions impacted the inhibitory effects of CBD on COX-1, COX-2, mPGES-1, and EP2. Furthermore, ELISA analysis revealed that the serum levels of PGE_2 and cAMP were significantly increased after TBI (Figure 7F,G) but were markedly decreased following CBD intervention. Notably, the regulatory effects of CBD were inhibited by the application of TG6-10-1 and H-89. The modulatory effects of CBD on inflammation and activated astrocytes were also significantly diminished following TG6-10-1 and H-89 intervention (Figure 7H–K). These results suggest that the neuroprotective effects of CBD on TBI may be mediated through the PGE_2-EP2-cAMP-PKA signaling pathway.

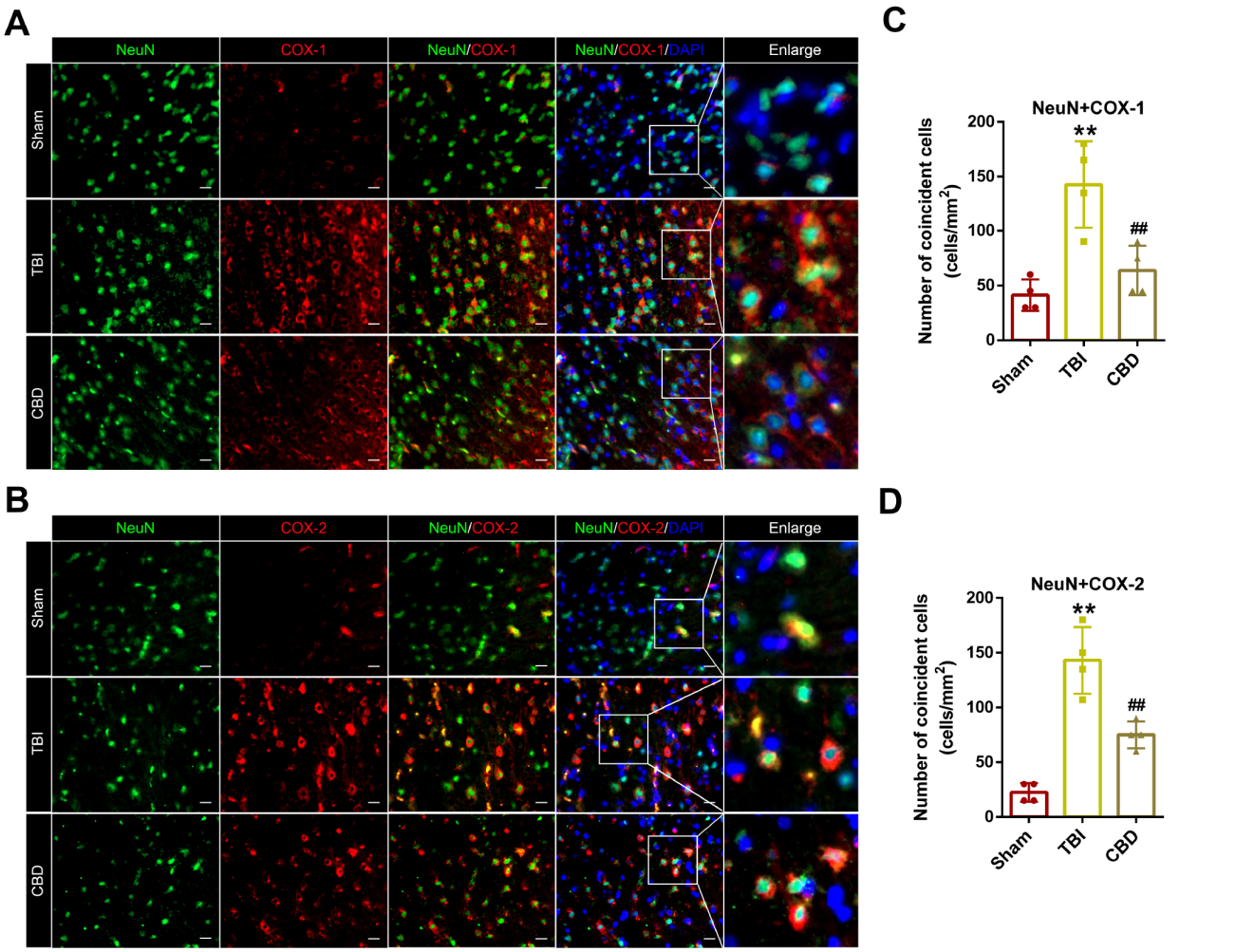

Figure 5. Double immunofluorescence staining of COX-1, COX-2, and NeuN in the injured ipsilateral cerebral cortex (A) Double immunofluorescence staining of NeuN (green)-marked neurons and COX-1 (red). (B) Double immunofluorescence staining of NeuN (green)-marked neurons and COX-2 (red). Scale bar: 20 μm. (C) Quantitative analysis of the density of cells coexpressing NeuN and COX-1. (D) Quantitative analysis of the density of cells coexpressing NeuN and COX-2. $^{**}P < 0.01$ vs Sham, $^{\#\#}P < 0.01$ vs TBI.

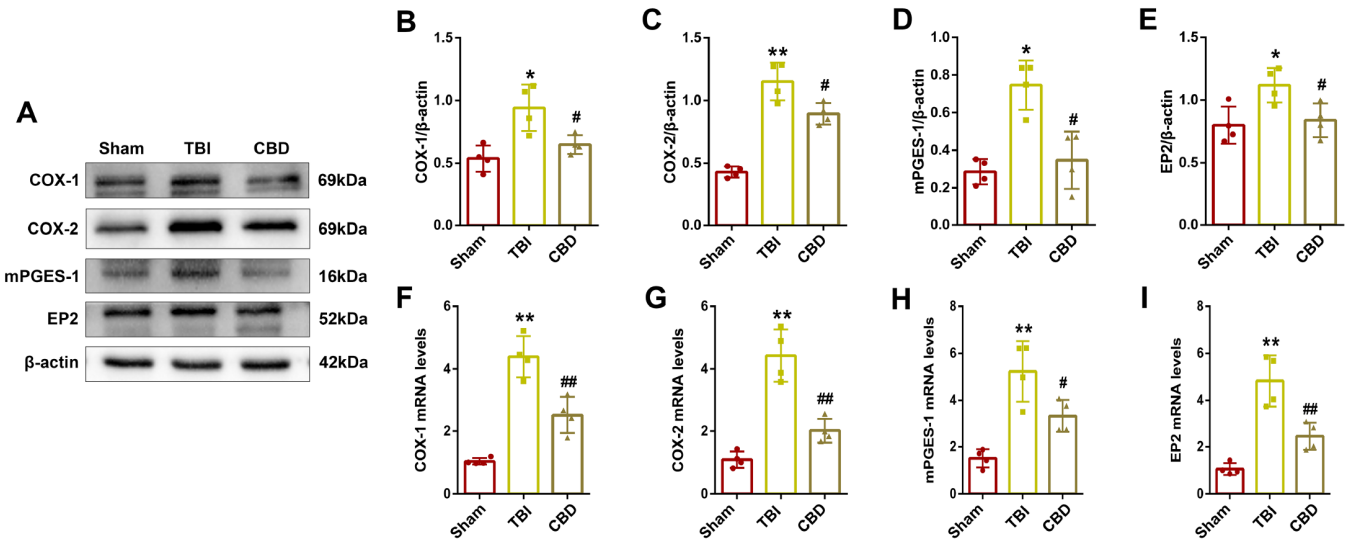

Figure 6. Effects of CBD on the protein expression and mRNA expression of COX-1, COX-2, mPGES-1, and EP2 in the injured ipsilateral cerebral cortex (A) Representative western blot images of COX-1, COX-2, mPGES-1, EP2 and β-actin. (B–E) Relative protein levels of COX-1, COX-2, mPGES-1, and EP2. (F–I) Relative mRNA levels of COX-1, COX-2, mPGES-1, and EP2. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TBI.

Discussion

TBI is widely acknowledged as a significant global public health concern. Despite substantial advancements in the diagnosis, monitoring, and clinical management of TBI, its pathophysiology remains incompletely understood. The window for therapeutic intervention following trauma, a crucial period to mitigate or prevent secondary injury, holds promise for improving long-term patient outcomes [18]. Hence, it is imperative to delve further into the pathogenesis and secondary pathological changes associated with TBI to develop novel therapeutic agents.

In the present study, we selected six distinct time periods for observation: 8 h, 1 d, 2 d, 3 d, 5 d, and 7 d. Assessments based on neurological deficit scores, H&E staining, and Nissl staining confirmed that TBI induced both neurological deficits and cytopathological changes in rats. The calcium-binding protein S-100β, which is mainly secreted by astrocytes and Schwann cells, is released into the bloodstream following injury to astrocytes or the BBB [19]. This protein serves as a recognized biomarker for determining both the severity and prognosis of TBI [20]. Additionally, elevated level of S-100β is associated with adverse outcomes in diverse neurological pathologies, including ischemic stroke, cerebral infarction, and brain tumors [21–23]. The glycolytic enzyme NSE, which is primarily localized in the cytoplasm of neurons and neuroendocrine cells, functions as a specific marker of neuronal damage [24]. The BBB is a specialized structure within the central nervous system that prevents the entry of large molecules from the peripheral blood into the brain parenchyma, thereby maintaining cerebral homeostasis through stringent regulation of molecular and ionic passages [25]. Brain injury can compromise the integrity of the BBB, leading to changes in its permeability. As an important method for detecting BBB permeability [16], EB binds to albumin upon administration to form macromolecules that are impermeable to the BBB. After brain injury occurs, these macromolecules permeate the blood and enter the interstitial space of the brain, leading to tissue staining as a marker of BBB disruption.

The neurological function scores, BBB permeability, histological staining, and ELISA results of the TBI rats suggested significant impairment within 7 d post-TBI. These indicators were alleviated at 3, 5, and 7 d, suggesting that pathological damage did not worsen over time and that the rats may possess some capacity for autonomic repair. After a thorough evaluation of the preliminary data, the 2 d time point was selected for subsequent experiments.

We established a CBD intervention group to determine the potential neuroprotective effects of the drug. The results showed that CBD ameliorated TBI-induced neurological dysfunction in rats and reduced the high expression of serum S-100β and NSE caused by TBI. Morphologically, CBD treatment mitigated the pathological changes in neurons, decreased the extent of nuclear pyknosis and necrosis, and reduced the number of necrotic neurons. Additionally, CBD administration reduced the exudation of EB stain, further corroborating its neuroprotective ability. Previous studies have also reported the neuroprotective capacity of CBD. For example, in newborn piglets subjected to hypoxia-ischemia, CBD administration can lower the concentrations of NSE and S-100β, as well as the number of TNF-α-positive cells [26]. Furthermore, oral CBD pretreatment can reduce short- and long-term high glutamate release after severe TBI and improve functional recovery [27]. Post-TBI administration of CBD can significantly reduce defecation scores, lesion volume, and loss of neurons in the ipsilateral and contralateral hippocampi, reverse TBI-induced GFAP upregulation and restore vestibulomotor and cognitive functions [28]. Oral CBD can also prevent allodynia and neurological dysfunctions in a mouse model of mild TBI [29]. Thus, on the basis of our experimental results and the above studies, CBD appears to offer measurable neuroprotection against secondary pathological damage caused by TBI.

Astrocytes interact with endothelial cells and pericytes to preserve the integrity of the BBB, and the activation of astrocytes leads to increased BBB permeability. As an intermediate filament protein present in the astrocyte cytoskeleton, GFAP serves as a representative marker of astrocytes [30]. In this study, immunohistochemical staining, immunofluorescence staining, western blot

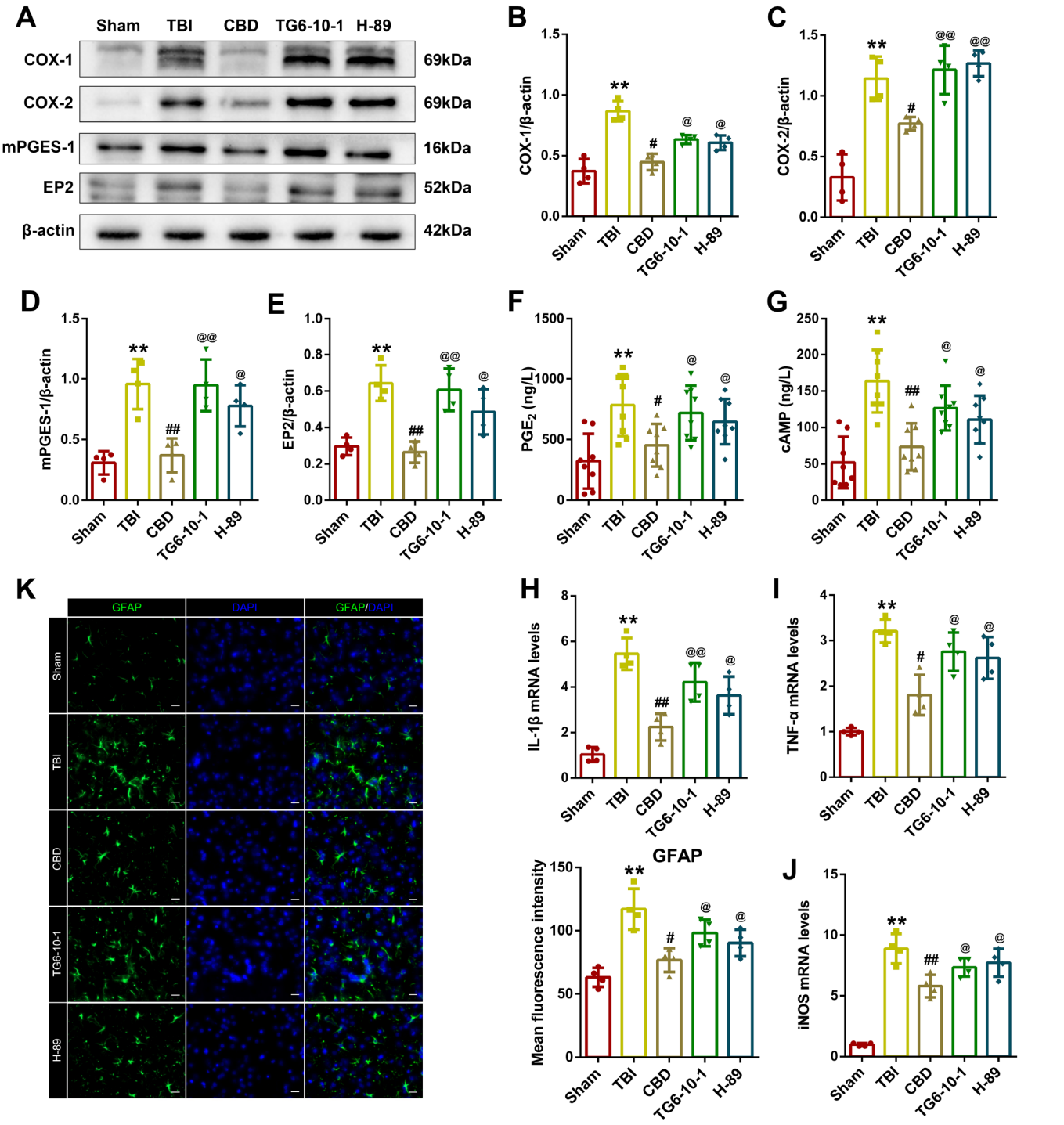

Figure 7. Effects of the EP2 inhibitor TG6-10-1 and the PKA inhibitor H-89 on the therapeutic effect of CBD (A) Representative western blot images of COX-1, COX-2, mPGES-1, EP2 and β-actin. (B–E) Relative protein levels of COX-1, COX-2, mPGES-1, and EP2. (F,G) Levels of PGE_2 and cAMP in the serum. (H–J) Relative mRNA levels of IL-1β, TNF-α, and iNOS. (K) Immunofluorescence staining of GFAP in the injured ipsilateral cerebral cortex and the mean fluorescence intensity of GFAP-positive cells. Scale bar: 20 μm. $^{**}P < 0.01$ vs Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TBI; $^{@}P < 0.05$, $^{@@}P < 0.01$ vs CBD.

analysis, and RT-PCR were employed to investigate astrocyte behavior. Morphological analysis revealed that post-TBI astrocytes presented enlarged cytosomes and thicker and more irregular foot protrusions, indicating an activated state. Western blot and RT-PCR analyses confirmed elevated levels of both GFAP protein and mRNA. Consistent with the morphological changes observed in our study, a prior rat controlled cortical impact injury (CCI) model revealed morphological changes in astrocytes in brain tissue at 2 d on the basis of immunohistochemical staining, with astrocytes showing yellowish or dark brown changes, larger cytosomes, and

thickened protrusions. Furthermore, in a previous rat model of TBI induced by Feeney's free-fall method, GFAP protein expression was found to be elevated on day 2 post-TBI [31], which is consistent with our molecular biology experiments. Astrocytes are over-expressed and morphologically activated after TBI. These reactive astrocytes are known to modulate BBB permeability under pathological conditions, thereby affecting neuroplasticity and central nervous system regeneration. As such, inhibiting astrocyte activation may be crucial for restoring BBB integrity and treating TBI. In the present study, CBD intervention attenuated astrocyte activation, as evidenced by reduced protein and mRNA expression levels. Previous studies have also shown that the intraperitoneal administration of CBD (5 mg/kg) can downregulate astrocyte activation, reduce ischemic brain injury, and improve functional recovery in neonatal rats [32]. Furthermore, treatment with 17β-estradiol (E2) can significantly alleviate TBI-induced neurological deficits, neuronal injuries, and brain edema while inhibiting the expressions of Iba1 and GFAP, which are markers of microglial and astrocyte activation, respectively [33]. Rosiglitazone can also exert neuroprotective effects by inhibiting the overactivation of astrocytes, thereby reducing inflammatory cytokine levels in cerebral ischemia/reperfusion injury models [34]. On the basis of these observations and prior experimental findings from our group [12], we hypothesize that CBD may downregulate TBI-induced astrocyte activation, thus providing a degree of neuroprotection.

We previously demonstrated a strong correlation between the prostaglandin metabolic system and secondary pathological changes due to hypoxia and concussion, revealing that modulation of the PGE_2-EP2-cAMP signaling pathway may inhibit excessive microglial activation and reduce the levels of tumor necrosis factor-α (TNF-α), inducible nitric oxide sythase (iNOS), and interleukin-1β (IL-1β) while also mitigating hippocampal neuronal degeneration and necrosis [35,36]. In the present study on closed brain trauma, TBI upregulated the expressions of three pivotal enzymes (COX-1, COX-2, and mPGES-1) and the EP2 receptor in the prostaglandin metabolic system. Concurrently, the serum levels of the metabolites PGE_2 and cAMP in the signaling pathway were also increased. These findings suggest the involvement of the prostaglandin metabolic system in the pathological progression of TBI, potentially in relation to the PGE_2-EP2-cAMP signaling pathway. Notably, CBD intervention downregulated the expressions of prostaglandin indicators. Previous studies have also reported the effects of CBD. For example, Zhang *et al.* [37] reported that CBD administration reduces the protein expression of COX-2 in myocardial tissue, contributing to its cardioprotective effects. Similarly, Genovese *et al.* [38] reported that CBD treatment in a rat model of endometriosis decreases the expression of COX-2 in lesions, decreases PGE_2 levels in the peritoneal fluid, and exerts anti-inflammatory and analgesic effects. Both the current experimental data and literature suggest that CBD can interfere with the prostaglandin metabolic system and downregulate specific biochemical indicators. To further validate the regulatory impact of CBD on TBI, we applied the EP2 inhibitor TG6-10-1 and the PKA inhibitor H-89. These inhibitors suppressed the downregulatory effects of CBD on prostaglandin indicators and inhibited the regulatory effects of CBD on astrocyte activation. Earlier research by Jiang *et al.* [13] reported that TG6-10-1 can diminish neurological impairments related to seizures, reactive gliosis, disruptions in the BBB, and damage to the hippocampus in a mouse model of status epilepticus induced by kainate alginate, suggesting that EP2 inhibition has both anti-inflammatory and neuroprotective effects. However, their study did not include pharmacological interventions, highlighting only the potential role of EP2 in the development of the epilepsy model. Furthermore, Ruan *et al.* [39] studied the antidepressant effects of oil from Fructus Gardeniae (OFG), derived from Gardenia jasminoides. They reported that OFG exerts antidepressant effects via the PKA-CREB-BDNF signaling pathway, with the application of the PKA inhibitor H-89 neutralizing its modulatory effects on the forced swimming test and expression of phosphorylated CREB and BDNF, thus supporting the role of the PKA-CREB-BDNF signaling pathway in the antidepressant effects of OFG. Collectively, these findings strongly suggest that the PGE_2-EP2-cAMP-PKA signaling pathway plays an important role in various brain injuries and neurodegenerative diseases. However, it remains unclear whether CBD directly acts on the EP2 receptor, and further validation via *in vitro* experiments will be considered in future studies.

In summary, this study confirmed the role of the prostaglandin system in the pathological mechanisms underlying TBI, as well as the modulatory and neuroprotective effects of CBD on nerve injury caused by TBI, which are likely due to the suppression of the PGE_2-EP2-cAMP-PKA signaling pathway and the inhibition of astrocyte activation. Overall, these findings advance our understanding of the mechanisms by which CBD provides neuroprotection following TBI, laying a new theoretical foundation for its potential clinical application.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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