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Minocycline attenuates neuronal apoptosis and improves motor function after traumatic brain injury in rats

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Abstract: Minocycline is a type of tetracycline antibiotic with broad-spectrum antibacterial activity that has been demonstrated to protect the brain against a series of central nervous system diseases. However, the precise mechanisms of these neuroprotective actions remain unknown. In the present study, we found that minocycline treatment significantly reduced HT22 cell apoptosis in a mechanical cell injury model. In addition, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining confirmed the neuroprotective effects of minocycline *in vivo* through the inhibition of apoptosis in a rat model of controlled cortical impact (CCI) brain injury. The western blotting analysis revealed that minocycline treatment significantly downregulated the pro-apoptotic proteins BAX and cleaved caspase-3 and upregulated the anti-apoptotic protein BCL-2. Furthermore, the beam-walking test showed that the administration of minocycline ameliorated traumatic brain injury (TBI)-induced deficits in motor function. Taken together, these findings suggested that minocycline attenuated neuronal apoptosis and improved motor function following TBI.
Key words: apoptosis, minocycline, motor function, traumatic brain injury (TBI)

Introduction

Traumatic brain injury (TBI) is a major cause of injury and death worldwide and represents a huge burden for society and the families of affected individuals [1, 2]. Thus TBI has become a serious public health problem. The biological mechanisms underlying TBI are complicated. When TBI occurs, the brain experiences two waves of injury in succession: the primary injury, caused by mechanical damage, and the secondary injury, which includes a complex cascade of pathophysiological processes, resulting in cell death, inflammation, oxidative stress, edema, blood–brain barrier disruption, endoplasmic reticulum stress, and immune responses[3, 4]. However, no effective pharmaceutical intervention currently exists to treat this type of trauma. Therefore, the exploration of novel pharmacotherapies that can effectively treat TBI patients and improve long-term outcomes remains necessary.

Increasing studies have confirmed that apoptosis plays an important role in the pathophysiological processes associated with TBI; therefore, the inhibition of apoptosis may represent a novel therapeutic strategy for TBI. Brain damage was significantly attenuated in a rodent model of controlled cortical impact (CCI) after treatment with an apoptosis inhibitor (z-DEVD-fmk) and following

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the overexpression of the anti-apoptotic protein Bcl-2 [5]. Similarly, pretreatment with a caspase-3 inhibitor not only reduced neurological deficits but also attenuated tissue damage following fluid percussion injury (FPI) in rats [6]. A previous study, using a rat TBI model, showed that activated caspase3 was significantly upregulated after TBI, indicating the occurrence of apoptosis. Furthermore, following traumatic injury, mild hypothermia has been shown to inhibit apoptosis and attenuate cell death within the hippocampus [7]. These results have supported the potential of exploring new drugs for TBI treatment that can effectively reduce apoptosis and are able to permeate the blood–brain barrier.

Minocycline is a type of tetracycline antibiotic with broad-spectrum antibacterial activity. Minocycline is highly fat-soluble and is capable of penetrating the blood–brain barrier. In addition to antibacterial activity, experimental studies have revealed that minocycline can protect the brain from a variety of central nervous system diseases [8–13]. Recently, it is reported that minocycline treatment was reported to significantly reduced intracranial hemorrhage (ICH)-induced perihematomal iron deposition. Further more, minocycline treatment also attenuated brain swelling, neuroinflammation, and neuronal loss and promoted the recovery of neurological function. In terms of mechanism, mechanistically, minocycline may act as an iron chelator of iron as well as anand an inhibitor of neuroinflammation [9]. Interestingly, minocycline also plays a similar role in TBI [14]. During the process of cerebral ischemia/reperfusion (I/R), minocycline treatment prevented pyramidal cell death and microglial activation in the hippocampus of a rat I/R model, in addition to reducing the levels of malonaldehyde (MDA) and pro-inflammatory cytokines [15]. As for apoptotic process, minocycline has been reported to regulate the balance between the pro-apoptotic and anti-apoptotic proteins. It induces the up-regulation of anti-apoptotic Bcl-2 and Bcl-xl, and decreases the expression of the pro-apoptotic proteins Bax, Bak, Bid, P53, Caspases, Apaf-1 and Fas[16].

However, the role played by minocycline in apoptosis following TBI remains unclear. In the present study, we attempted to determine the effectiveness of minocycline treatment for the inhibition of apoptosis following TBI, using flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and examined changes in molecular biomarkers after TBI. Furthermore, we tested the therapeutic efficacy of minocycline for improving cognitive function in a rat model of TBI. The results from the present study will bring hope for the treatment of TBI.

Materials and Methods

Mechanical cell injury

HT22 cells were purchased from American Type Culture Collection (ATCC) and cultured with Dulbecco's Modified Eagle's Medium (DMEM; Gibco Lab., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and penicillin. A mechanical cell injury model was prepared as previously described, with slight modifications [17, 18]. Briefly, HT22 cells were plated on BioFlex six-well culture plates containing collagen-coated Silastic membranes (Flexcell International Corp., Hillsborough, NC, USA). After the HT22 cells reached confluence, a stretch injury was applied using a Cell Injury Controller IIa system (Virginia Commonwealth University), with a 50 ms burst of nitrogen gas, resulting in the HT22 cells being injured with 7.5-mm deformation. Following injury, the cells were incubated for 24 h. For the minocycline group, the cells were treated with 4 μM minocycline 30 min after the injury. The dose was decided as previously reported [19].

Flow cytometry

HT22 cells were cultured in an incubator for 24 h following mechanical injury. The cells were digested using 0.25% trypsin without ethylenediaminetetraacetic acid (EDTA), washed twice with cold phosphate-buffered saline PBS, and resuspended in binding buffer. Next, 100 μL of the cell suspension was combined with a 5 μL mixture containing annexin V, Alexa Fluor 488, and propidium iodide (PI; Component B) in a 1.5-ml centrifuge tube. After the solution was mixed evenly, the mixture was incubated for 10 min at room temperature in the dark, followed by the addition of 400 μL binding buffer to each tube. Apoptotic cells were calculated using flow cytometry. Flow cytometry was performed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

Animals

The animal procedures of this study were approved by the Animal Care and Experiment Committee of Shanghai Health Medical College (No. 201701). Male Sprague Dawley rats (250–300 g), 6–8 weeks old, breeding by Shanghai SLAC Laboratory Animal Corporation (Shanghai, China), were housed for one week before surgery in a animal device with temperature- (22–25°C) and humidity-controlled (50% relative). All rats were specific pathogen free. All animals were free to eat and drink except for eight hours before surgery.

CCI model and drug administration

In the present study, moderate CCI models of TBI were generated, as previously described, with slight modifications [20]. In brief, the rats were intraperitoneally (i.p.) anesthetized with 0.4% sodium pentobarbital (P3761, Sigma, St. Louis, MO, USA), at a dose of 50 mg/kg body weight, and then fixed in a stereotaxic frame (Stoelting, Varese, Italy). A midline scalp incision was made using a scalpel, and a round craniotomy (6-mm diameter, centered 2.8 mm posterior to bregma and 3 mm from midline) was made on the left side of the midline. The bone was gently removed, taking care to avoid damaging the dura mater. Before impact, a 5.0-mm rounded tip was connected to an motor unit device (PinPoint™ PCI3000 Precision Cortical Impactor™, Hatteras Instruments, Cary, NC, USA). To ensure the tip was perpendicular to the dura, and the angle relative to the vertical plane was usually adjusted at 10° to 15°. The rats were then underwent to a moderate cortical injury through a single contusion to the dura (deformation depth: 2.0 mm, piston velocity: 3.0 m/s, dwell time: 180 ms). In the sham group, the rats experienced identical procedure, except for the contusion injury. In the TBI + Vehicle group, the rats received an i.p. saline injection followed by CCI treatment, whereas the rats in the TBI + MINO group received a CCI treatment and an i.p. injection of 40 mg/kg bodyweight of minocycline. The dose was decided as previously reported [14]. The injection of minocycline (Sigma) carried out on day 0 after CCI (2 h post-injury), and on days 1, 2 post-TBI on day- time.

Slice preparation

Rats were perfused transcardially with 0.9% saline (4°C) and 4% paraformaldehyde (4°C), in succession, after being anesthetized with sodium pentobarbital (70 mg/kg body weight, i.p.) 3 days post-injury. The brains were removed and immersed in 4% paraformaldehyde at 4°C. Eight hours later, the brains were covered by 20% and 30% sucrose solutions respectly untill the brains sank to the bottom. The samples were then embedding, cut into slices and stored at −80°C until use.

TUNEL staining

TUNEL staining was performed using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, slides were incubated in the indicated reaction mixture in the dark for 60 min at 37°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Jiangsu, China). A fluorescent microscope (20× objective, Nikon 90i, Tokyo, Japan) was used to perform image capture.

Western blotting

Rats were sacrificed at 3 days postinjury, and tissue samples of the injury cortex were dissected for protein extraction. Protein were sepertated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride made membranes (Millipore, Merck KGaA, Darmstadt, Germany). Membranes were blocked for 2 h at room temperature using 5% non-fat milk powder and then immersed in primary antibodies at 4°C for at least 12 h. After that, they were incubated with corresponding secondary antibodies. The blots were detected using an enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL, USA). Band densities were quantified by NIH ImageJ software (Bethesda, MD, USA), and the densities were normalized against that for β-actin. The primary antibodies used in this study were as follows: monoclonal rabbit anti-Bax (1:2,000, ab32503, Abcam, Cambridge, UK); polyclonal rabbit anti-Bcl-2 (1:2,000, ab194583, Abcam); monoclonal rabbit anti-cleaved caspase-3 (1:5,000, ab214430, Abcam); monoclonal rabbit anti-caspase-3 (1:3,000, ab184787, Abcam) and monoclonal rabbit anti-β-actin (1:2,000, #4970, Cell Signaling Technology, Beverly, MA, USA).

Beam-walking test

The rat motor function was evaluated by the beam-walking test, as described previously [21]. The beam-walking test was performed on day 0 presurgery, and on days 1, 2, 3, 7, 14, 21, and 28 post-TBI.

Statistical analysis

Data are presented as the mean ± SD. Statistical analyses were performed using SPSS 16.0. Comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA). Tukey's post hoc test or Dunnett's post hoc test was used to determine specific differences between groups. Significance was defined as P values less than 0.05.

Results

Minocycline attenuates neuronal apoptosis induced by stretch injury in HT22 cells

Flow cytometry was used to assess the level of apoptosis among HT22 cells. Cell apoptosis was determined according to the quantity of annexin V-positive cells detected during flow cytometry-based assays. In the present study, 100 μM minocycline treatment significantly reduced the numbers of early-stage apoptotic cells, as determined by the detection of annexin V-positive/ PI-negtive cells by flow cytometry. These findings demon-

strated that stretch-induced injury could trigger early-stage apoptosis in HT22 cells, which could be attenuated by minocycline treatment (Figs. 1A and B).

Minocycline reduced apoptosis in a rat model of TBI

To evaluate whether minocycline treatment could protect the cortex from apoptosis *in vivo,* TUNEL staining was performed in a rat model of TBI. Almost no TUNEL-positive nuclei were observed in the sham TBI group (Fig. 2). In contrast, TBI resulted in a significant increase in apoptotic cells (P<0.05, n=5). The number of TUNEL-positive nuclei in the contusion margins was reduced significantly when minocycline treatment was administered following TBI (P<0.05, n=5).

Minocycline treatment modulated the expression of pro-apoptotic proteins and reduced caspase-3 activation after TBI

The upregulation of the pro-apoptosis protein BAX or the downregulation of the anti-apoptotic protein BCL-2 are indicators that the mitochondrial membrane permeability has increased, and cell apoptosis is likely to occur. In the present study, as expected, the protein levels of Bax increased significantly following TBI, which coincided with reduced BCL-2 protein levels (Fig. 3A). However, minocycline treatment reversed the expression levels of Bax and Bcl-2 in the contusion margins. The levels of cleaved caspase-3, which is a key apoptotic executor, were significantly upregulated after TBI, which is consistent with the results of TUNEL-staining. The minocycline treatment group showed significantly reduced cleaved caspase-3 levels compared with the vehicle treatment group (P<0.05, n=5; Fig. 3B).

Treatment with minocycline ameliorated TBI-induced deficits in motor function

The beam-walking test was performed to evaluate motor functional recovery after TBI. Compared with the sham group, the foot faults of rats subjected to TBI were significantly increased at all time-points post-injury (P<0.001), indicating obvious motor defects following TBI. Furthermore, after treatment with minocycline, the l foot faults were significantly reduced compared with the corresponding values in vehicle-treated rats on day 21 and 28 post-TBI (14 days, 28 days P<0.05; Figs. 4A and B).

Discussion

Minocycline is a second-generation tetracycline derivative that has been used as a unique antimicrobial agent since it was first introduced in 1967 [22]. Minocycline is characterized by broad-spectrum antimicrobial activity, excellent organelle penetration due to high hydrophobicity, superior oral absorption, and a long half-life. Therefore, an increasing number of researchers have begun to explore new uses for this old drug. The high lipid solubility of minocycline enables it to cross the blood–brain barrier effectively. Recently, minocycline has been reported to play neuroprotective roles in a variety of nervous system diseases, including intracerebral hemorrhage, glial cell-mediated bilirubin neurotoxicity, ethanol-induced damage, neonatal hypoxic insult, and transient cerebral I/R [8, 11–13, 15, 23]. In our study, minocycline significantly attenuated neurological impairment caused by TBI and increased neuronal viability. Minocycline regulates iron metabolism by chelating Fe^{2+} and Fe^{3+} *in vitro,* reducing the iron concentration in both the cerebral spinal fluid and brain tissues (particularly the cortex and hippocampus), attenuating the

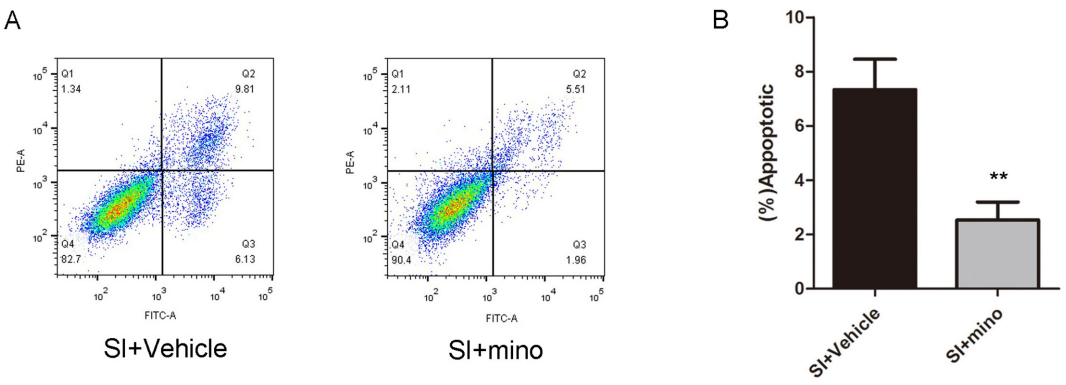

Fig. 1. Minocycline treatment inhibits apoptosis in HT22 cells after stretch injury (SI). (A) Flow cytometry-based assay was performed to determine the level of cell apoptosis. Minocycline treatment significantly decreased the quantity of annexin V-positive/ PI-negtive cells (Q3 quadrant). (B) Bar graph showing the percentage of annexin V-positive cells. **P<0.01 for minocycline vs. vehicle group.

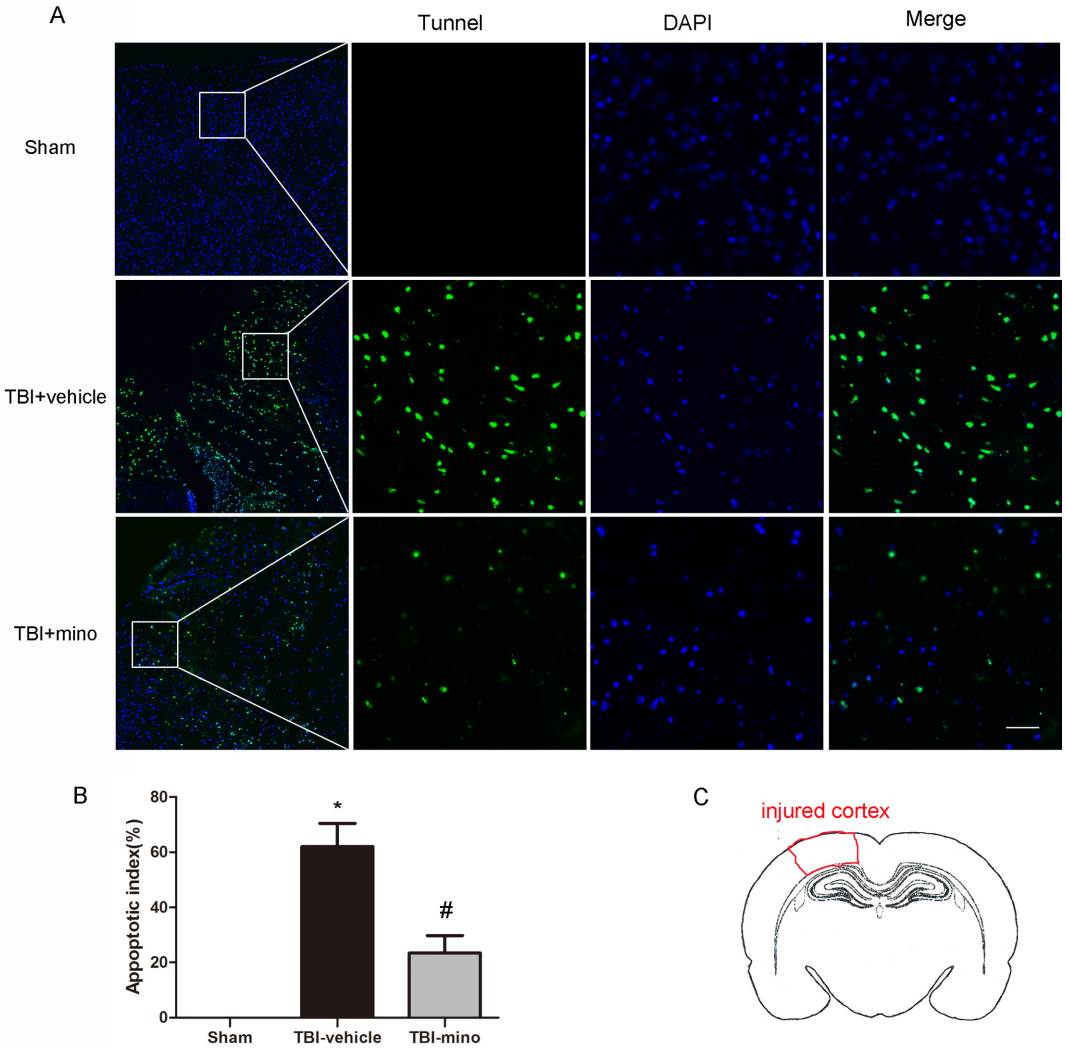

Fig. 2. Minocycline treatment inhibited TBI-induced apoptosis *in vivo*. (A) Representative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, green) and 4',6-diamidino-2-phenylindole (DAPI, blue) staining in cortical brain sections from the three groups. The scale bar is 50 μm. (B) Quantification showed that minocycline significantly reduced the apoptotic index following TBI. Data are presented as the mean ± SD (n=5 per group); **P*<0.05, vehicle vs. sham group; #*P*<0.05, v minocycline vs. vehicle group. (C) Graphical illustration of injured brain region.

neurological impairment caused by TBI [14]. However, the precise role played by minocycline in neural apoptosis remains unclear. In the present study, using a mechanical cell injury model, we found that minocycline significantly reduced apoptosis in HT22 cells. In addition, using a rat CCI model of TBI, we confirmed the neuroprotective effects of minocycline treatment *in vivo*. Furthermore, the administration of minocycline ameliorated TBI-induced deficits in motor function. Taken together, these findings suggested that minocycline can attenuate neuronal apoptosis and improve motor function after TBI; therefore, minocycline may represent a new drug for the treatment of TBI patients.

The mechanical cell injury model is a classic *in vitro* TBI model, and apoptosis represents a major mechanism associated with stretch injury. Stretch injury has been reported to stimulate apoptosis by regulating the c-Jun-N-terminal kinase (JNK)/c-Jun signaling pathway. However, treatment with glibenclamide was shown to reverse stretch injury-induced damage [17]. In the present study, flow cytometry-based assays showed that the early-stage apoptosis of HT22 cells was induced by stretch injury, whereas this pathological process was attenuated by minocycline treatment.

Generally, apoptosis is considered to be a programmed cell death pathway, mediated by mitochondria, and is characterized by internucleosomal DNA fragmentation. Cells that undergo apoptotic process can be detected *in situ* using the TUNEL-staining method [24]. It is repoted that approximately 50–75% of TUNEL-positive cells were neurons that appeared to be undergoing apoptosis. The excessive activation of apoptosis leads to massive

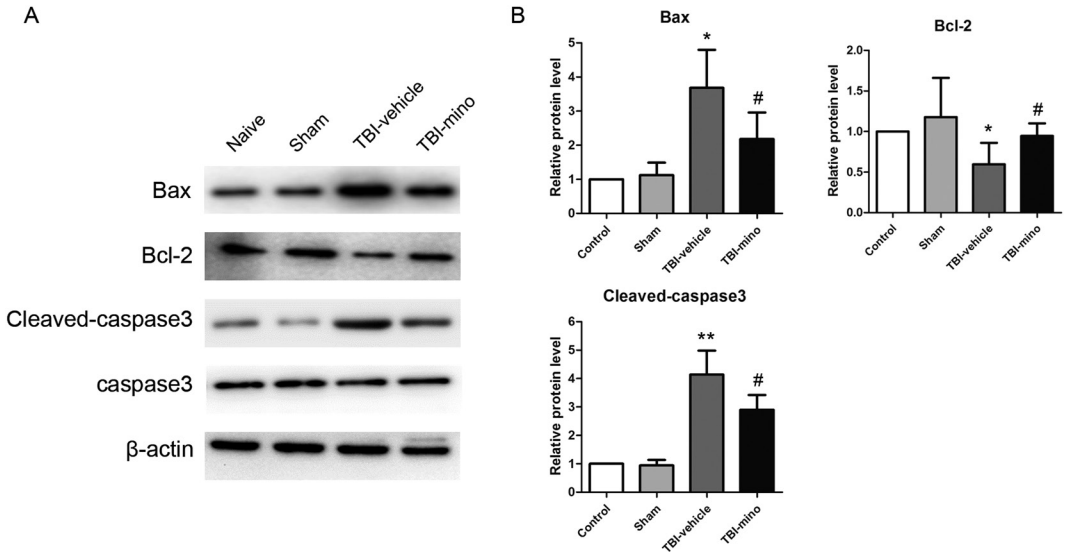

Fig. 3. Effects of minocycline administration on anti-apoptotic and pro-apoptotic proteins in a TBI rat model (A) Western blot analysis showing the protein expression levels of BAX, BCL-2, cleaved caspase-3, and caspase-3. (B) Bar graphs showing the quantification of BAX, BCL-2, and cleaved caspase-3 levels. Data are presented as the mean ± SD (n=5 per group), ***P*<0.01, **P*<0.05 vehicle vs. sham group, #*P*<0.05 minocycline vs. vehicle group.

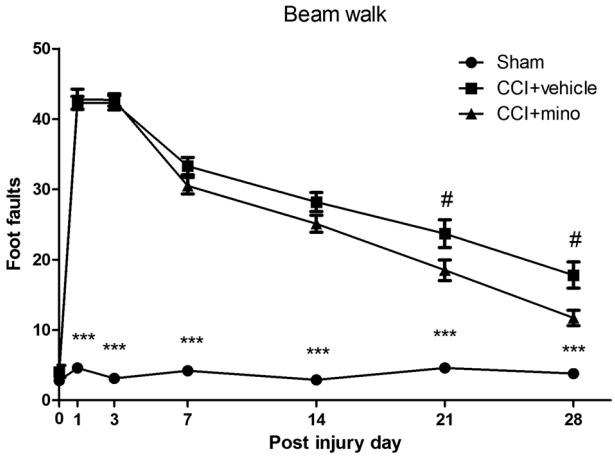

Fig. 4. Minocycline treatment attenuated TBI-induced deficits in motor function. Line chart showing that TBI-induced significant deficits in motor function compared with sham-injured rats and there were significant differences between the CCI+vehicle and the sham group at all time-points post-injury. There was a significant difference between the CCI+mino group and the CCI+vehicle group at 21, 28 d post-injury. Data are showed as mean ± SD. Data were analyzed using ANOVA with Tukey's post hoc test (n=10 each). ****P*<0.001 vs. the sham group; #*P*<0.05, vs. CCI+vehicle.

nerve cell loss, which can result in motor deficiencies [21]. Therefore, apoptosis represents an important therapeutic target for TBI. A series of animal studies have confirmed that neuronal cell apoptosis occurs in the pericontusional cortex and the hippocampus. In a rat lateral FPI brain injury model, apoptotic cells in the ipsilateral hippocampus could be observed as early as 24 h after injury by TUNEL staining [25]. Similar results were observed in a CCI model [21, 26]. Meanwhile, a series of durgs were confirmed to inhibit apoptosis after TBI. Astaxanthin, a carotenoid pigment, ameliorates neuronal apoptosis via SIRT1/NRF2/Prx2/ASK1/p38 after traumatic brain injury in mice, and thus improved the neurological functions [27]. In the present study, apoptosis was observed at 3 days after CCI in the pericontusional cortex, and the administration of minocycline significantly reduced the number of apoptotic cells, ameliorated TBI-induced deficits in motor function.

In general, the activation of caspase-3 is associated with apoptosis in a variety of pathophysiological processes, and an increase in cleaved caspase-3 levels is considered to be an important biomarker of apoptosis [6, 28]. Our study found that cleaved caspase-3 was significantly upregulated in the CCI rat model, whereas the administration of minocycline attenuated the activation of caspase-3. The elevated expression of the pro-apoptosis protein BAX or the repression of the anti-apoptotic protein BCL-2 has also been associated with the occurrence of apoptosis [29]. In the present study, the expression of BAX was elevated, and the expression of BCL-2 was reduced after TBI in the rat model. However, treatment with minocycline reversed the levels of BAX and BCL-2, which suggested that this drug may attenuate tissue damage after TBI via the repression of apoptosis.

In conclusion, our data provided ample evidence to confirm the role played by minocycline in the inhibition of apoptosis, resulting in improved motor function in an animal model of TBI.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

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