Research Article

Inhibition of nitric oxide synthase aggravates brain injury in diabetic rats with traumatic brain injury

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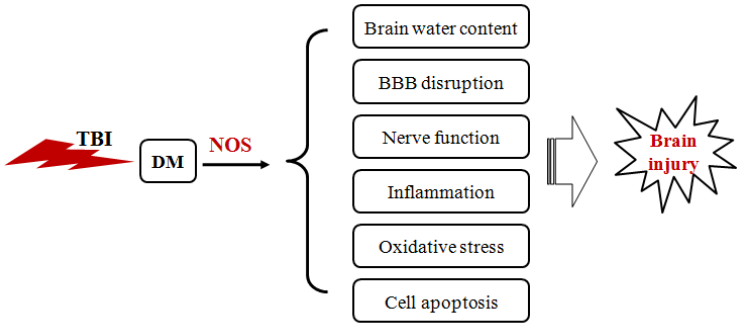
Graphical Abstract *Nitric oxide synthase (NOS) plays a benefical role in maintaining cerebral microcirculation, anti-inflammation, anti-oxidative stress and anti-apoptosis in diabetes mellitus (DM) rats with traumatic brain injury (TBI)*



Abstract

Studies have shown that hyperglycemia aggravates brain damage by affecting vascular endothelial function. However, the precise mechanism remains unclear. Male Sprague-Dawley rat models of diabetes were established by a high-fat diet combined with an intraperitoneal injection of streptozotocin. Rat models of traumatic brain injury were established using the fluid percussion method. Compared with traumatic brain injury rats without diabetic, diabetic rats with traumatic brain injury exhibited more severe brain injury, manifested as increased brain water content and blood-brain barrier permeability, the upregulation of heme oxygenase-1, myeloperoxidase, and Bax, the downregulation of occludin, zona-occludens 1, and Bcl-2 in the penumbra, and reduced modified neurological severity scores. The intraperitoneal injection of a nitric oxide synthase inhibitor N(5)-(1-iminoethyl)-L-ornithine (10 mg/kg) 15 minutes before brain injury aggravated the injury. These findings suggested that nitric oxide synthase plays an important role in the maintenance of cerebral microcirculation, including anti-inflammatory, anti-oxidative stress, and anti-apoptotic activities in diabetic rats with traumatic brain injury. The experimental protocols were approved by the Institutional Animal Care Committee of Harbin Medical University, China (approval No. ky2017-126) on March 6, 2017.
Key Words: apoptosis; blood-brain barrier; brain edema; diabetes mellitus; inflammation; injury; neurological function; nitric oxide synthase; traumatic brain injury

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Introduction

Traumatic brain injury (TBI) is characterized by injury due to the mechanical effects of shear force on blood vessels, neurons, and glial cells, which can cause axonal injury, vascular rupture and hemorrhage, resulting in enhanced neuronal necrosis (Rodriguez-Grande et al., 2017). After the initial brain injury, a signaling cascade is triggered, resulting in secondary injury, including the aggravation of brain edema, decreased cerebral blood flow perfusion, the dysregulation of brain self-regulation, and an increase in the permeability of the blood-brain barrier (BBB) (Prins et al., 2013; Schwarzmaier et al., 2015; McGinn and Povlishock, 2016). The structure and function of the BBB ensure the normal operation of neurons and glial cells in a relatively stable microenvironment (Xu et al., 2017). Therefore, the destruction of the BBB is likely to worsen the condition of TBI patients. The risk of developing many neurodegenerative diseases, such as Alzheimer's disease and cognitive impairment, is also increased among TBI patients (Gardner and Yaffe, 2015).

A study has shown that hyperglycemia can aggravate brain damage by affecting vascular endothelial function, and nitric oxide (NO), produced by NO synthase (NOS), plays a key role in vascular/endothelial function (Manwani et al., 2015). The primary neuroprotective mechanisms through which NO exerts its effects include the relaxation of endothelium-dependent blood vessels, the promotion of vascular regeneration, and the inhibition of inflammation, oxidative stress, thrombosis, and cell apoptosis (Xu et al., 2018).

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Diabetes mellitus (DM) and TBI are serious social and public health problems, and patients with DM who experienced TBI require extensive attention (Wei et al., 2019). The role played by NOS in the development of vascular complications in DM patients has received increasing attention (Hoang et al., 2013), and damage to the vascular endothelium following TBI is the pathological basis underlying changes in BBB permeability. Increased inflammation and oxidative stress disrupt the stability of endothelial NOS mRNA, significantly shortening its half-life and degrading endothelial NOS (Yoshizumi et al., 1993). However, due to the co-activity of other NOS isomers (such as inducible NOS and neuronal NOS) *in vivo*, the factors that drive changes in NO content after injury are complex. The interactions between DM and TBI and the contributions of NOS remain unclear. Therefore, in the present study, a rat model of DM complicated with TBI was established to investigate the role played by NOS in the mechanism of injury by determining the pathophysiological and neurological changes that occur in DM model rats after TBI.

Materials and Methods

Animals and study design

A total of 128 specific pathogen-free grade male Sprague-Dawley (SD) rats (weighing 40–60 g, approximately 3 weeks old) were obtained from the Department of Experimental Animals, Harbin Medical University [License No. SCXK (Hei) 2006-010], and male rats were selected due to hormone levels and gender differences, particularly to avoid the influence of estrogen on the experimental results (Koellhoffer and McCullough, 2013; Manwani et al., 2015). Animals were housed under a 12-hour light/dark cycle, at a constant room temperature of 18–22°C, with an indoor ammonia concentration < 20 ppm, relative humidity of 40–70%, with food and water freely available and frequently changed bedding, in a well-ventilated and dry environment. A power calculation was performed using OpenEpi software (Open Source Epidemiologic Statistics for Public Health; www.openepi.com) to calculate the number of necessary animals (Greco et al., 2018) to observe a significant effect, and at least 5 rats per experimental group were determined to be necessary to achieve our aims. The animals were randomly divided into four groups: (1) Sham operation (Sham, n = 32); (2) TBI (n = 32); (3) DM with TBI (TBI + DM, n = 32); and (4) Inhibitor-treated DM with TBI (TBI + DM + L-NIO, n = 32): 10 mg/kg N(5)-(1-iminoethyl)-L-ornithine (L-NIO; Sigma-Aldrich, Shanghai, China), a NOS inhibitor that was intraperitoneally injected 15 minutes before TBI (Greco et al., 2017). The experimental protocols were approved by the Institutional Animal Care Committee of Harbin Medical University (approval No. ky2017-126) on March 6, 2017, and all procedures were conducted in strict accordance with the guidelines for the care and use of laboratory animals of Harbin Medical University as well as the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines for animal research. The experimental protocol is shown in **Figure 1A**.

DM model

The DM rat model was induced as described previously (Duca et al., 2015; Govindaraj and Sorimuthu Pillai, 2015). The rats were fed with a high-sugar, high-fat diet (20% lard, 20% sucrose, 2.5% cholesterol, and 57.5% base diet) for 4 weeks, fasted for 12 hours (no water prohibition), and weighed. The DM rat model was induced by the intraperitoneal injection of a small dose of streptozotocin [35–40 mg/kg, soluble in 0.1 M citric acid-sodium citrate buffer solution, pH 4.5; Sigma-Aldrich (Shanghai) Trading Co., Ltd., Shanghai, China]. All injections were completed within 1 hour to prevent the degradation of streptozotocin (which was freshly prepared as needed). After the streptozotocin injection, rats continued to be fed with a high-sugar and high-fat diet for 7 days. Blood was collected from the rat tail vein, and the blood glucose concentration was detected using a blood glucose meter. Rats with blood glucose levels greater than 11.1 mM were considered to have been successfully modeled (Zeng and Liu, 2019). Sham rats were fed with a base diet and were given similar volumes of 0.1 M citric acid-sodium citrate buffer solution by intraperitoneal injection, based on weight.

TBI model

The TBI model was induced as described previously (Kabadi et al., 2010; Brizuela et al., 2017). The DM model rats were intraperitoneally injected with 3% pentobarbital sodium (30 mg/kg; Abbott, North Chicago, IL, USA) for anesthesia. Endotracheal intubation was performed after anesthesia to provide mechanical ventilation for 1 hour. A catheter puncture in the caudal artery was performed for arterial blood pressure detection and blood gas analysis (Rapidlab248; Bayer, Leverkusen, Germany). Then, 2% lidocaine was used for infiltration anesthesia in the middle of the rat scalp, and the head of the rat was fixed in the stereotaxic frame (SR-6N; Narishige, Tokyo, Japan). Following a midline incision, the scalp was cut open in the middle of the scalp, and the outer membrane of the skull was isolated. The skull and the anterior fontanelle were exposed. A hole was drilled, 4 mm in diameter, 2 mm behind the anterior fontanelle, and 2.5 mm on the left side of the sagittal suture, and the dura mater was exposed. The instrument used for fluid percussion injury in rats was closely connected with the exposed dura mater, and cyanoacrylate glue was used to seal the joint to form a closed cavity. After the cyanoacrylate glue dried, the pendulum of the instrument was fixed at 17°. When the pendulum was loosened, the pendulum fell naturally, the saline impingement in the liquid tank impacted the rat dura mater, and the pressure ensured 1.7 atm of feedback from the computer, which is consistent with moderate brain injury (1.5–2 atm). Then, the rats were removed from the stereotaxic frame, cleaned the cyanoacrylate glue around the brain injury, fully disinfected with iodine, and finally the damaged area was sutured layer by layer. Inclusion criteria for the TBI group were adult male rats weighing 250–300 g, random blood sugar < 11.1 mM, and impact of 1.7 atm pressure for brain injury.

Brain water content measurement

Rats were sacrificed under deep anesthesia, using 3% pentobarbital sodium, 48 hours after TBI. The brains were removed and divided into two hemispheres, along the midline, and the cerebella were removed. The left hemispheres were collected immediately. After weighing the wet weight, the samples were dried at 80°C for 24 hours to obtain the dry weight. The brain water content (%) was calculated using the following formula: (wet weight – dry weight)/(wet weight) × 100 (Cheng et al., 2010).

Evans blue staining

The permeability of the BBB was evaluated at 48 hours after TBI by measuring the extravasation of Evans blue dye (Wang et al., 2018). The Evans blue dye (2%, 4 mL/kg; Sigma-Aldrich, St. Louis, MO, USA) was administered through the femoral vein. In our studies, Evans blue dye circulated for 30 minutes before the rats were perfused. Following the anesthetization, the rats were perfused with physiological saline solution through the left ventricle of the heart until the complete removal of the intravascular-localized dye was achieved. Then, the animals were decapitated, and the brains were removed. After that, damage to the cerebral tissue BBB was observed.

Sodium fluorescein

Sodium fluorescein is a low-molecular-weight tracer that has been widely used in a variety of model systems for the evaluation of BBB permeability. We evaluated the BBB permeabilization with sodium fluorescein 48 hours after TBI. The basic method for sodium fluorescein was the same as for

Evans Blue. The sodium fluorescein dye (2%, 5 mL/kg; Sigma-Aldrich) was administered through the femoral vein. Later, each brain region was weighed to determine the quantitative measurement of sodium fluorescein extravasation. Brain tissues were homogenized in 2 mL phosphate-buffered saline and then mixed with a vortex for 2 minutes after the addition of 2 mL 60% trichloroacetic acid at 4°C to precipitate proteins. Homogenized samples were maintained in a cold room (4°C) for 30 minutes and centrifuged at 18,000 r/min at 4°C for 10 minutes. A 250 mL volume of each supernatant was placed into microplate containers, and the concentration of sodium fluorescein in the supernatant was analyzed at an excitation wavelength of 440 nm and an emission wavelength of 525 nm using a spectrophotometric microplate reader (DTX 880; Multimode Detector, BioTek Instruments, Inc., Winooski, VT, USA). The tissue contents of sodium fluorescein were quantified according to a linear standard curve, derived from the dye and expressed in ng per mg of brain tissue using the software application (Multimode Analysis Software) that accompanied the microplate reader (Kaya and Ahishali, 2011).

Immunohistochemistry

Under deep anesthesia, using 3% pentobarbital sodium, rats were transcardially perfused at 48 hours after TBI with physiological saline and 4% paraformaldehyde. The rats were decapitated, and the brain was removed and placed in 4% paraformaldehyde at 4°C for 24 hours. The brain tissue (penumbra area around the brain injury tissue) was embedded in paraffin and cut into continuous coronal sections (3 μm). After dewaxing with xylene and alcohol, the sections were dyed in hematoxylin and eosin. The dyed sections were baked for 20 minutes and, finally, sealed with gum. The Nissl dyeing method was performed as previously described (Chen et al., 2014). Alternatively, a selection of the cryostat sections obtained from each of the four groups were dewaxed, blocked, antigen retrieved, and immunostained. The sections were blocked in phosphate-buffered saline containing 1.5% normal goat serum and 1% bovine serum albumin for 2 hours at room temperature, incubated with rabbit anti-heme oxygenase-1 (HO-1, 1:100; Cat# AF5393; Affinity Biosciences, Cincinnati, OH, USA) (Bharucha et al., 2010), rabbit anti-myeloperoxidase (MPO, 1:200; Cat# PAA601Ra01; Cloud-Clone Corp., Houston, TX, USA) (Jang and Rabb, 2009), rabbit anti-occludin (1:200; Cat# DF7504; Affinity Biosciences) (Yu et al., 2005), and rabbit anti-zonula occludens-1 (ZO-1, 1:200; Cat# PAC262Ra01; Cloud-Clone Corp.) (Jiao et al., 2011) antibodies at 4°C overnight. The secondary antibody (1:5000; Rockland Inc., Gilbertsville, PA, USA) was added at room temperature for 30 minutes, and 3,3′-diaminobenzidine staining and hematoxylin staining were performed. After immunopositive cells were observed under the microscope, the staining was terminated, and the sections were dehydrated and sealed with neutral glue. The sections were observed under a light microscope (BX51; Olympus, Tokyo, Japan). Under low-power microscopy (40×), the area of each localized section was identified in the same plane as the cortical injury. Neuron damage was observed under high magnification (400×).

Western blot analysis

The peri-lesion area of the ipsilateral hemisphere, defined as the "penumbra," was removed and immediately stored at –80°C for later use during western blot analysis. The proteins were extracted from brain tissue (penumbra area), and the protein concentrations were determined using a bicinchoninic acid (BCA) kit. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with Tris-buffered saline/0.1% Tween-20 containing 5% non-fat dry milk for 2 hours. The membranes were incubated overnight at 4°C with primary antibodies rabbit anti-β-actin (1:400; Cat# PR-0255; ZSGB Biotechnology, Beijing, China), rabbit anti-Bcl-2 (1:1000; Cat# ab59348; Abcam Biotechnology, Cambridge, UK), rabbit anti-Bax (1:2500; Cat# ab32503; Abcam Biotechnology), rabbit anti-MPO (1:2000; Cat# PAA601Ra01; Cloud-Clone Corp.), and rabbit anti-occludin (1:2000; Cat# DF7504; Affinity Biosciences). After incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000; Cat# ZB-2301; ZSGB Biotechnology) at room temperature for 1 hour, the protein bands were visualized by an enhanced chemiluminescent kit using a gel imaging system acquisition (Smartchemi; Beijing Saizhi Pioneering Technology Co., Ltd., Beijing, China). β-Actin was used to normalize the protein levels as a loading control. The optical density was analyzed by the electrochemiluminescence kit and gel imaging system (Smartchemi; Beijing Saizhi Pioneering Technology Co., Ltd., Beijing, China).

Modified neurological severity score test

To investigate the functional neurological changes, the modified neurological severity score (mNSS) test was performed 1, 3, 5, and 7 days after TBI (Wang et al., 2012). The mNSS test is graded on a scale from 1–18, correlating with neurological deficits. Scores from 1–6 points indicate mild injury, 7–12 points indicate moderate injury, and 13–18 points indicate severe injury.

Statistical analysis

Statistical analysis was performed using SPSS software (version 21; IBM SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Normal distribution was determined by the Kolmogorov-Smirnov test. Comparisons of blood glucose levels between preoperative and intraoperative time points were performed by Student's *t*-test. Differences among groups were determined using a one-way analysis of variance or repeated-measures analysis of variance followed by *post hoc* analysis with Bonferroni's test. All reported *P*-values are two-tailed, and $P < 0.05$ was considered significant.

Results

Changes in the blood glucose levels of rats with DM complicated with TBI

The blood glucose levels were significantly higher in rats from the TBI + DM and TBI + DM + L-NIO groups compared with those in the sham and TBI groups ($P < 0.05$). The blood glucose level of rats detected 30 minutes after TBI was significantly decreased than that before the TBI in the TBI, TBI + DM and TBI + DM + L-NIO groups ($P < 0.05$; **Table 1**).

Table 1 | **Blood glucose levels (mM) of rats with DM complicated with TBI**

	Sham	TBI	TBI + DM	TBI + DM + L-NIO
Preoperative	7.47±0.81	7.70±1.05	16.89±2.87$^{\#\dagger}$	17.32±3.11$^{\#\dagger}$
Intraoperative	6.47±1.50	5.87±1.69$^{\$}$	12.38±3.17$^{\#\dagger\$}$	13.15±3.97$^{\#\dagger\$}$

Data are expressed as the mean ± SD ($n = 6$). #$P < 0.05$, *vs*. sham group; †$P < 0.05$, *vs*. TBI group; \$$P < 0.05$, *vs*. preoperative (one-way analysis of variance followed by Bonferroni *post hoc* test).

Brain water contents increase in rats with DM complicated with TBI

The severity of brain injury was judged by measuring changes in the brain water contents of rats following TBI induction. The water contents of the ipsilateral cortical tissue significantly increased in rats 48 hours after TBI compared with the sham group ($P < 0.05$). Rats in the TBI + DM and TBI + DM + L-NIO groups showed significantly increased water contents compared with that in the TBI group (both $P < 0.05$). However, no significant difference was observed between the TBI + DM + L-NIO and TBI + DM groups ($P > 0.05$; **Figure 1B**).

BBB permeability increases in rats with DM complicated with TBI

The BBB is an important component of the brain, and changes in BBB permeability reflect the degree of brain injury (Abou-El-Hassan et al., 2017). To evaluate BBB permeability after TBI, the permeability of Evans Blue Dye was evaluated (**Figure 2A**). Compared with the sham group, the TBI group showed an increase in the Evans blue permeability of the damaged brain tissue 2 days after TBI. Hyperglycemia significantly increased the Evans blue permeability of damaged brain tissue. Rats exposed to TBI also displayed increased levels of fluorescein sodium contents in the ipsilateral cortical and hippocampal tissues compared with those in the sham group 48 hours after TBI ($P < 0.05$). The DM + TBI group showed a remarkable and significant increase in the fluorescein sodium contents in the ipsilateral cortex compared with the TBI group ($P < 0.05$; **Figure 2B**).

Pathological changes in the penumbra brain region in rats with DM complicated with TBI

The brain tissue lesions and the associated neuronal damage both directly reflected the degree of brain injury. Hematoxylin-eosin staining showed that the cortical cells in the sham group maintained their normal cell structures, with abundant cytoplasm and intact nucleus. Nissl staining showed that there were larger and more complete Nissl bodies in the sham group compared with the other groups. The degree of damage observed in the brain tissue of rats in the TBI, TBI + DM, and TBI + DM + L-NIO groups was categorized as serious, based on the identification of massive hemorrhagic foci, loss and edema of tissues, and reduced cytoplasmic Nissl staining (**Figure 3**). Immunohistochemistry analysis of HO-1, MPO, occludin, and ZO-1 immunopositivity was performed, as these proteins play extremely important roles in the maintenance of the BBB and the regulation of inflammation. The results showed that the percentages of HO-1- and MPO-positive cells significantly increased in the TBI + DM + L-NIO group compared with those in the other groups (all $P < 0.05$). The percentages of ZO-1- and occludin-positive cells significantly decreased in the TBI + DM + L-NIO group compared with those in the other groups (all $P < 0.05$; **Figure 4**).

Protein expression levels of Bax, Bcl-2, occludin, and MPO in the penumbra area of rats with DM complicated with TBI

The expression levels of apoptosis- (Bax and Bcl-2) (Cory and Adams, 2002), BBB- (occludin) (Ni et al., 2017), and oxidative stress-related proteins (MPO) (Demir et al., 2019) were determined to further explain the severity of brain injury. As shown in **Figure 5**, all four groups showed the upregulation of Bax and MPO (sham < TBI < TBI + DM < TBI + DM + L-NIO) protein expression, and the downregulation of Bcl-2 and occludin (sham > TBI > TBI + DM > TBI + DM + L-NIO) protein expression.

Neurological functions of rats with DM complicated with TBI

The mNSS score reflects the neurological function of rats, and the severity of neurobehavioral defects is positively correlated with the score (Wang et al., 2012). Compared with the rats in the sham group, the rats in other groups showed a decrease in food intake and activity levels and presented with symptoms of stress unresponsiveness and limb hemiplegia. The scores for each parameter and the mNSS score were the lowest for the TBI group and the highest for the TBI + DM + L-NIO group, which indicated the development of increasingly serious signs of neurological deficits in the TBI, TBI + DM, and TBI + DM + L-NIO groups. In addition, the total scores (sum of all parameters) decreased with increased time after TBI (**Table 2**).

Table 2 | Modified neurological severity test subscores and total scores for rats with DM complicated with TBI

Group	1 d	3 d	5 d	7 d
Raising rat by the tail				
Sham	0.8±0.4	0.4±0.5	0.6±0.5	0.4±0.5
TBI	2.0±0.0$^{\#}$	1.6±0.5$^{\#}$	1.2±0.4$^{\#}$	1.0±0.0$^{\#}$
TBI + DM	2.4±0.5†	2.0±0.0†	2.0±0.0†	1.2±0.4†
TBI + DM + L-NIO	2.8±0.4§	2.4±0.5§	2.4±0.5§	1.8±0.4§
Placing rat on the floor				
Sham	0	0	0	0
TBI	1.0±0.0$^{\#}$	0.8±0.4$^{\#}$	1.0±0.0$^{\#}$	0.6±0.5$^{\#}$
TBI + DM	1.2±0.4†	1.0±0.0†	1.0±0.0	0.8±0.4†
TBI + DM + L-NIO	1.6±0.5§	1.2±0.4§	1.0±0.0	0.8±0.4§
Sensory tests				
Sham	0.4±0.5	0.8±0.4	0.8±0.4	1.0±0.0
TBI	1.0±0.0$^{\#}$	1.0±0.0$^{\#}$	1.0±0.0$^{\#}$	0.6±0.5
TBI + DM	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0†
TBI + DM + L-NIO	1.8±0.8§	1.0±0.0	1.0±0.0	1.0±0.0
Beam balance tests				
Sham	0.2±0.4	0.2±0.4	0.0±0.0	0
TBI	2.6±1.1$^{\#}$	0.4±0.5$^{\#}$	0.2±0.4$^{\#}$	0
TBI + DM	3.8±0.8†	2.2±0.8†	1.0±0.7†	0.2±0.4†
TBI + DM + L-NIO	3.4±0.5	3.4±0.9§	2.2±0.8§	0.4±0.5§
Reflexes absent and abnormal movements				
Sham	0	0	0	0
TBI	0	0	0	0
TBI + DM	0	0	0	0
TBI + DM + L-NIO	0	0	0	0
Total				
Sham	1.4±0.5	1.4±0.5	1.4±0.5	1.4±0.5
TBI	6.6±1.1$^{\#}$	3.8±0.8$^{\#\ddagger}$	3.4±0.5$^{\#}$	2.2±0.8
TBI + DM	8.4±1.1†	6.2±0.8†	5.0±0.7†	3.2±0.4‡
TBI + DM + L-NIO	9.6±1.1§	8.0±1.0§‡	6.6±1.1§‡	4.0±1.0$^{\#}$

Data are expressed as the mean ± SD ($n = 5$). #$P < 0.05$, *vs*. sham group; †$P < 0.05$, *vs*. TBI group; §$P < 0.05$, *vs*. TBI + DM group; ‡$P < 0.05$, *vs*. previous time point (repeated measures analysis of variance followed by Bonferroni *post hoc* test). DM: Diabetes mellitus; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; TBI: traumatic brain injury.

Discussion

Diabetic patients experience a high incidence of retinopathy, ketoacidosis, and disturbances in consciousness, which can lead to a significant increase in the incidence of brain damage. Similarly, DM can also accelerate the progression of brain injury. A study showed that, compared with non-diabetic mice, diabetic mice have been found to have increased neurological damage, mortality, and cerebral infarction areas (Desposito et al., 2017). The mortality rate of DM patients after TBI was significantly increased compared with that of non-diabetic patients (Ley et al., 2011). After TBI occurs in diabetic patients, due to the presence of active inflammatory cytokines in the body, insulin levels can become relatively insufficient, which results in the interruption of the glucose supply and the inability to meet the energy demands of cells (Bosarge et al., 2015; Martinez and Peplow, 2019). In the present study, the results showed that compared with those in TBI rats, the levels of brain edema, vascular permeability, oxidative stress, apoptosis-related protein expression, and neurological dysfunction in TBI + DM rats were aggravated after injury. This change was significantly aggravated by the administration of a NOS inhibitor. Thus, NOS plays important regulatory and protective roles in oxidative stress, the inflammatory response, BBB permeability, vascular endothelial function, and neuronal apoptosis in diabetic rats after trauma.

NOS is an isoenzyme expressed in neurons, and three NOS subtypes have been identified: neuronal NOS and endothelial NOS are expressed under normal conditions, whereas inducible NOS is expressed after injury. NO is synthesized by

NOS in endothelial cells and plays important neuroprotective roles in the maintenance of cerebral microcirculation, the inhibition of platelet aggregation, the expansion of blood vessels, the prevention of inflammation and apoptosis, and the promotion of angiogenesis (Sauvant et al., 2009; De Cillà et al., 2017; Xu et al., 2018). Studies have also found that NO plays a dual role in cerebral ischemia. When hypoxic-ischemic brain injury occurs, inducible NOS and neuronal NOS produce excessive NO in neurons. Excessive NO combines with superoxide anions to produce cytotoxic peroxynitrite anions and accelerates tissue lipidization. This peroxidation reaction depletes thiols such as glutathione, produces excessive free radicals, induces oxidative stress damage in the body, and leads to neuronal apoptosis (Kashihara et al., 2010). Vascular endothelial dysfunction is a primary cause of poor prognosis in patients with DM and TBI. On the one hand, the high expression of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha in diabetic patients induces Notch signal and blunts the activation of the NOS-related signaling pathway, which results in the reduction of NO production and the aggravation of vascular endothelial dysfunction (Sawada et al., 2014). On the other hand, 24 hours after TBI, endothelium-dependent dilatation decreased sharply, due primarily to NOS-mediated small artery dilatation and endothelial cell-mediated vasodilation. During traumatic neuronal injury, NOS phosphorylation levels increase significantly, and NO production decreases, which may cause

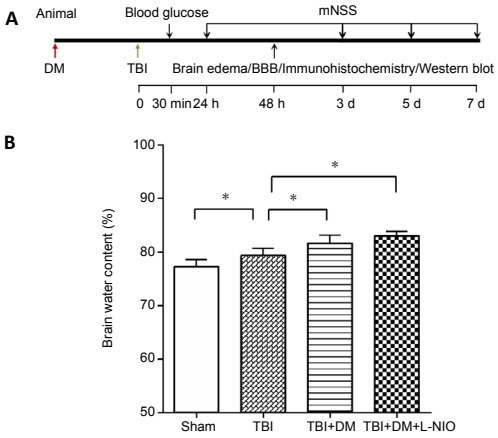

Figure 1 | A diagram illustrating the protocol and changes in brain water contents in rats with DM complicated with TBI.
(A) A diagram illustrating the protocol. The bottom horizontal axis represents time, and the arrows indicate the experimental operation performed at each time. (B) Water contents of damaged ipsilateral brain tissues, 48 hours after TBI, detected by dry-wet weight method. Brain water content (%) = (wet weight − dry weight)/(wet weight) × 100. Data are expressed as the mean ± SD (n = 6). *P < 0.05 (one-way analysis of variance followed by Bonferroni *post hoc* test). BBB: Blood-brain barrier; DM: diabetes mellitus; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; mNSS: modified neurological severity score; TBI: traumatic brain injury.

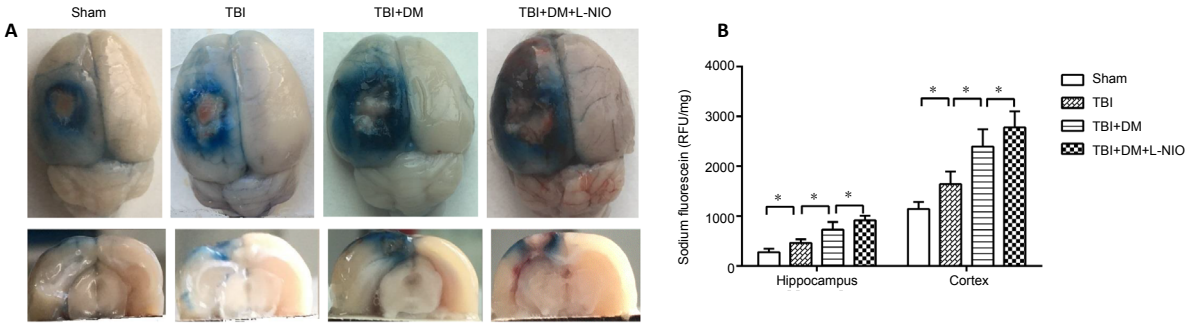

Figure 2 | Changes in the blood-brain barrier permeability in the injured penumbra and hippocampus of rats with DM complicated with TBI.
The blood-brain barrier permeability was evaluated by Evans blue staining. (A) The permeability of Evans blue dye in rat brain tissues. Compared with the Sham group, rats in the TBI group demonstrated an increased Evans blue penetration rate on the injured side of the brain, 2 days after surgery. Hyperglycemia significantly increased the penetration rate of Evans blue in the injured brain tissue. (B) Quantitative results of sodium fluorescein levels. Data are expressed as the mean ± SD (n = 5). *P < 0.05 (one-way analysis of variance followed by Bonferroni *post hoc* test). DM: Diabetes mellitus; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; RFU: relative fluorescence unit; TBI: traumatic brain injury.

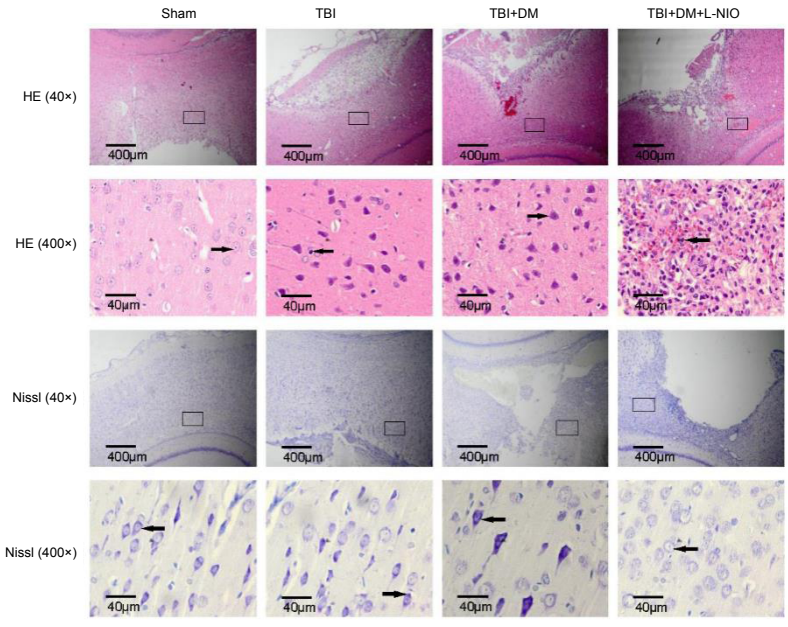

Figure 3 | Pathological changes in the penumbra region of rats with DM complicated with TBI.
Hematoxylin and eosin (HE) staining and Nissl staining in brain tissues from the rats in the four experimental groups (n = 6). The results showed that the brain tissue of rats in the Sham group displayed a regular cellular morphology, with uniform distribution, clear nucleoli and borders, rich cytoplasm, and no obvious neuronal degeneration and necrosis. The brain tissues from the other three groups of rats showed varying degrees of brain tissue destruction, massive hemorrhage, tissue relaxation and edema, and decreased cytoplasmic neighbors. Arrows indicate pathological changes. Scale bars: 400 μm and 40 μm (as labeled). DM: Diabetes mellitus; HE: hematoxylin-eosin; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; TBI: traumatic brain injury.

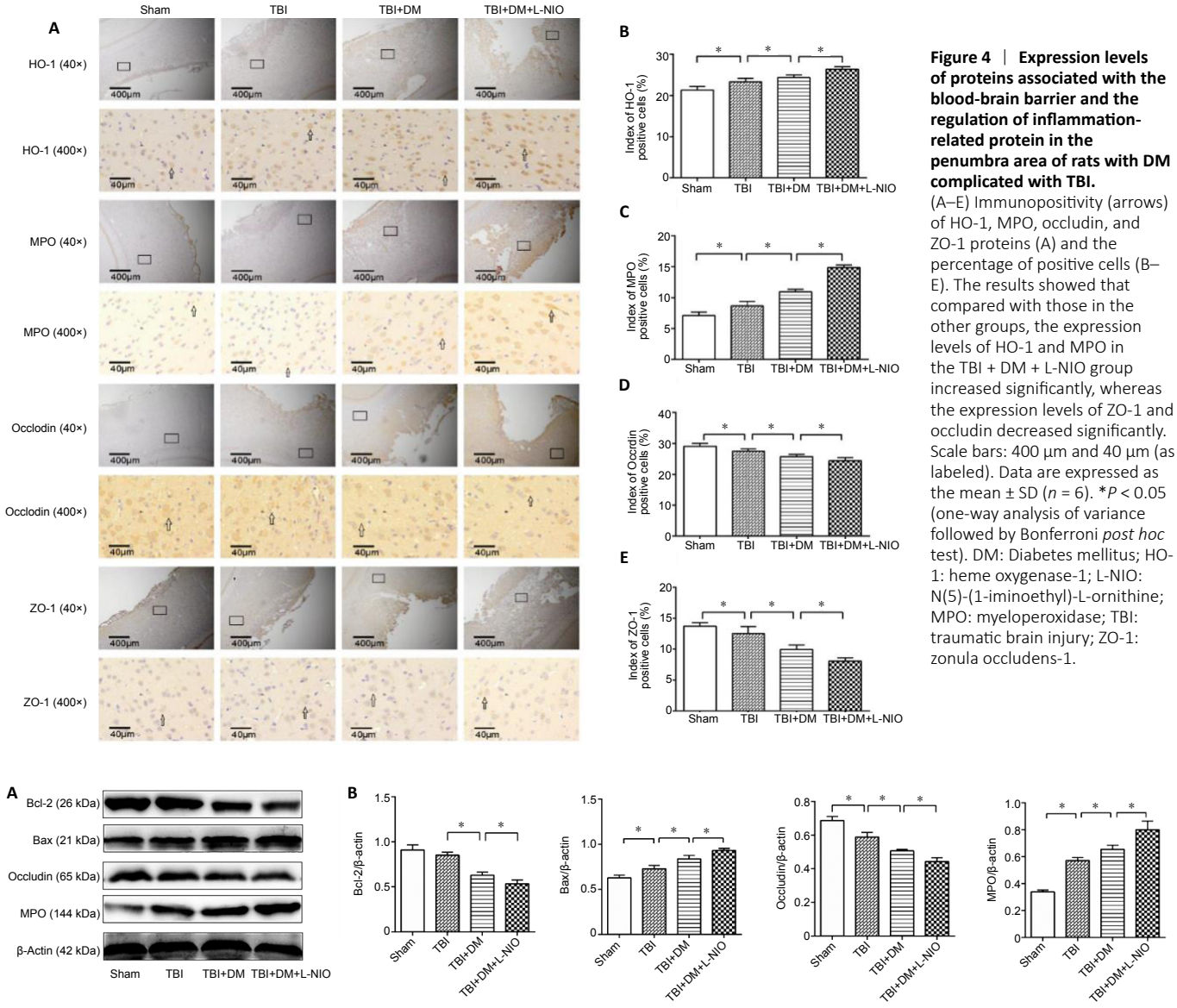

Figure 4 | **Expression levels of proteins associated with the blood-brain barrier and the regulation of inflammation-related protein in the penumbra area of rats with DM complicated with TBI.**
(A–E) Immunopositivity (arrows) of HO-1, MPO, occludin, and ZO-1 proteins (A) and the percentage of positive cells (B–E). The results showed that compared with those in the other groups, the expression levels of HO-1 and MPO in the TBI + DM + L-NIO group increased significantly, whereas the expression levels of ZO-1 and occludin decreased significantly. Scale bars: 400 μm and 40 μm (as labeled). Data are expressed as the mean ± SD (n = 6). *P < 0.05 (one-way analysis of variance followed by Bonferroni *post hoc* test). DM: Diabetes mellitus; HO-1: heme oxygenase-1; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; MPO: myeloperoxidase; TBI: traumatic brain injury; ZO-1: zonula occludens-1.

Figure 5 | **Protein expression levels of Bax, Bcl-2, occludin, and MPO in the penumbra area of rats with DM complicated with TBI.**
(A) The detection of Bax, Bcl-2, occludin, and MPO protein expression by western blot. (B) Quantification of Bax, Bcl-2, occludin, and MPO protein expression levels. Data are expressed as the mean ± SD (n =5). *P < 0.05 (one-way analysis of variance followed by Bonferroni *post hoc* test). DM: Diabetes mellitus; L-NIO: N(5)-(1-iminoethyl)-L-ornithine; MPO: myeloperoxidase; TBI: traumatic brain injury.

endothelial dysfunction after brain injury (Zhou et al., 2014; Schwarzmaier et al., 2015; Villalba et al., 2017). Therefore, NOS plays an important role in TBI and DM, through the downstream molecule NO.

Occludin is one of the most representative proteins found in tight junctions (TJs) and is directly involved in the barrier functions of TJ proteins, regulating structural changes in TJs, together with ZO-1 (Yu et al., 2005; Li and Wang, 2020). ZO-1 is one of the primary components of TJs that form between cells. Changes in ZO-1 expression levels are closely related to changes in the permeability of brain microvessels and the degree of cerebral edema (Jiao et al., 2011). HO-1 is an endogenous protein involved in the regulation of brain-protective factors and the inhibition of neuronal apoptosis (Bharucha et al., 2010). MPO is a specific marker of myeloid cells, which plays roles in the production and regulation of inflammatory responses. MPO can catalyze the production of excessive oxidants, causing oxidative stress and tissue damage at the inflammation site (Jang and Rabb, 2009). The Bcl-2 and Bax have been identified as typically anti- and pro-apoptotic proteins, respectively, and their protein expression levels have been closely associated with apoptosis (Volkmann et al., 2014; Kosuru et al., 2018). In the present study, the results suggested that the mechanism through which hyperglycemia affects brain injury may involve changes to TJs and active transport mechanisms that lead to the down-regulation of occludin and ZO-1. These changes result in the opening of TJs, increasing BBB permeability and promoting neuronal apoptosis, oxidative stress, and inflammation through the increased expression of HO-1, MPO, and Bax and the decreased expression of Bcl-2. Moreover, the mNSS results associated with different treatments were consistent with changes in the expression of proteins involved in BBB permeability. The mNSS includes the evaluation of motor, sensory, reflex, and balance systems (Chen et al., 2001), which can reflect the neurological status of the rat.

During the experiment, we were surprised to find that the blood glucose level was significantly reduced 30 minutes after brain injury, which may be associated with glucose being the primary energy source for the brain. Increased energy consumption during the early stages of TBI may result in reduced extracellular glucose levels after TBI. The level of endogenous glucose may not be sufficient to meet

the increased energy consumption required by the impaired brain, which may lead to secondary damage associated with dysregulated metabolism (Bosarge et al., 2015). A rodent TBI model provided additional support for this hypothesis (Moro et al., 2013). After injury, exogenous glucose administration was shown to provide an important cellular protective effect and improved brain metabolism. Another study suggested that severe TBI can trigger stress and metabolic reactions in patients, which can lead to the release of catecholamines and increase blood glucose levels (Alvis-Miranda et al., 2014; Khajavikhan et al., 2016). Hill et al. (2010) studied the effects of persistent hyperglycemia on diabetic patients and streptozotocin-induced animal models of type 1 DM. The results showed that hyperglycemia was able to alleviate cerebral edema. Glucose in the serum acts as a penetrant, forming a concentration gradient that may reduce the water contents of the damaged brain (Hill et al., 2010). Differences in experimental results may be due to differences in the animal models used. In the present study, a type 2 DM model was generated, after which a TBI treatment was administered, which reduces the likelihood of persistent hyperglycemia. In this experiment, we found that the increased glucose level aggravated the degree of brain injury, which may be related to the insulin response and the inability to absorb glucose in DM rats, which require further confirmation. In addition, the time points used differed between the two studies were different. We primarily observed the indicators and long-term neurological functions 48 hours after TBI, without examining the early post-traumatic effects.

The present study had some limitations. Because of the limited experimental conditions, the intracranial pressure, blood pressure, and NO and NOS contents were not monitored in the rats in this study, which could provide additional information to inform the pathophysiological changes that occur after TBI. In addition, only a moderate TBI animal model was established in this study. Further studies remain necessary to assess the effects of DM on TBI prognosis in mild, moderate, and severe TBI animal models, to simulate diabetic patients with varying degrees of TBI.

In summary, the present data demonstrated that hyperglycemia associated with DM could stimulate inflammation, affect microcirculation, and increase the permeability of BBB, which can aggravate TBI injury in rats, and ultimately affecting the prognosis of rats with DM and TBI. NOS plays important roles in the maintenance of cerebral microcirculation, anti-inflammation, anti-oxidative stress, and anti-apoptosis. These findings can deepen our understanding of the pathology of TBI and provide new ideas for effective treatments and interventions in patients with DM combined with TBI.

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