Enriched environment may improve secondary brain injury after traumatic brain injury by regulating the TLR2/NF-κB signaling pathway

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ABSTRACT

BACKGROUND: Traumatic brain injury (TBI) can cause damage to the blood-brain barrier, resulting in neuroinflammatory reactions and brain edema that seriously affect the recovery of neurological function. We hypothesize that an enriched environment (EE) regulates the TLR2/NF-κB signaling pathway and thereby modulates the integrity of the blood-brain barrier to achieve neuroprotective effects.

OBJECTIVE: This study evaluated the expression of toll-like receptor (TLR)-2 after TBI in a rat model, with the aim of determining whether TLR2/NF-κB improves secondary brain injury by inhibiting the release of inflammatory factors and reducing brain edema.

METHODS: We established a TBI model using Sprague-Dawley rats and implemented EE intervention or TLR2 siRNA to reduce TLR2. Western-blot analysis, real-time PCR, immunofluorescence staining, ELISA, TUNEL and FJC staining, wet–dry methods, rotarod testing, and neurological scoring were then applied for analysis.

RESULTS: Our results revealed that TLR2 was activated after TBI in rats and that EE or silencing of TLR2 with TLR2 siRNA reduced the level of inflammation, significantly alleviating brain edema, neuronal apoptosis, and degeneration. TBI exacerbated brain edema and nerve damage caused by TLR2/NF-κB signaling, and EE appeared to regulate neuroinflammation and brain edema by reducing TLR2.

CONCLUSIONS: Inhibition of TLR2 with EE might constitute a successful approach in the management of TBI.

PLAIN LANGUAGE SUMMARY

Background: Traumatic brain injury (TBI) can cause damage to the blood-brain barrier, resulting in neuroinflammatory reactions and brain edema that seriously affect the recovery of neurological function. We hypothesize that an enriched environment (EE) regulates the TLR2/NF-κB signaling pathway and thereby modulates the integrity of the blood-brain barrier to achieve neuroprotective effects. Objective: This study evaluated the expression of toll-like receptor (TLR)-2 after TBI in a rat model, with the aim of determining whether TLR2/NF-κB improves secondary brain injury by inhibiting the release of inflammatory factors and reducing brain edema. Methods: We established a TBI model using Sprague-Dawley rats and implemented EE intervention or TLR2 siRNA to reduce TLR2. Western-blot analysis, real-time PCR, immunofluorescence staining, ELISA, TUNEL and FJC staining, wet–dry methods, rotarod testing, and neurological scoring were then applied for analysis. Results: Our results revealed that TLR2 was activated after TBI in rats and that EE or silencing of TLR2 with TLR2 siRNA reduced the level of inflammation, significantly alleviating brain edema, neuronal apoptosis, and degeneration. TBI exacerbated brain edema and nerve damage caused by TLR2/NF-κB signaling, and EE appeared to regulate neuroinflammation and brain edema by reducing TLR2. Conclusions: Inhibition of TLR2 with EE might constitute a successful approach in the management of TBI.

KEYWORDS: Enriched environment, brain injury, TLR2, blood-brain barrier, neuroinflammation

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Introduction

Traumatic brain injury (TBI) is a type of brain injury that causes nerve and neuropsychologic abnormalities due to impact, penetration, or vibration, and affects the normal functioning of the brain.[1,2] The annual incidence of TBI worldwide is 349/100,000 individuals each year, constituting the third-highest

cause of death globally.[3] Secondary brain injury following TBI arises from damage to the brain's vessels and tissue; mechanisms include oxidative stress, inflammatory reaction, excitotoxicity, an imbalance in calcium homeostasis, heightened vascular permeability, destruction of the blood-brain barrier (BBB), and other pathologic mechanisms.[4]

The enriched environment (EE) is defined as the complex of various stimuli—such as the enlargement of the space for animal breeding and built-in objects that are rich and novel—that not only provide a variety of physical stimulation and sports opportunities but also engender the possibility of mutual social behavior.[5] In comparison to the typical feeding environment, EE offers increased physical and social interactions for animals.[6] Previous studies have shown uses for EE in therapeutic applications in central nervous system injuries such as cerebral ischemia, Parkinson's disease, and Alzheimer's disease.[7] EE can inhibit the chronic inflammatory response, induce cerebral vascular neogenesis to reduce BBB leakage in brain tissue, and improve neurological function in rats with brain injury.[5,8,9]

Toll-like receptors (TLRs) are a class of transmembrane receptors that recognize patterns, and they are found in both immune and non-immune cells in mammals—as well as in brain cells such as astrocytes, microglia, oligodendrocytes, neurons, and endothelial cells.[10,11] Some authors have reported that TLR2 expression was up-regulated in a cerebral ischemia model, exacerbating brain injury.[12,13] In an intracerebral hemorrhage model, TLR2 was involved in BBB damage, while TLR2 knockout in mice reduced bleeding volume, neurological damage, and BBB damage, neutrophil infiltration, and the expression of inflammatory factors.[14,15] Although these findings indicate that TLR2 is involved in the pathologic progression of BBB breakdown and inflammation, it is not known whether EE induces changes in BBB permeability and inflammation by regulating TLR2.

Materials and Methods

Animals

A total of 75 male Sprague-Dawley rats weighing between 250 and 280 g were obtained from the Zhaoyan New Drug Research Center in Suzhou, China, and we included 72 in our analysis. The rats had unrestricted access to food and water and were housed in a setting that maintained consistent temperature and humidity and was exposed to a 12-h light/dark schedule.

Study design and animal grouping

We employed a TBI rat model for this study. The rats did not show any noticeable variations in weight, food consumption, or physical abilities during the study. A total of 72 rats (those surviving from an original group of 75) underwent randomization into Sham, TBI 14 d, TBI + Con-siRNA, TBI + TLR2 siRNA, TBI + EE, and TBI + EE + TLR2 siRNA groups. After 14 days of TBI modeling and EE intervention, the brain tissue adjacent to the affected area was collected and divided. Tissue samples from the front of the injured area were analyzed using western-blot (WB) analysis and real-time PCR, while samples from the rear area were used for staining analyses. Six rats in each group were evaluated using WB, real-time PCR, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining, and Fluoro-Jade C (FJC) staining. Three additional rats from each group were assessed for brain edema, and six rats from each group were chosen at random for neurological score and rotarod testing before euthanasia. The experimental protocol timeline is shown in Figure 1. This study lasted for 14 months (from April 2023 to May 2024).

Establishment of TBI rat model

Experimental TBI in rats was established as described in a previous report.[16] Rats were anesthetized intraperitoneally with 1% sodium pentobarbital at 40 mg/kg and secured to a stereotaxic device (Yuyan, China). A parietal window measuring 5 mm was created on the right side of the midline and posterior to the coronal suture using a bone drill, leaving the dura undisturbed. A copper weight (4 mm wide and 5 mm high) was inserted into the bone opening, and a 40-g steel rod was released from a distance of 25 cm onto the copper weight to cause severe TBI. A brief interruption in the rat's heart rate and respiration signified a successful simulation. Following disinfection and stitching procedures, the rats were allotted time to recuperate in a heated environment. The Sham rats were also exposed to the same procedure, except for the steel rod insertion.

Housing conditions

After recovery from anesthesia, rats treated with EE were immediately placed in EE cages for 14 days, with six rats per cage. The EE cage (92 × 78 × 51 cm) was equipped with horizontal and inclined platforms and various toys such as balls, ladders, blocks, tunnels, and bridges; these items were rearranged daily. The rats in the Sham and TBI groups were raised separately in regular cages (37 × 25 × 18 cm).

TLR2 siRNA injection

Twenty-four hours prior to TBI, the TLR2 siRNA and control siRNA groups underwent intracerebroventricular injection (500 pmol; Thermo Fisher, USA) of an in vivo RNA transfection reagent (Engreen, China) according to a previously described method.[17]

Tissue collection and sectioning

Anesthesia with sodium pentobarbital was administered 14 days following the injury. Rats were injected with 200 mL of 0.9% saline solution through the heart, and samples of the cortex near

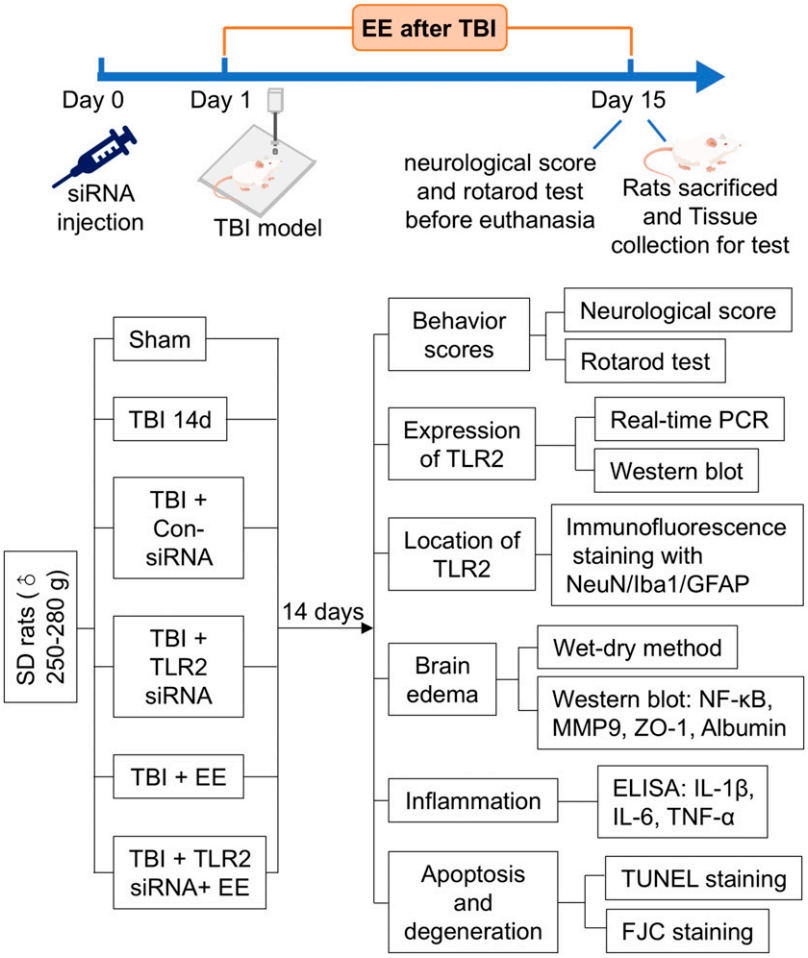

Figure 1. The experimental protocol timeline of this study.

the injury site were collected and kept on ice. Some samples were rapidly frozen and stored at −80°C for WB and real-time PCR. After preservation in 4% paraformaldehyde for more than 24 h, the remaining samples were encased in paraffin and sectioned at 5 μm using a paraffin slicer (SLEE medical GmbH, Germany) for immunofluorescence, TUNEL, and FJC staining. Two pathologists who had not seen the process extracted and chose the tissue samples.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and complementary DNA (cDNA) was synthesized using 1 μg of total RNA. Real-time PCR was conducted using the SYBR™ Green Master Mix kit (Thermo Fisher) on a QuantStudio™ RT-PCR Instrument (Thermo Fisher). The cycle conditions were denaturation at 95°C for 2 min, followed by amplification for 40 cycles (95°C for 15 s, 60°C for 15 s, and 72°C for 1 min), and extension at 72°C for 10 min; samples were then maintained at 4°C. We adopted GAPDH as the internal reference and analyzed the data using the $2^{-\Delta\Delta Ct}$ method. The sequences of each gene were as follows:

TLR2: FORWARD: 5′-TGGAGGTCTCCAGGTCAAATC-3’

REVERSE: 5′-ACCAGCAGCATCACATGACA-3’

GAPDH: FORWARD: 5′-TGTGAACGGATTTGGCCGTA-3’

REVERSE: 5′-GATGGTGATGGGTTTCCCGT-3′

Western-blot analysis

We performed WB analysis using a previously published method.[18] Brain tissues were mixed with a tissue protein extraction reagent (CWBIO, China) containing protease inhibitors before being homogenized on ice for 20 min. Centrifugation at 12,000 g and 4°C was used to isolate the homogenates. We quantified protein with a Pierce™ BCA Protein Assay Kit (Thermo Fisher). Equivalent quantities of total protein were separated using SDS-PAGE and then transferred electrically onto a PVDF membrane (Millipore, USA). Following membrane blocking using QuickBlock™ Blocking Buffer (Beyotime, China) at room temperature for 1 h, primary antibodies were added, and membranes were incubated

overnight at 4°C. Goat anti-rabbit or anti-mouse IgG-HRP (Invitrogen) was subsequently added, and membranes were incubated at room temperature for 1 h. Finally, the membranes were analyzed with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and an imaging system (BioRad, CA, USA) for viewing, and quantification was performed using ImageJ (National Institutes of Health, USA) with GAPDH used as control. The primary antibodies were rabbit anti-TLR2 (Abcam, ab213676,1:1000), rabbit anti-NF-κB (Cell Signaling, 8242S, 1:1000), rabbit anti-phospho-NF-κB (Cell Signaling, 3033S, 1:1000), rabbit anti-MMP9 (Abcam, ab76003, 1:2000), rabbit anti-Albumin (Abcam, ab207327, 1:2000), rabbit anti-ZO-1 (Proteintech, 21773-1-AP, 1:5000), and rabbit anti-GAPDH (Sigma, G9545-200UL, 1:10000).

Immunofluorescence staining

We implemented double-immunofluorescence staining according to the methods outlined in a previous study.[19] After dewaxing, the paraffin sections were treated with immunostaining permeabilization solution (Beyotime) to rupture the cell membranes and rinsed three times with PBS. Immunostaining block solution (Beyotime) was used to block sections for 30 min, followed by overnight incubation at 4°C with primary antibodies that included rabbit anti-TLR2 (Abcam, 1:200), mouse anti-NeuN (Abcam, 1:1000), mouse anti-GFAP (Millipore, 1:800), and mouse anti-Iba1 (Abcam, 1:1000). After washing three times with PBS, the sections were exposed to secondary antibodies that included Alexa Fluor 488 donkey anti-rabbit IgG antibody (Invitrogen) and Alexa Fluor 555 donkey anti-mouse IgG antibody (Invitrogen) for 1 h at room temperature. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was then applied for counterstaining, and the sections were observed using a fluorescence microscope (OLYMPUS, Japan).

ELISA

We utilized ELISA to measure the levels of IL-1β (BOSTER, China), IL-6 (YIFEIXUE, China), and TNF-α (BOSTER) near the damaged brain region in rats, following the manufacturer's instructions.

Neurological score

Neurological score evaluation was performed before the rats were sacrificed.[16] There were seven elements in the scoring system, including spontaneous activity, axial sensation, vibrissae proprioception, limb movement symmetry, lateral turning, forelimb outstretching, and climbing. Each subtest received a score ranging from 0 to 3, with a total possible score of 21.

Rotarod test

The rats were subjected to an accelerated rotarod test prior to sacrifice. Rats were placed on an accelerated rotation cylinder with multiple runways in which the speed was increased from 4 to 30 r/min within 60 s; rotational velocity was maintained at 30 r/min for 5 min, during which time the rats were allowed to walk on the device to prevent falling.[20] The trial ended if the animal fell off the pedal, and the time the rats spent on the device was ultimately recorded.

Brain edema

Evaluation of brain edema was performed using the wet–dry method.[21] After separating rat brains, they were split into ipsilateral and contralateral sides, and the wet weight was immediately determined. The brain tissues were subsequently dehydrated at a temperature of 100°C for 24 h, after which the weights of the dried tissues were recorded. The brain's water content was measured by calculating the percentage of water in relation to the wet weight after subtracting the dry weight.

TUNEL staining

Apoptosis was measured using a TUNEL kit (Beyotime). Following dewaxing in xylene, paraffin sections underwent a 20-min treatment with DNase-free proteinase K (20 μg/mL) at 37°C. A 1-h incubation was then conducted using the TUNEL working solution at 37°C in the absence of light. Next, DAPI Fluoromount-G™ (YEASEN, China) was applied for counterstaining before observation under a fluorescence microscope (OLYMPUS). We calculated the apoptotic index as the percentage of TUNEL-positive cells divided by the total number of cells.

Fluoro-Jade C staining

The extent of neuronal degeneration was assessed using an FJC staining kit (Biosensis, USA). After dewaxing in xylene, the paraffin sections were moved to solution B that consisted of potassium permanganate and distilled water in a 1:9 ratio, and left to incubate for 10 min. The sections were then treated with a mixture of FJC solution and distilled water (1:9, vol/vol) for 30 min in the absence of light, followed by rinsing with distilled water. The samples were dried at 60°C for 10 min, immersed in xylene for 5 min, and covered with neutral balsam (YEASEN) before examination under a fluorescence microscope (OLYMPUS).

Statistical analyses

We presented our data as mean ± standard deviation and conducted the analysis with GraphPad Prism 8.0 (USA). Multiple groups were compared using a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's multiple-comparison test. We analyzed brain edema using a two-way ANOVA followed by post-hoc Tukey's multiple-comparison test. Statistical significance is indicated at the $P < 0.05$ level.

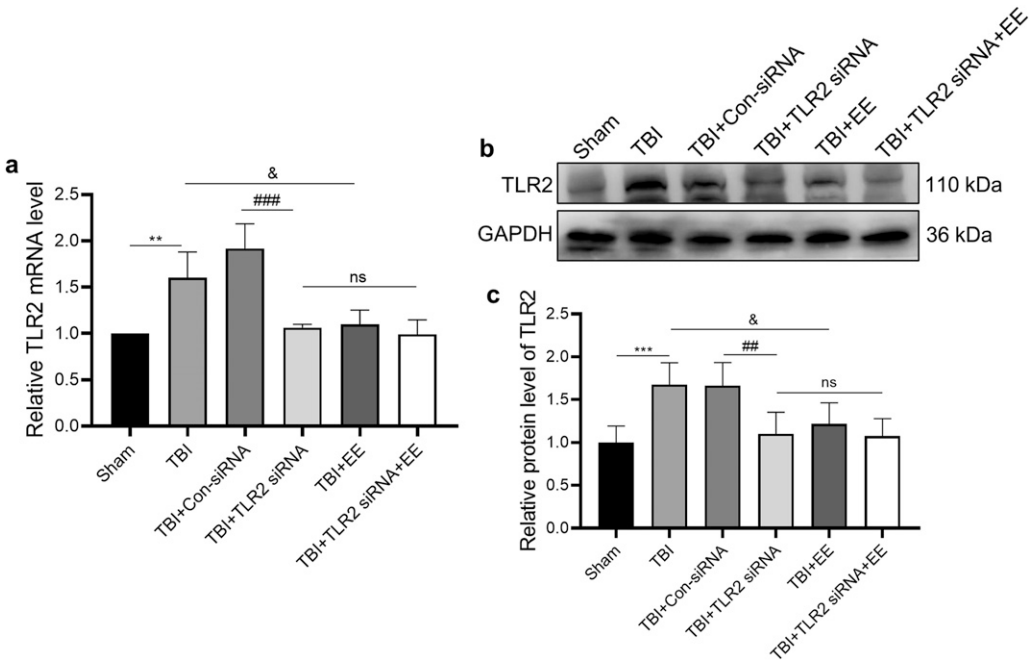

Figure 2. mRNA and protein levels of TLR2 in the brain of TBI rats. (A) Real-time PCR was performed to assess mRNA levels of TLR2. (B, C) Western-blot analysis was conducted to assess TLR2 protein levels in the Sham group, TBI group, TBI + Con-siRNA group, TBI + TLR2 siRNA group, TBI + EE group, and TBI + TLR2 siRNA + EE group. We statistically analyzed our data with one-way ANOVA and Tukey's post-hoc test (n = 6/group).

Results

TLR2 mRNA and protein levels in the brains of TBI rats after EE or TLR2 siRNA administration

We noted that TLR2 mRNA expression in the brain tissue around the TBI injury increased 14 days after TBI compared with the Sham group and that TLR2 protein expression was consistent with mRNA levels. Following TLR2 siRNA treatment or exposure to EE, we observed that the mRNA and protein levels for TLR2 fell in both the TLR2 siRNA and EE groups relative to the Con-siRNA group, with a slightly lesser reduction in the EE group compared with the TLR2 siRNA group (Figure 2).

Localization of TLR2 in the brains of rats with TBI

Immunofluorescence was employed to clearly indicate the presence of TLR2 by staining with NeuN as a neuronal marker (Figure 3(A)), GFAP as an astrocyte marker (Figure 3(B)), and Iba1 as a microglial marker (Figure 3(C)). Our findings indicated that TLR2 was present in neurons, astrocytes, and microglia as shown by co-staining with NeuN/GFAP/Iba1 (arrow).

Expression of NF-κB and MMP9 in TBI brains after EE or TLR2 siRNA injection

The expression of NF-κB (Figure 4(A) and (C)) and MMP9 (Figure 4(B) and (D)) in the TBI group was higher than in the Sham group, but after intervention with TLR2 siRNA or EE, their expression diminished compared with the Con-siRNA group. When TLR2 siRNA and EE were applied in combination, the expression of NF-κB and MMP9 was reduced to a greater extent than with either alone.

Brain edema in TBI rats after EE or TLR2 siRNA injection

Tight junction protein ZO-1 (Figure 5(A)-(C)) and albumin (Figure 5(B)-(D)) were adopted as indicators of cell membrane integrity, and we observed reduced expression of ZO-1 in the TBI group relative to the Sham group, whereas albumin expression reflected an increase. EE or TLR2 siRNA administration of resulted in augmented ZO-1 expression compared with the Con-siRNA group, while albumin expression was diminished. Furthermore, when the wet–dry method was employed to assess brain edema (Figure 6(E)), our findings indicated a rise in brain water content on the damaged side of TBI and that this was reversed by TLR2 siRNA or EE.

Levels of inflammatory factors in brain tissue of rats with TBI after EE or TLR2 siRNA injection

ELISA results indicated that levels of IL-1β (Figure 5(E)), IL-6 (Figure 5(F)), and TNF-α (Figure 5(G)) were elevated in the TBI group compared with the Sham group. However, following EE or injection of TLR2 siRNA, the expression of these cytokines was reduced in both groups compared with the Con-siRNA group.

Neuronal apoptosis or degeneration in TBI rats after EE or TLR2 siRNA injection

TUNEL staining was used to reflect neuronal apoptosis (Figure 6(A)), and our results revealed that the number of TUNEL-positive cells rose after TBI, while number of

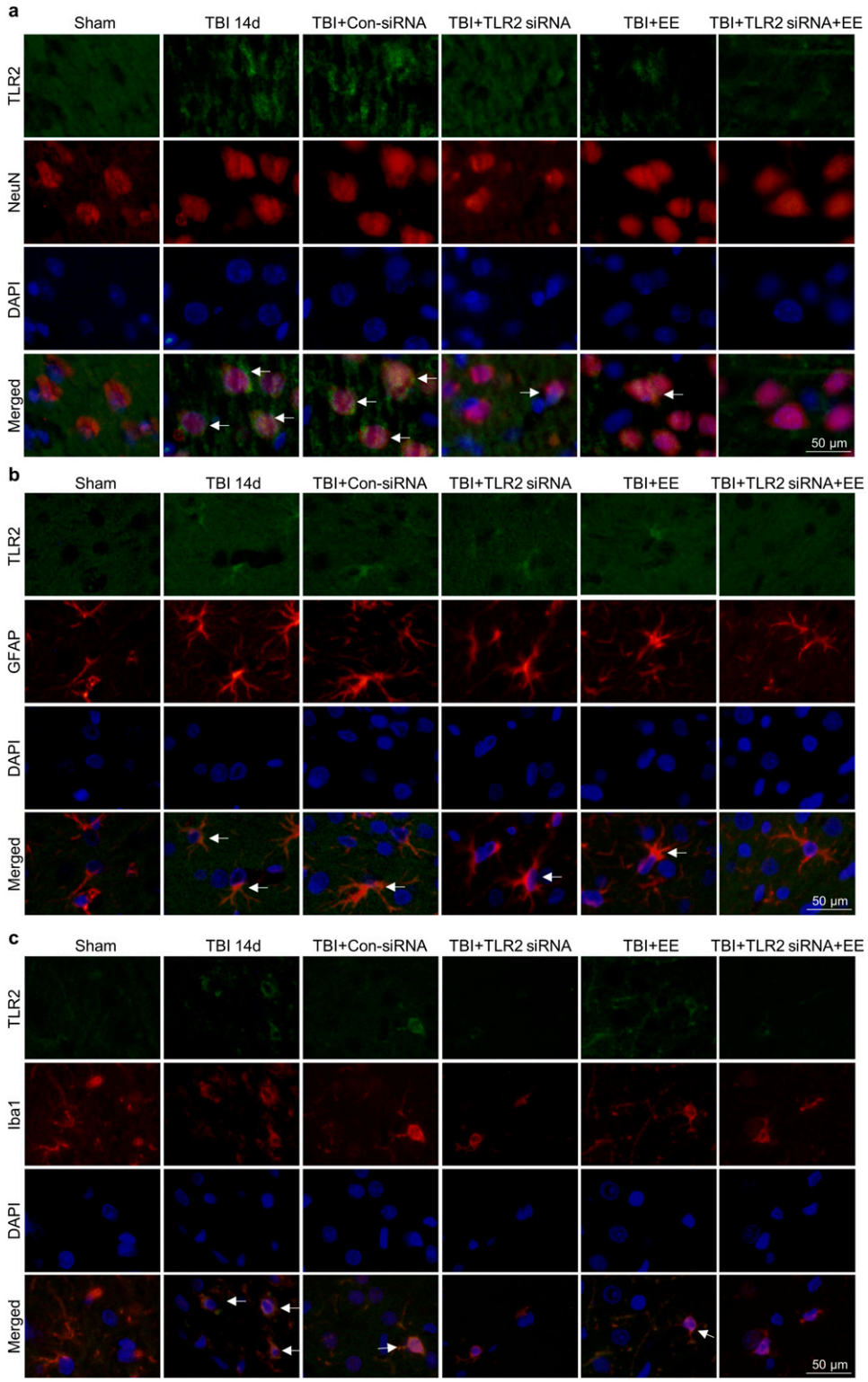

Figure 3. Localization of TLR2 in neurons/astrocytes/microglia of TBI rats Double-immunofluorescent staining images of green-marked TLR2 and red-marked NeuN (2a)/GFAP (2b)/Iba1 (2c) in the Sham group, TBI 14 d group, TBI + Con-siRNA group, TBI + TLR2 siRNA group, TBI + EE group, and TBI + EE + TLR2 siRNA group; the nuclei were fluorescently labeled with DAPI (blue). Arrows indicate the co-localization of TLR2 and neurons/astrocytes/microglia (scale bar = 50 μm).

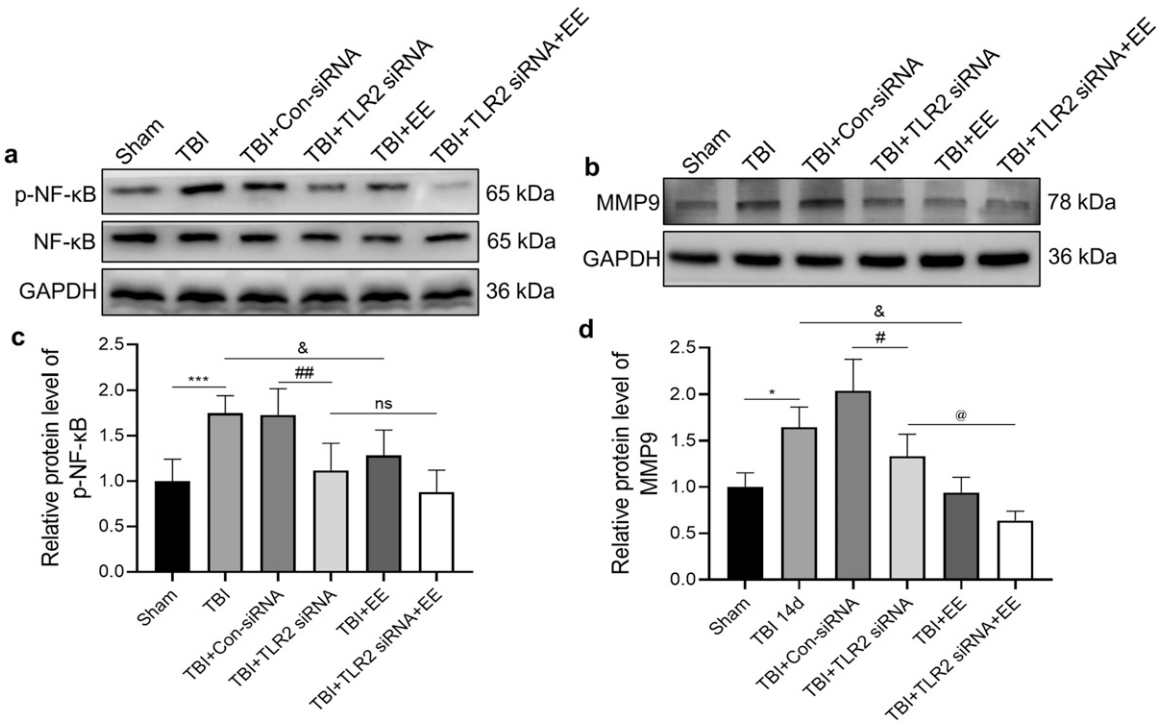

Figure 4. p-NF-κB and MMP9 protein levels in the brain of TBI rats (A, C) Western-blot analysis was performed to assess p-NF-κB protein levels in the Sham group, TBI group, TBI + Con-siRNA group, TBI + TLR2 siRNA group, TBI + EE group, and TBI + TLR2 siRNA + EE group. (B, D) Western-blot analysis was conducted to assess MMP9 protein levels in the Sham group, TBI group, TBI + Con-siRNA group, TBI + TLR2 siRNA group, TBI + EE group, and TBI + TLR2 siRNA + EE group. Statistics were analyzed with one-way ANOVA and Tukey's post-hoc test (n = 6/group).

TUNEL-positive cells fell in the EE and TLR2 siRNA groups compared with the Con-siRNA group. FJC staining was used to ascertain cellular degeneration (Figure 6b) and showed an elevation in the number of degenerating cells following TBI, which was then reduced after EE treatment or administration of TLR2 siRNA.

Neurological scores and motor function in rats after EE or TLR2 siRNA injection

The neurological scores of rats were decreased after TBI, but EE or TLR2 siRNA injection reversed this drop in scores ((Figure 6(C)). The rats' motor function was assessed using the rotarod test, and results indicated that TBI rats possessed a reduced drop time compared with Sham rats and that the rats' retention time on the rotarod increased following EE or TLR2 siRNA injection relative to the TBI rats (Figure 6(D)).

Discussion

We herein assessed intervention with EE or TLR2 siRNA after TBI in rats to verify the effects of EE on secondary brain injury after TBI. Our findings indicated that TBI enhanced the levels of TLR2 and activated the NF-κB/MMP9 pathway to aggravate neuroinflammation and brain edema that resulted from BBB disruption. EE decreased the levels of TLR2 and NF-κB/MMP9 signal-pathway transduction, leading to a reduction in inflammatory factors and nerve apoptosis and necrosis; this ultimately enhanced the recovery from secondary brain injury in rats with TBI. EE and TLR2 siRNA also showed equivalent effects. EE refers to a living environment composed of multiple stimuli and social interactions, unaffected by the effects of sex, age, or injury.[22] Increasing evidence indicates that EE enhances functional recovery following TBI and that exposing TBI rats to EE can facilitate the improvement of their neuromotor impairment.[23] Our research also showed that EE improved the rats' neurologic scores and motor function after TBI (Figure 6(C)-(D)).

Neuroinflammation and BBB dysfunction are two important mechanisms underlying the effects of secondary brain injury in TBI. Previous studies have shown that TLRs play an important role in the initiation and spread of neuroinflammation caused by TBI.[24,25] For example, TLR2 levels were significantly elevated in TBI patients, but neuronal cell death caused by neuroinflammation and apoptosis was ameliorated through the upregulation of TNF-α, IL-1β, IL-6, and NF-κB levels.[26] TLR2 knockout in mice mitigated the harmful consequences of a range of secondary injuries resulting from ischemic injury and reduced overall mortality.[27] The results of the present study indicated that EE could reverse neuronal apoptosis and necrosis caused by the elevation of TLR2 after TBI (Figure 6(A)-(B)). In addition, studies have shown that the activation of microglia in the cerebral cortex persists for a lengthy period after injury[28,29] and

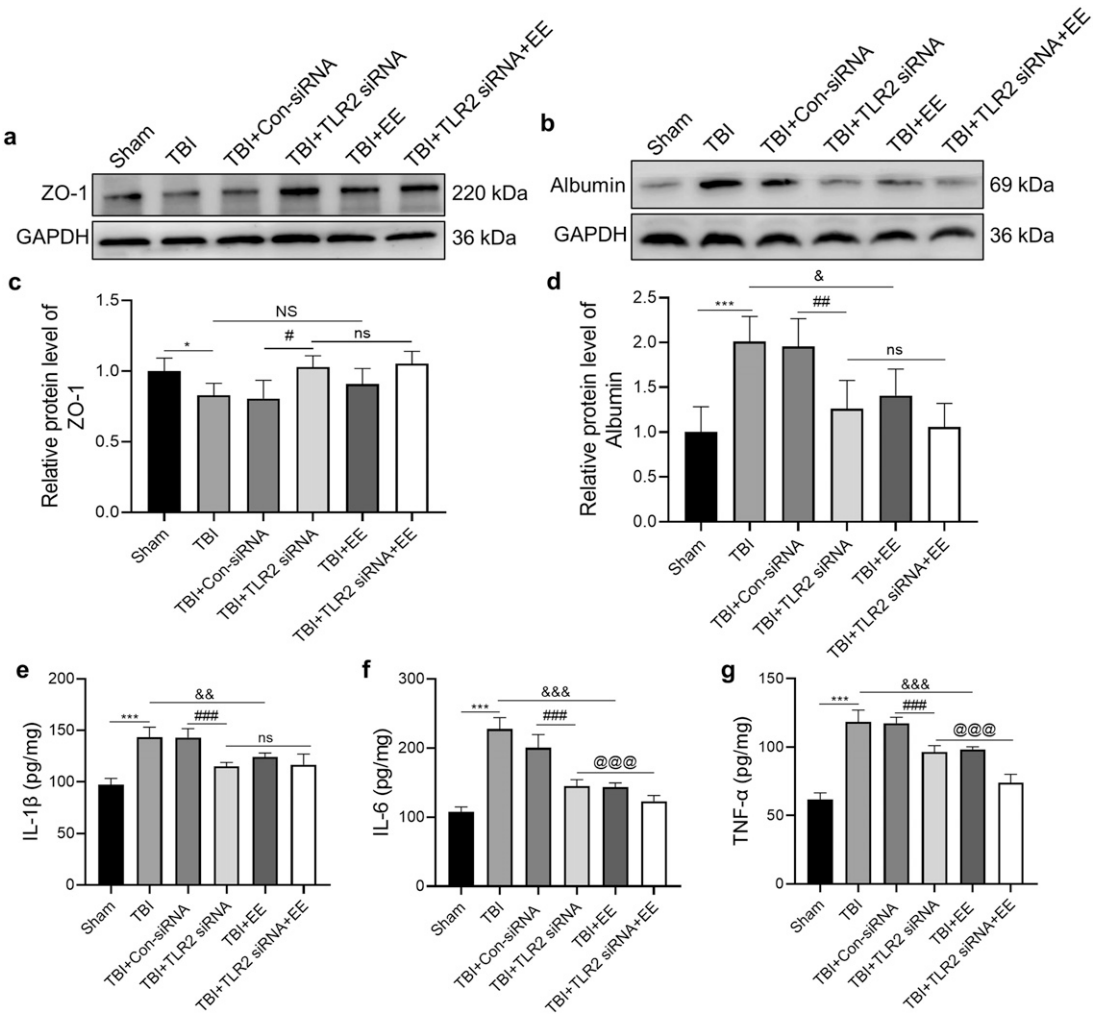

Figure 5. Injury to the blood-brain barrier (BBB) and inflammatory levels in TBI rats. Protein levels for ZO-1 (A, C) and albumin (B, D) in the Sham group, TBI group, TBI + Con-siRNA group, TBI + TLR2 siRNA group, TBI + EE group, and TBI + TLR2 siRNA + EE group were determined to reflect BBB injury. ELISA was used to quantify the expression levels of inflammatory factors IL-1β (E), IL-6 (F), and TNF-α (G) in the surrounding brain tissue after TBI. Statistical analysis entailed one-way ANOVA and Tukey's post-hoc test (n = 6/group).

that this long-term inflammation affects neuronal repair and functional recovery after TBI. We demonstrated that the expression of TLR2 increased after TBI (Figure 2) and was co-localized with the marker Iba1 in microglia (Figure 3(C)). EE also reduced the overexpression of TLR2 after TBI while lowering the concentrations of inflammatory factors (Figure 5(E)-(G)).

The BBB is an essential barrier that regulates the biochemical environment in the brain to maintain homeostasis,[4] and its permeability is principally determined by the basement membrane and extracellular matrix of blood vessels.[30] MMP9 belongs to the matrix metalloproteinase group that is capable of breaking down the tight junctions between endothelial cells and extracellular matrix, causing the disruption of the BBB and resulting in brain edema.[31] After cerebral ischemia, down-regulation of MMP9 expression reduced the loss of the tight junction protein ZO-1 and the closure protein occludin to improve BBB permeability.[32] Inhibition of MMP9 also protects the integrity of the BBB after cerebral hemorrhage, thereby reducing the degree of brain injury.[33] Our previous investigation also revealed that MMP9 was upregulated in neurons and glial cells of the injured peripheral brain tissue after TBI and that it reduced the integrity of the BBB by degrading the tight junction protein collagen-IV and the basement membrane protein occludin, thus participating in the pathologic process of secondary brain injury.[34] In this study, we discerned that MMP9 expression rose (Figure 4(B) and (D)) and ZO-1 expression declined after TBI (Figure 5(A)-(C)). We additionally noted an elevation in albumin exudation (Figure 5(B)-(D)), indicating that the stability and integrity of intercellular connections were disrupted. EE reduced MMP9 levels and increased ZO-1 levels, reduced albumin exudation, and improved brain edema in our study (Figure 6(E)). MMP9 activity decreased significantly with EE, portraying a positive role in the reconstruction of dendrites in the cerebral cortex.[35] The tight-junction gaps between BBB vascular endothelial cells then

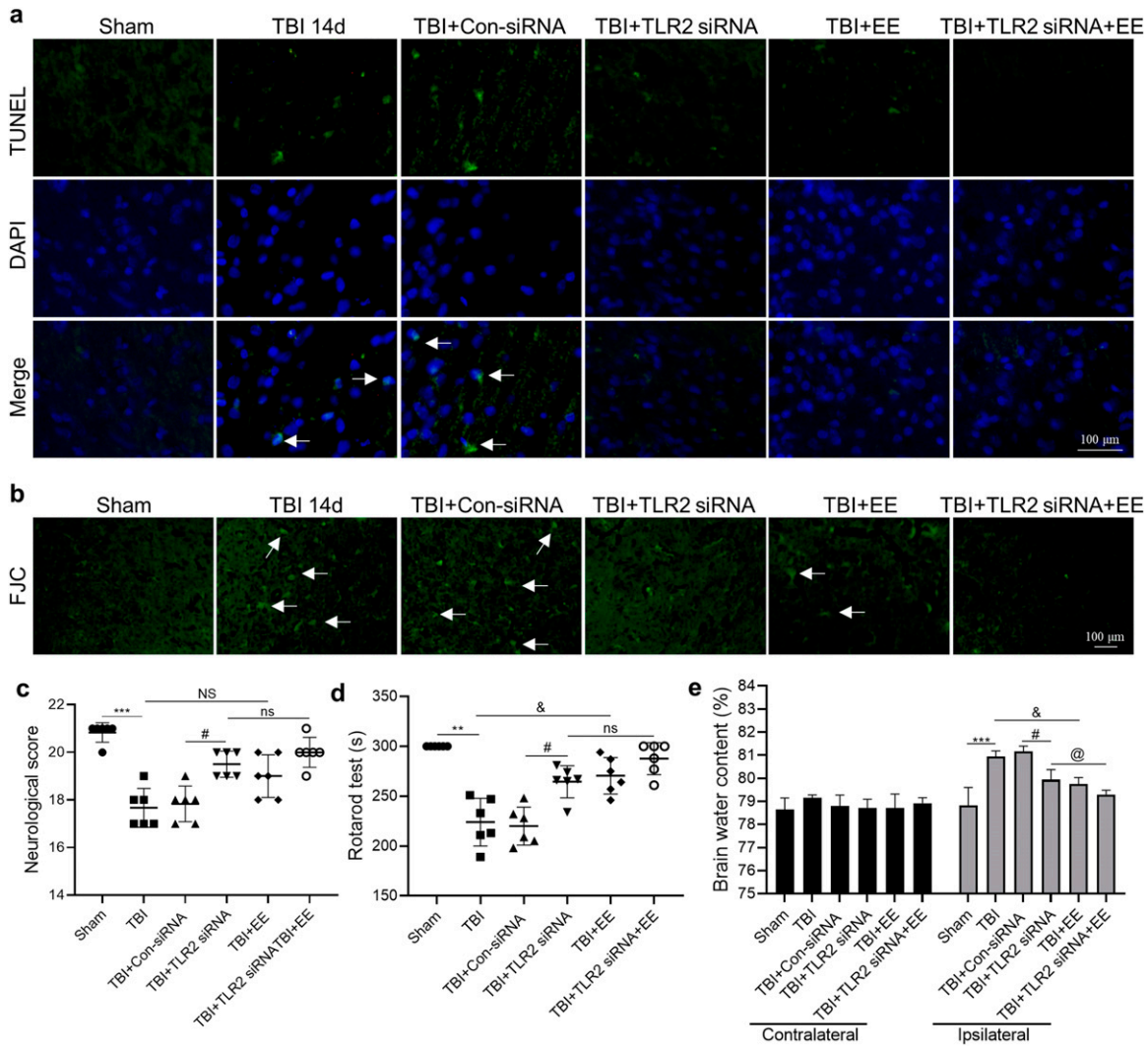

Figure 6. Neurological damage and brain edema in TBI rats (A) We detected neuronal apoptosis with TUNEL staining, and the arrows indicate the TUNEL-positive cells. (B) Denaturation was evaluated with FJC staining, and the arrows indicate the FJC-positive cells. (C) Neurological scores in TBI rats. (D) Motor function was reflected with the rotarod test. (E) Brain edema was measured using the wet–dry method. Statistical analysis was performed using one-way ANOVA and Tukey's post-hoc test, except for brain edema data. We analyzed brain edema using two-way ANOVA and Tukey's post-hoc comparison test (n = 6/group).

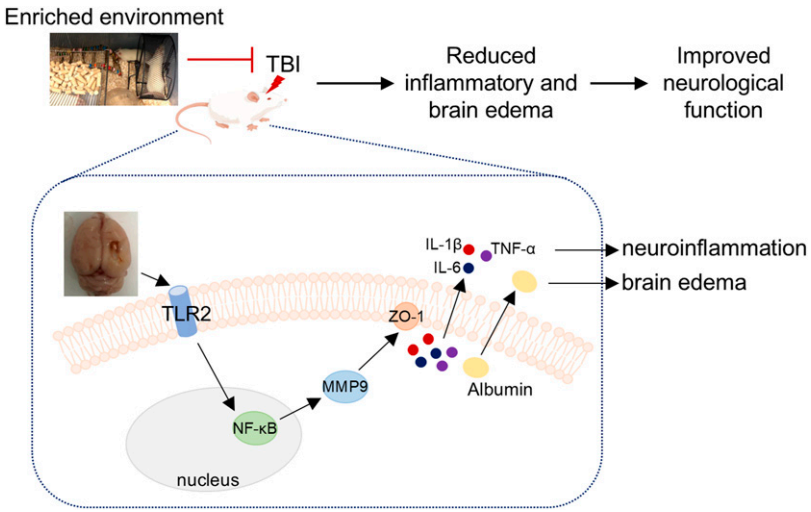

Figure 7. The mechanism of EE improving secondary brain injury after TBI through the TLR2/NF-κB/MMP9 pathway. EE treatment reduced TLR2 overexpression after TBI and inhibited the activation of the NF-κB/MMP9 pathway, reduced the release of inflammatory factors and albumin leakage, and improves secondary brain injury after TBI.

become smaller, and the swelling of astrocyte processes is significantly improved,[36] and this may promote the permeability of the blood brain barrier after cerebral ischemia.[5]

There were some limitations to the present study. First, the sample size of this study was relatively small (with nine rats per group), resulting in no statistical significance in the TBI + TLR2 siRNA and TBI + TLR2 siRNA + EE groups. However, the results of the comparison between the TBI and TBI + EE groups were statistically significant, and we therefore considered the effects of EE on the improvement of secondary brain injury in TBI rats as clear and distinct. In addition, only male adult Sprague-Dawley rats were selected in this study, and the effect of sex difference on EE was not considered.

Conclusions

In summary, this study provided important evidence for EE treatment of rats with TBI and secondary brain injury. Following TBI, TLR2 quickly rose and triggered the NF-κB/MMP9 pathway, leading to the breakdown of the BBB and resulting in the secretion of inflammatory factors and brain edema. Treatment with EE led to a notable diminution in TLR2 expression and in the activation of the NF-κB/MMP9 pathway, resulting in reduced inflammatory factor release, severity of brain edema, and neuronal cell death, as well as improved neurological function following TBI (Figure 7). We posit that blocking TLR2 with EE might constitute a successful approach in the prevention and management of TBI. In this research analysis, we investigated the impact of EE on secondary brain injury following TBI via animal experiments, offering experimental support for potential clinical use.

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Author contributions

Conception and design by MW, FG and BD. Material preparation, data collection and analysis were performed by MW, XH, YG, CW, YH and FG. The first draft of the manuscript was written by MW and XH and all authors commented on previous versions of the manuscript.

Ethical statement

Ethics approval

The Animal Ethics and Welfare Committee (AEWC) of Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine in Zhangjiagang, China, approved the experimental protocols (approval no. KY2023-0316-1) on March 16, 2023, which adhered to the Guidelines for Animal Care and Use promulgated by the National Institutes of Health (NIH), USA.

Consent for publication

All authors read and approved the final manuscript for publication.

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