Acta Pharmacologica Sinica 2017: 1445–1455
 1671-4083/17
www.nature.com/aps

Original Article

Sesamin alleviates blood-brain barrier disruption in mice with experimental traumatic brain injury

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Abstract

Sesamin, a major lignan of sesame oil, was reported to have neuroprotective effects in several brain injury models. However, its protective action in maintaining blood-brain barrier (BBB) integrity has not been studied. In this study we investigated the effects of sesamin on the BBB in a mouse model of traumatic brain injury (TBI) and explored the underlying mechanisms. Adult male C57BL/6 mice were subjected to a controlled cortical impact (CCI) injury and then received sesamin (30 mg·kg^{-1}·d^{-1}, ip). The mice were euthanized on the 1st and 3rd days after CCI injury and samples were collected for analysis. Sesamin treatment significantly attenuated CCI-induced brain edema on the 1st and 3rd days after the injury, evidenced by the decreases in water content, tissue hemoglobin levels, Evans blue extravasation and AQP4 expression levels in the ipsilateral cortical tissue compared with the vehicle-treated group. Furthermore, sesamin treatment significantly alleviated CCI-induced loss of the tight junction proteins ZO-1 and occludin in the brain tissues. The neuroprotective mechanisms of sesamin were further explored in cultured mouse brain microvascular bEnd.3 cells subjected to biaxial stretch injury (SI). Pretreatment with sesamin (50 μmol/L) significantly alleviated SI-induced loss of ZO-1 in bEnd.3 cells. Furthermore, we revealed that pretreatment with sesamin significantly attenuated SI-induced oxidative stress and early-stage apoptosis in bEnd.3 cells by decreasing the activation of ERK, p38 and caspase-3. In conclusion, sesamin alleviates BBB disruption at least partly through its anti-oxidative and anti-apoptotic effects on endothelial cells in CCI injury. These findings suggest that sesamin may be a promising potential therapeutic intervention for preventing disruption of the BBB after TBI.

Keywords: sesamin; traumatic brain injury; CCI injury; bEnd.3 cells; oxidative stress; apoptosis; BBB; tight junction proteins

Acta Pharmacologica Sinica advance (2017) 38: 1445–1455; doi: 10.1038/aps.2017.103; published online 3 Aug 2017

Introduction

Traumatic brain injury (TBI), a major cause of death and disability, also leads to long-term serious neurological impairment in survivors[1, 2]. In the United States alone, nearly 1 500 000 people have suffered from TBI, and approximately one third of trauma-related deaths are attributed to TBI[3], thus making it a serious issue for public health. TBI consists of a primary injury from the immediate mechanical insult and a secondary injury due to the pathology of blood brain barrier (BBB) disruption, inflammatory responses, cell apoptosis, excitotoxic damage, and free radical production, which evolve over minutes to months after the primary injury[4]. Nevertheless, few clinical treatments for TBI have been shown to be effective or to reverse the pathology[1, 5, 6]. Therefore, novel pharmacotherapies that can promote neural function in TBI patients are needed.

The BBB plays an important role in the brain in maintaining the biochemical environment for basic neural function by preventing potentially neurotoxic plasma components, pathogens, and blood cells from permeating the brain[7]. The main component of the BBB is cerebrovascular endothelial cells (ECs), which are connected to an integrated vascular system by continuous intercellular tight junctions (TJs). Under precise regulation, ECs transport waste products from the brain's interstitial fluid into the blood and deliver oxygen and other substances essential for neural tissue into the brain. Unfortunately, primary and secondary injury from TBI may result in necrosis, inflammation, oxidative stress, and apoptosis in ECs, leading to BBB disruption and the destruction of the proper microenvironment for normal neural function.

Sesame oil is a common edible fat that has been used in China for centuries. Sesamin, which is a major lignan of ses-

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Received 2016-11-30 Accepted 2017-03-12

ame oil, has been shown to have anti-oxidative, anti-apoptotic, anti-inflammatory, atherosclerosis-lowering, and anti-hypertension functions[7-10]. Its anti-oxidative function was demonstrated in multiple models of neuronal cell injuries induced by ischemia or oxidative stress[11, 12]. Its effects have not been explored in cerebrovascular ECs, which arean important component of the neurovascular system. In this study, we focused on the anti-oxidative and anti-apoptotic functions of sesamin in attenuating BBB disruption after TBI.

Materials and methods

Experimental design and animals

This study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. One hundred sixty adult male C57BL/6 mice (Shanghai SLAC Laboratory Animal Corporation; Shanghai, China) weighing 20–25 g under standard nutritional and environmental conditions were used in all animal experiments in this investigation. Sesamin was diluted with corn oil containing 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MO, USA). The dose (30 mg/kg) was chosen based on an earlier study[13]. Mice were randomly divided into three major groups as follows. Group I: control (mice received no treatment); Group II: mice received sesamin at a dose of 30 mg/kg[8]; Group III: mice were treated with vehicle. Each mouse in groups II and III received a TBI induced by a controlled cortical impact (CCI) injury 30 min after an intraperitoneal injection with sesamin or vehicle, and injections were given once a day until the animals were sacrificed. Mice were euthanized on the 1st and 3rd days after CCI injury and samples were collected for further analysis.

Surgical procedures and CCI injury model

After anesthetization with xylazine (10 mg/kg) and ketamine (75 mg/kg), adult male C57BL/6 mice were secured in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) with a hot pack placed under the body to maintain body temperature at 37 °C. The surgical area was cleaned with ethanol swabs and then a midline incision (10 mm long) was made over the head. After retracting the skin and fascia, a craniotomy was performed using a 4-mm trephine over the central aspect of the right parietal bone, 1 mm lateral to the sagittal suture. The surgical operation was very subtle to maintain an intact dura, and mice were excluded from the study if dural integrity was breached. The intact dura matter was impacted using a 3-mm-diameter rounded steel impactor tip of a CCI device (PinPoint Precision Cortical Impactor PCI3000; Hatteras Instruments Inc, Cary, NC, USA), which many labs have employed to imitate a TBI[14, 15], and the impactor was maintained perpendicular to the cortical surface to mimic an experimental focal TBI.

A CCI injury of moderate severity was induced by an impact velocity of 1.5 m/s, a deformation depth of 1.5 mm, and a dwell time of 100 ms. After the injury was induced, sterile cotton was used to control bleeding on the injured cortical surface, and the cranial opening was sealed with bone wax. Silk sutures (6-0) were used to close the incision. The animal was then placed in a heated cage to regain full consciousness then placed in its home cage. The exact same procedure was performed in sham animals as in the injured mice, except for CCI injury. Drug treatment and CCI injury were performed by the same person to minimize variance.

Measurement of brain edema

Brain edema was estimated through the water content in brain tissue using the wet-dry method, which was introduced in our earlier study. Briefly, a 3-mm coronal section of tissue from the ipsilateral cortex (or corresponding contralateral cortex), centered on the impact site, was evaluated. After removal, the tissue sample was immediately weighed to determine wet weight, then dried in an oven (100 °C) for 24 h to obtain its dry weight. Tissue water content (%) was calculated as follows: (wet weight–dry weight)/(wet weight)×100.

Evans blue (EB) extravasation

BBB permeability was evaluated onthe 1st and 3rd days after CCI injury by measuring the extravasation of EB dye (Sigma Aldrich). The EB dye (2%, 4 mL/kg) was injected intravenously 2 h prior to the sacrificing of mice. Following euthanasia, mice were transcardially perfused with phosphate buffered saline (PBS) through the left ventricle of the heart to sufficiently eliminate the intravascular-localized dye. The brain was then removed and divided into two hemispheres. Each sample was immediately weighed and homogenized in 1 mL of 50% trichloroacetic acid solution. The homogenate was centrifuged (12 000×*g*, 20 min), and the supernatant was transferred to a new tube and diluted 1:3 with ethanol. Its absorbance was determined at 610 nm using a spectrophotometer (BioTek, Winooski, VT, USA). A standard curve was used to calculate the quantity of dye, which was expressed as micrograms per gram of brain tissue.

Measurement of tissue hemoglobin level

Brain hemorrhage was quantified through the detection of the hemoglobin level in tissue, which was used as an index. After perfusion, approximately 100 mg of brain tissue from the traumatic focus was removed and homogenized in Drabkin's color reagent according to the manufacturer's instructions (Analisa). Samples were centrifuged at 3000×*g* for 15 min. The supernatant was pipetted out and filtered using 0.2 μm filters, and the extravasated blood in the tissue homogenates was quantified at 520 nm with a spectrophotometer (BioTek).

Mechanical cell injury

Stretch-induced cell injury, which has been introduced by our group and other researchers[1, 16], was used to imitate cellular mechanical injury during CCI injury in this study. Mouse brain microvascular ECs bEnd.3 cells were grown to confluence in BioFlex six-well culture plates with collagen-coated Silastic membranes (Flexcell International Corp, Burlington, NC, USA), and the experimental group was treated with sesamin (50 μmol/L) for 1 h. After sufficient preparation, a Cell Injury Controller II system (Virginia Commonwealth Univer-

sity, Richmond, VA, USA) was used to generate biaxial stretch on the cells in the plate, delivering a 50 ms burst of nitrogen gas that produced a downward deformation of the Silastic membrane and adherent cells. In our study, cells were injured through a 7.5-mm membrane deformation (analogous to the mechanical stress range exerted on the human brain), consistent with a previous study[1]. Cells were ready for examination after 24 h of continuous incubation.

Cell culture

The mechanism underlying the protective action of sesamin was investigated at the cellular level by culturing bEnd.3 cells, which were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 μg/mL penicillin. For the experiments, cells were seeded at a density of $(0.14–0.6)\times10^5/cm^2$ on collagen-coated glass cover-slips or BioFlex elastic membrane supports (Flexcell International Corp). Then, the cultures were maintained in an anaerobic chamber infused with a gas mixture containing 5% CO_2 and 95% N_2. Cells were pretreated with 50 μmol/L vehicle[8] or sesamin for 1 h before undergoing stretch injury.

Lactate dehydrogenase (LDH) release assay

The degree of cytotoxicity was determined by measuring the release of LDH[17, 18]. bEnd.3 cells cultured on a BioFlex plate were stretch injured. After 24 h, the supernatant was transferred to assay LDH activity. The reaction mixture (0.1 mL; Roche, Mannheim, Germany) containing catalyst (diaphorase/NAD^+) and dye solution (lodotetrazolium chloride and sodium lactate) was mixed with cell-free supernatant to a final volume of 0.2 mL in a 96-well plate. The rate of absorbance was read at 490/630 nm on a SpectraMAX 340 instrument. Data were expressed as the mean percent of viable cells *vs* control.

Detection of intracellular reactive oxygen species (ROS)

To estimate intracellular ROS generation, dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) fluorescent staining[19], which can detect intracellular superoxide anions (the most common being ROS), was applied to cultured bEnd.3 cells that were stretch injured. Cells 6 h post-injury were added to 10 μmol/L DHE at 37 °C for 30 min and washed twice with PBS. The intensity of DHE fluorescence was measured using an inverted fluorescence microscope (Leica, Solms, Germany), with the intensity being proportional to intracellular superoxide anion generation.

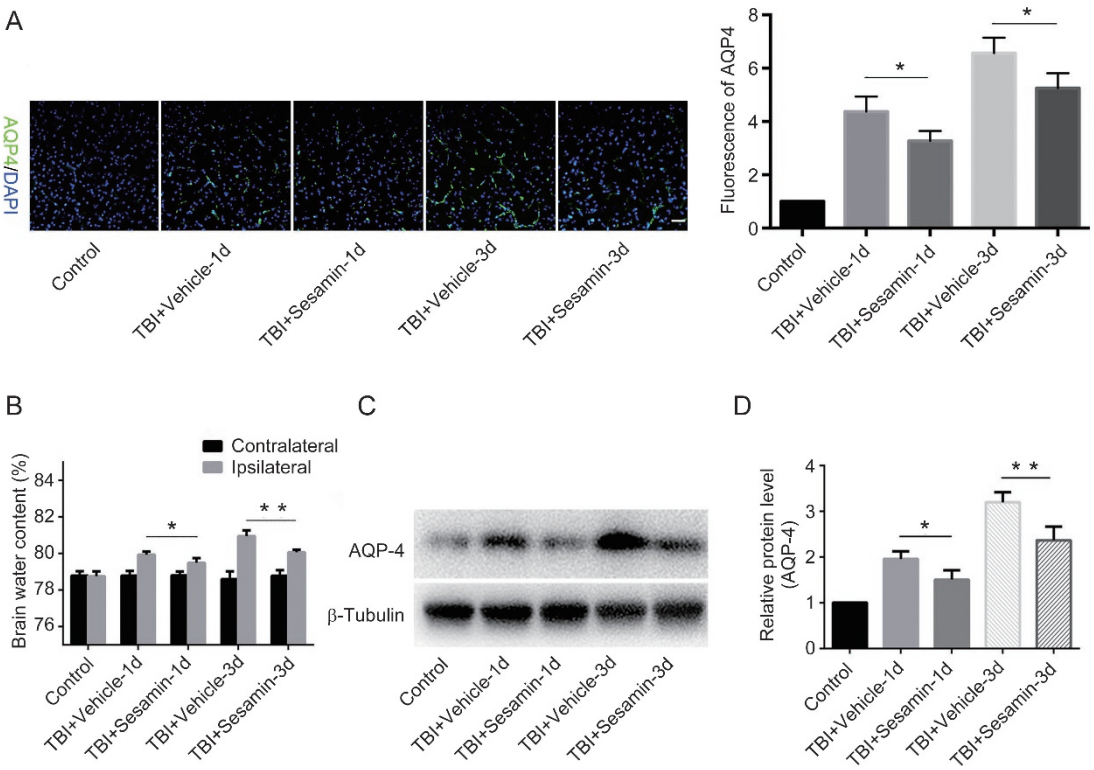

Figure 1. Sesamin inhibits brain edema after traumatic brain injury (TBI). (A) Representative aquaporin 4 (AQP4) immunofluorescence staining in brains and its quantification in control, TBI+vehicle, and TBI+sesamin groups. (B) Bar graph of the quantification analysis of brain water content. (C) Western blot analysis showing a significant increase in AQP4 protein expression in the pericontusional area on the 1st and 3rd days following TBI. (D) Sesamin treatment reduced AQP4 expression, compared with the vehicle treatment group. n=5 per group; data are presented as the mean±SD; $^*P$<0.05, sesamin versus vehicle group; $^{**}P$<0.01, sesamin versus vehicle group. Scale bar=30 μm.

Immunostaining

Double-staining with ZO-1 (Invitrogen, Carlsbad, CA, USA)/CD31 (BD Biosciences, San Jose, CA, USA) and occludin (Invitrogen)/CD31 (BD Biosciences) was performed as previously described[1, 6]. Briefly, brain sections (20 μm thick) were blocked with 10% fetal bovine serum for 1 h and then incubated with antibodies (1:(100–200) dilution) overnight at 4 °C. After washing, the brain sections were incubated with the appropriate secondary antibodies for 1 h at 37 °C. Finally, a confocal microscope (Leica) was used to examine the brain sections and photographs were taken for further analysis.

Western blot analysis

For Western blot analysis, we used a previously described method[1] in which samples were lysed in a radioimmunoprecipitation assay (RIPA; Millipore, Bedford, MA, USA) supplemented with 1 mmol/L of PMSF protease inhibitor (Thermo, Waltham, MA, USA), Halt™ protease inhibitor cocktail (Thermo), and a phosphatase inhibitor (Thermo).Following sufficient homogenization, insoluble material was removed by centrifugation at 12 000×*g* at 4 °C for 30 min. After denaturation, samples that contained equal amounts of protein were loaded onto a resolving gel (Promoton, Shanghai, China) for electrophoresis. The proteins were then transferred onto a nitrocellulose membrane (Whatman, Piscataway, NJ, USA) and blocked with 5% nonfat milk. The membranes were incubated with primary antibodies overnight at 4 °C at the following dilutions: ZO-1 (1:500), occludin (1:500), ERK, p-ERK, P38, P-P38, JNK, and p-JNK (1:1000; Cell Signaling Technology, Beverly, MA, USA), and β-tubulin and GAPDH (1:1000; Santa Cruz Technology, Santa Cruz, CA, USA). After washing, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and then reacted with an enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA). The results were recorded using Quantity One image software (Bio-Rad, Hercules, CA, USA), and the relative intensity was calculated usingthe Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD, USA).

Flow cytometry

To detect cell apoptosis, bEnd.3 cells grown in BioFlex six-well culture plates (Flexcell International Corp) were subjected to mechanical injury induced by biaxial stretching. After a subsequent 24-h incubation (37 °C, 5% CO_2), cells were treated with 0.25% trypsin without ethylenediaminetetraacetic acid

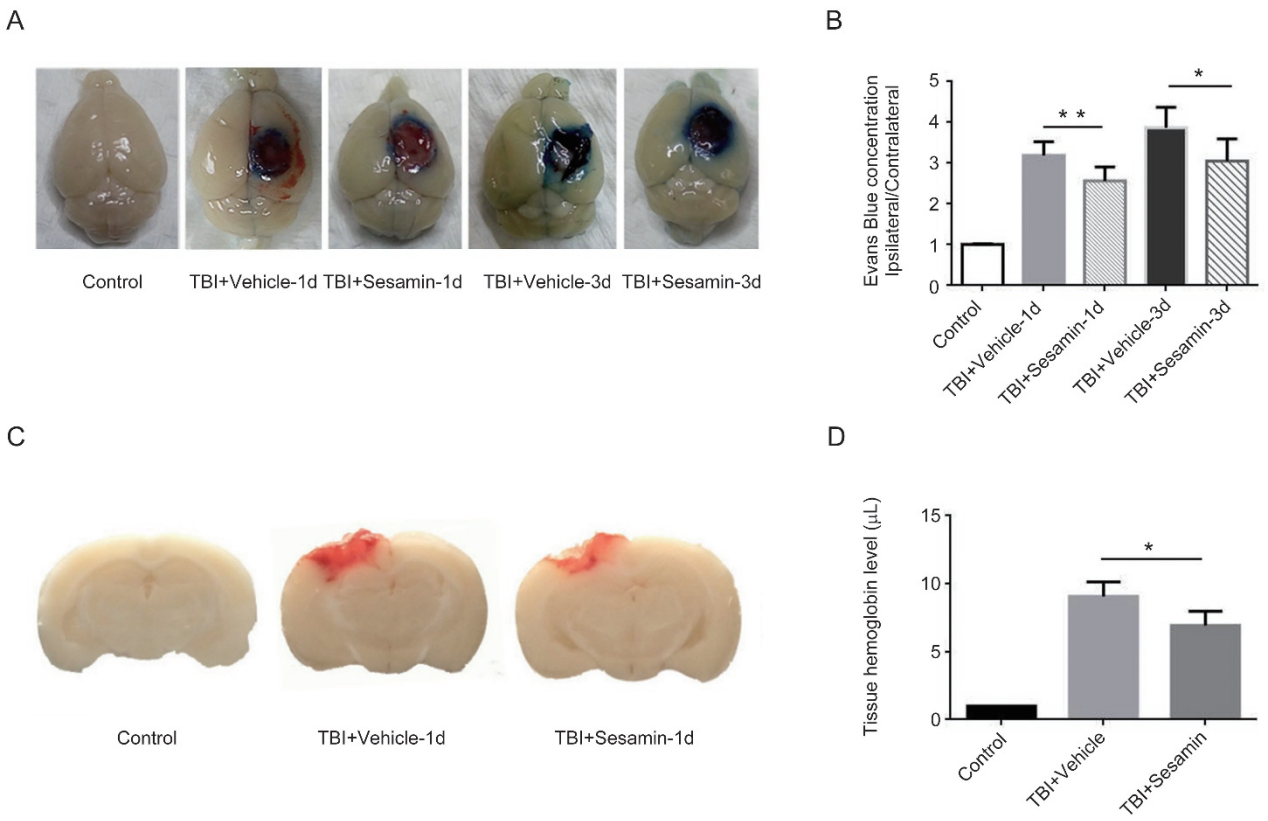

Figure 2. Sesamin lowers Evans blue extravasation and tissue hemorrhaging following traumatic brain injury (TBI). (A) Images showing Evans blue extravasation in control, TBI+vehicle, and TBI+sesamin groups on the 1st and 3rd days after TBI. Dark areas indicate the extravasation of Evans blue. (B) Bar graph showing the quantification of Evans blue content in the brain. (C) The amount of extravasated blood present in contused brains on the 1st day after injury in mice. (D) Bar graph showing the quantification of hemoglobin content in brain tissue. *n*=5 per group; data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group; $^{**}P<0.01$, sesamin versus vehicle group.

(EDTA), washed twice with cold PBS, and then resuspended in the binding buffer. Then, annexin V, Alexa Fluor 488, and propidium iodide (PI; Component B) were mixed in a 5-μL volume of solution, which was then added to a 100-μL solution in a 1.5-mL centrifuge tube. The solution was then gently mixed with the cells, which were further incubated for 10 min at room temperature in the dark. Binding buffer (250 μL) was then added to each well of a 96-well plate. Finally, apoptotic cells were assessed using flow cytometry after 1 h, and stained cells and controls were analyzed with an Accuri C6 flow cytometer (BD Biosciences).

To detect ROS in cells, bEnd.3 cells were cultured for 6 h after stretch injury. Then, cells were suspended in serum-free DMEM (DCFH-DA 10 μmol/L) and incubated (37 °C, 5% CO_2) for 20 min. After washing in PBS, cells were ready to be examined in a flow cytometer.

Statistical analysis

All data are presented as the mean±SD. Equality of variance was assessed by Levene's test. T-tests were used to compare means between groups. *P* values of <0.05 were considered to indicate statistical significance. Graphic representations of the data were produced using Graph Pad Prism 6 (GraphPad Software, San Diego, CA, USA), and SPSS 20.0 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analyses.

Results

Sesamin reduced brain edema in a mouse model of CCI injury

Brain tissue samples from the same set of mice were weighed and dehydrated. As shown in Figure 1B, significant increases in water content in the ipsilateral cortical tissue samples (79.9% and 80.9%) and the control group (78.8%; $P<0.01$ *vs* vehicle) were observed on the 1st and 3rd days after the CCI procedure, and there was a significant reduction in brain water content in mice treated with 30 mg/kg sesamin on the 1st and 3rd days after CCI injury (79.5% and 80.1%; $P<0.01$ *vs* vehicle).

The increase in aquaporin 4 (AQP4) expression in the cytomembraneis correlated withthe severity of brain edema[1]. In this study, the level of AQP4 expression was examined using immunofluorescence and Western blot analyses. To assess the biological effects of sesamin on the formation of brain edema after CCI injury, mice were treated with 30 mg/kg of sesamin

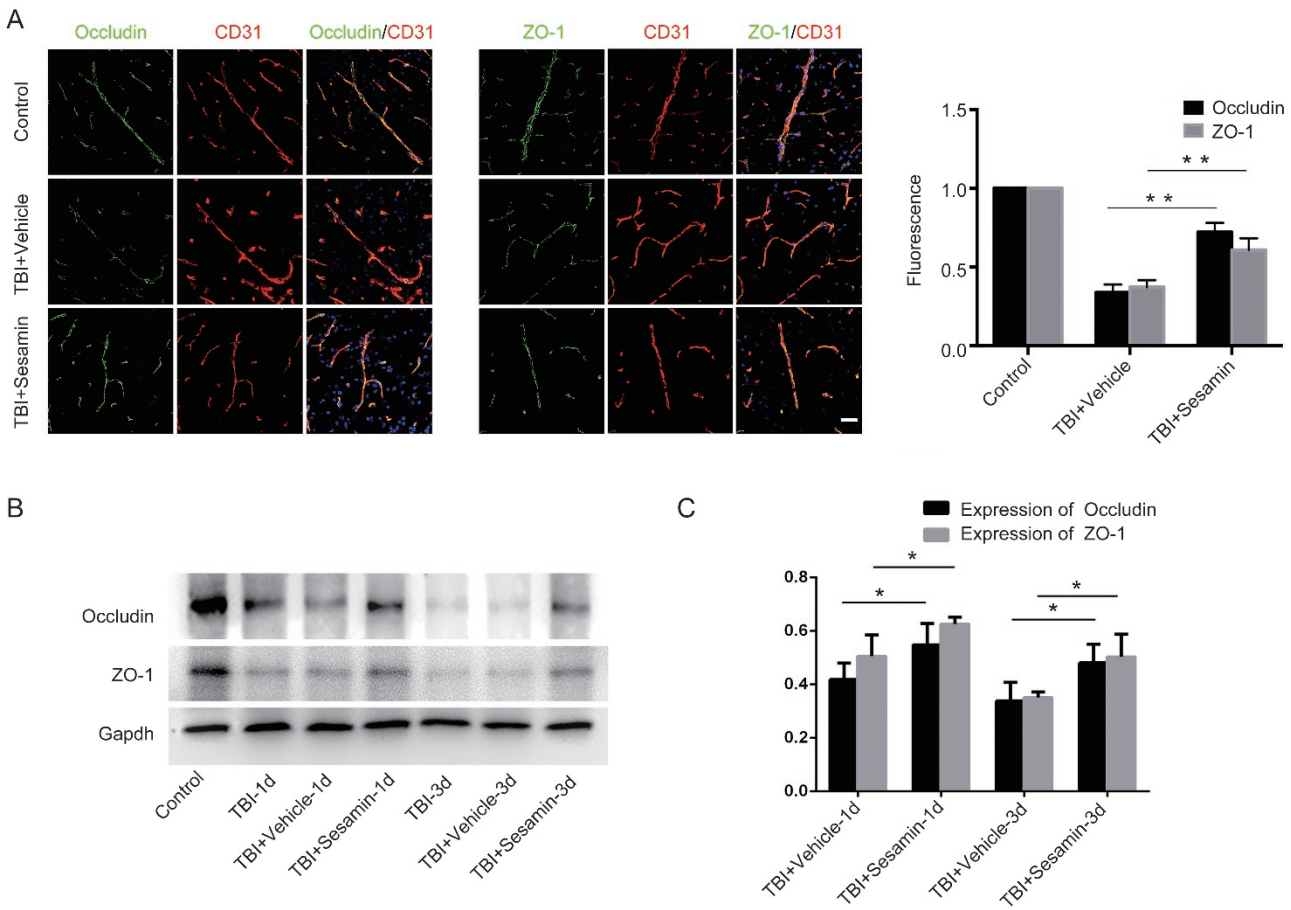

Figure 3. Sesamin alleviates the decrease in ZO-1 and occludin expression induced by traumatic brain injury (TBI). (A) Expression of ZO-1 and occludin in control, vehicle, or sesamin-treated groups on the 3rd day following TBI. Scale bar=30 μm. (B) Representative Western blot results of ZO-1 and occludin expression on the 1st and 3rd days after TBI. (C) Bar graphs showing quantification of ZO-1 and occludin expression. *n*=5 per group; data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group.

and euthanized on the 1st and 3rd days after the CCI injury procedure. Both immunofluorescence on frozen brain sections and Western blotting of protein samples revealed that AQP4 protein levels increased after CCI injury but were significantly reduced in the sesamin-treated group compared with the vehicle group (Figure 1A, 1C–1E).

Taken together, these results suggest that sesamin plays a role in reducing cerebral edema following CCI injury, at least partly via the downregulation of AQP4 expression.

Sesamin reduced BBB disruption in a mouse model of CCI injury

To assess the protective function of sesamin on BBB disruption, an Evans blue extravasation test was performed after the CCI injury procedure. The sesamin-treated group exhibited a lower level of EB leakage in the ipsilateral cortex compared with the vehicle group on the 1st and 3rd days post-injury (Figure 2A), and further analysis indicated that sesamin significantly alleviated CCI injury-induced BBB disruption on the 1st and 3rd days post-injury (Figure 2B, $P<0.01$ and $P<0.05$ *vs* vehicle group). Furthermore, we quantified the amount of extravasated blood present in ipsilateral contused tissues 1 d after injury (Figure 2C) and found that the amount of extravasated blood in the vehicle-treated group was significantly greater than in the sesamin-treated group (Figure 2D, $P<0.05$ *vs* vehicle group).

To evaluate the integrity of the BBB after CCI injury, the distribution of TJs was measured using an *in situ* double-staining procedure with ZO-1/CD31 and occludin/CD31. The staining results showed that occludin and ZO-1 were consistently located on the margin of the ECs in the control group, whereas a few gaps were present in TJs in the post-injury group (Figure 3A). The sesamin-treated group had fewer gaps than did the vehicle-treated group on the 3rd day after CCI injury. Additionally, Western blot analysis confirmed that sesamin-treated mice exhibited lower reductions in the expression of ZO-1 and occludin than did the vehicle-treated group on the 1st and 3rd

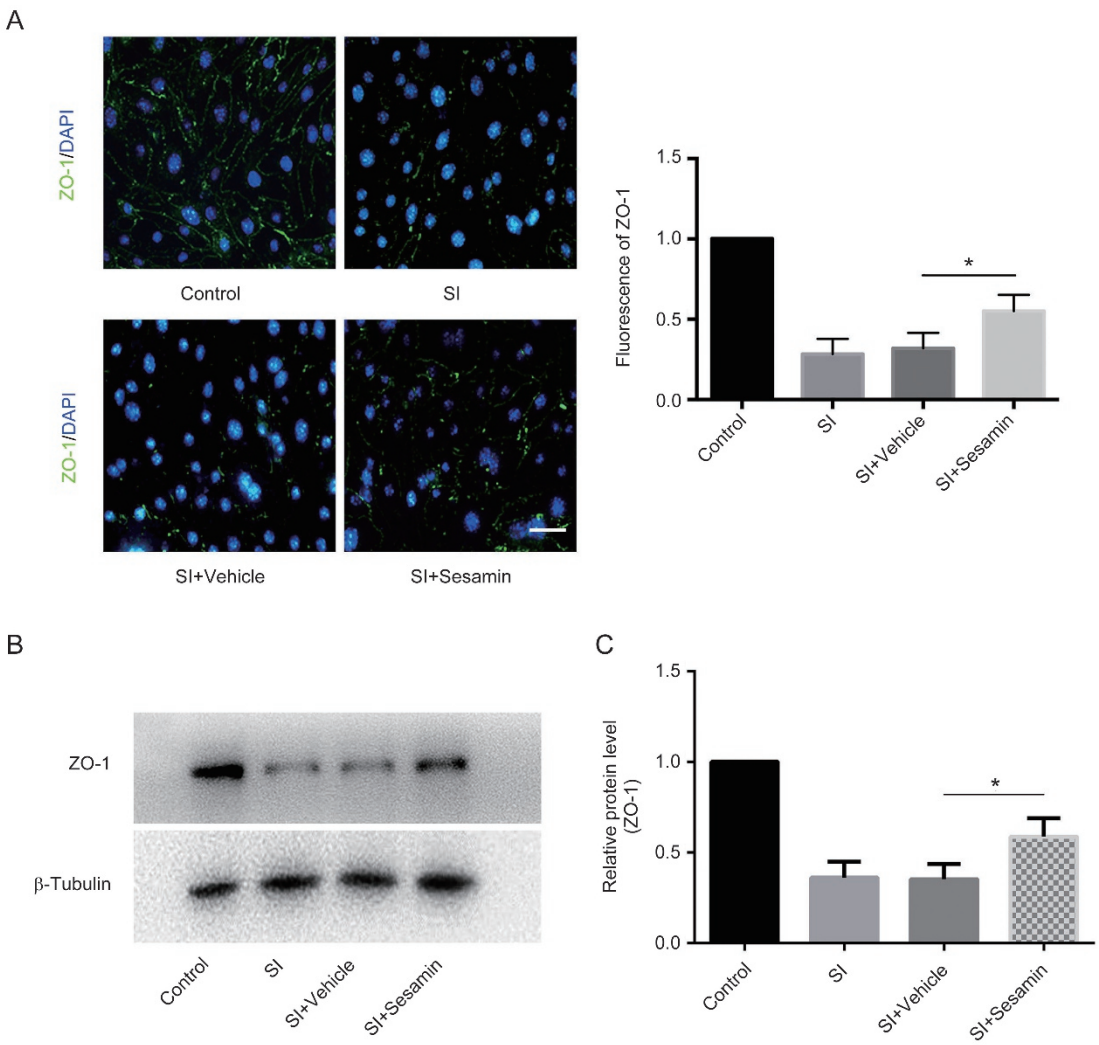

Figure 4. Sesamin (50 μmol/L) inhibits the decrease in tight junction proteins in endothelial cells after stretch injury (SI). (A) Immunocytochemistry from fixed bEnd.3 cells 24 h after stretch injury. Scale bar=30 mm. (B) Representative results of ZO-1 expression 24 h after stretch injury. (C) Bar graphs showing the quantification of ZO-1 expression. Data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group.

days post-injury (Figure 2B, 2C; $P<0.05$ *vs* vehicle group).

Sesamin treatment can alleviate the loss of ZO-1 induced by stretch injury in bEnd.3 cells

To evaluate the protective effect of sesamin in alleviating the loss of ZO-1, bEnd.3 cells were subjected to stretch injury and ZO-1 was examined using immunofluorescence. For further assessment, protein samples were collected for Western blotting 24 h after the stretch injury. As shown in Figure 4A, the expression of ZO-1 was consistently high at the edge of cells, but was markedly reduced after injury. Western blotting indicated that the loss of ZO-1 was lower in the sesamin-treated group than in the vehicle-treated group after stretch injury (Figure 4B, 4C; $P<0.05$ *vs* vehicle group).

Sesamin inhibited oxidative stress after stretch injury of bEnd.3 cells

Oxidative stress is known to cause brain damage by increasing ROS levels in a number of neurological disorders[13]. To evaluate intracellular ROS production induced by stretch injury, the amount of superoxide anion (O_2^-) was examined using the DHE staining method in bEnd.3 cells 6 h post-injury. The results indicated that stretch injury induced high levels of oxidative stress but that sesamin markedly alleviated oxidative stress compared to no treatment (Figure 5A, 5B; $P<0.01$ *vs* vehicle group).

For further analysis, ROS was quantified via combination with a DCFH-DA probe using flow cytometry-based assays. As shown in Figure 5C, sesamin-treated cells tended to generate significantly less ROS than vehicle-treated cells after stretch injury (Figure 5C, $P<0.01$ *vs* vehicle group).

Sesamin reduced early-stage apoptosis induced by stretch injury in bEnd.3 cells

To determine the level of apoptosis in bEnd.3 cells induced by stretch injury, we examined LDH release. The results showed that the concentration of LDH released by post-injury cells was much higher than in normal cells. Injured cells that were treated with sesamin released less LDH than vehicle-treated cells (Figure 6A, $P<0.05$ *vs* vehicle group), showing that sesamin can alleviate stretch injury-induced apoptosis in ECs.

Flow cytometry-based assays can also be used to examine apoptosis in cells through the level of annexin V-positive cells. In our study, a 50-μmol/L sesamin pretreatment significantly reduced the number of annexin V-positive bEnd.3 cells undergoing early-stage apoptosis (Figure 6B, 6C; $P<0.05$ *vs* vehicle group). These results suggest that sesamin can reduce stretch injury-induced early-stage apoptosis in ECs.

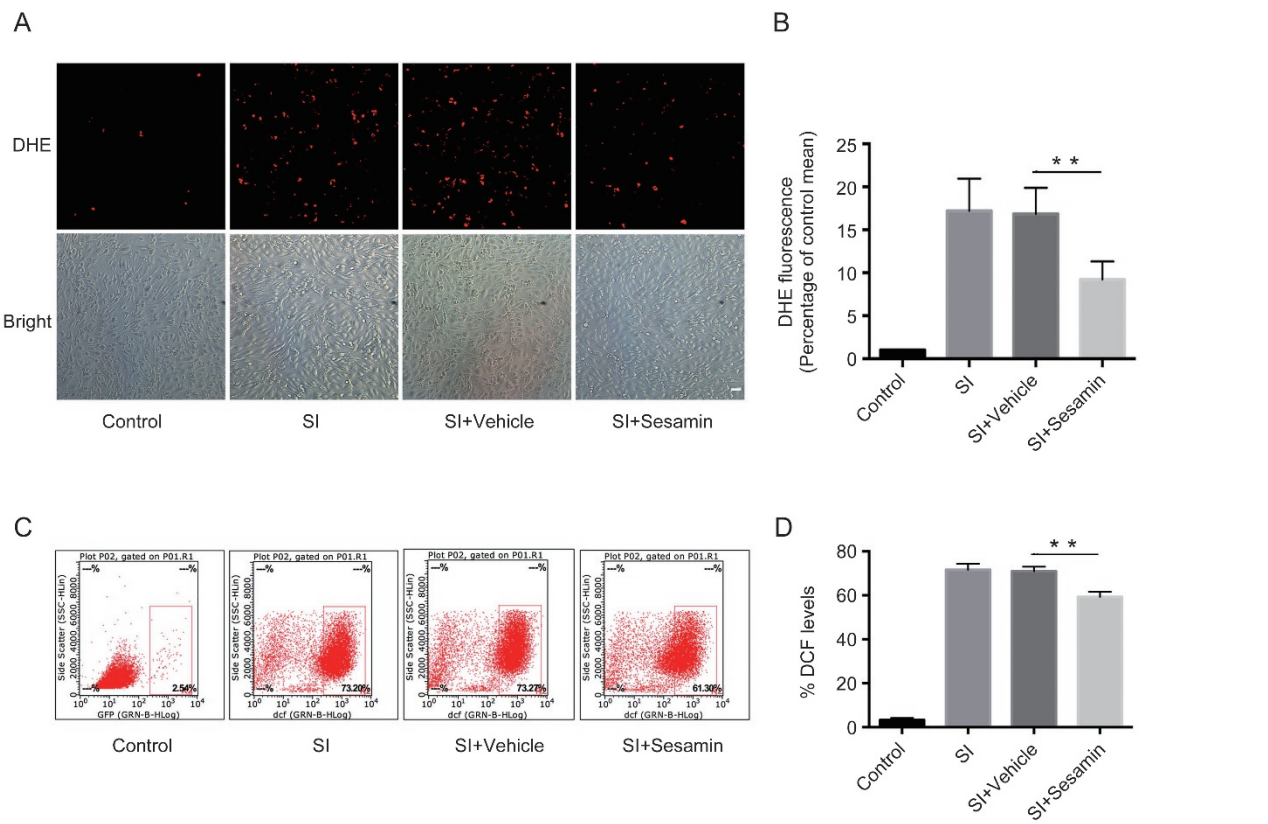

Figure 5. Sesamin (50 μmol/L) inhibits oxidative stress in endothelial cells after stretch injury (SI). (A) DHE fluorescent staining of bEnd.3 cells 6 h after SI. Scale bar=30 mm. (B) Bar graphs showing the quantification of DHE fluorescence. (C) Sesamin-treated bEnd.3 cells have lower fluorescence intensity compared to cells treated with vehicle. (D) Bar graphs showing the quantification of the DCF intensity of cells treated with sesamin and vehicle. Data are presented as the mean±SD; $^{**}P<0.01$, sesamin versus vehicle group.

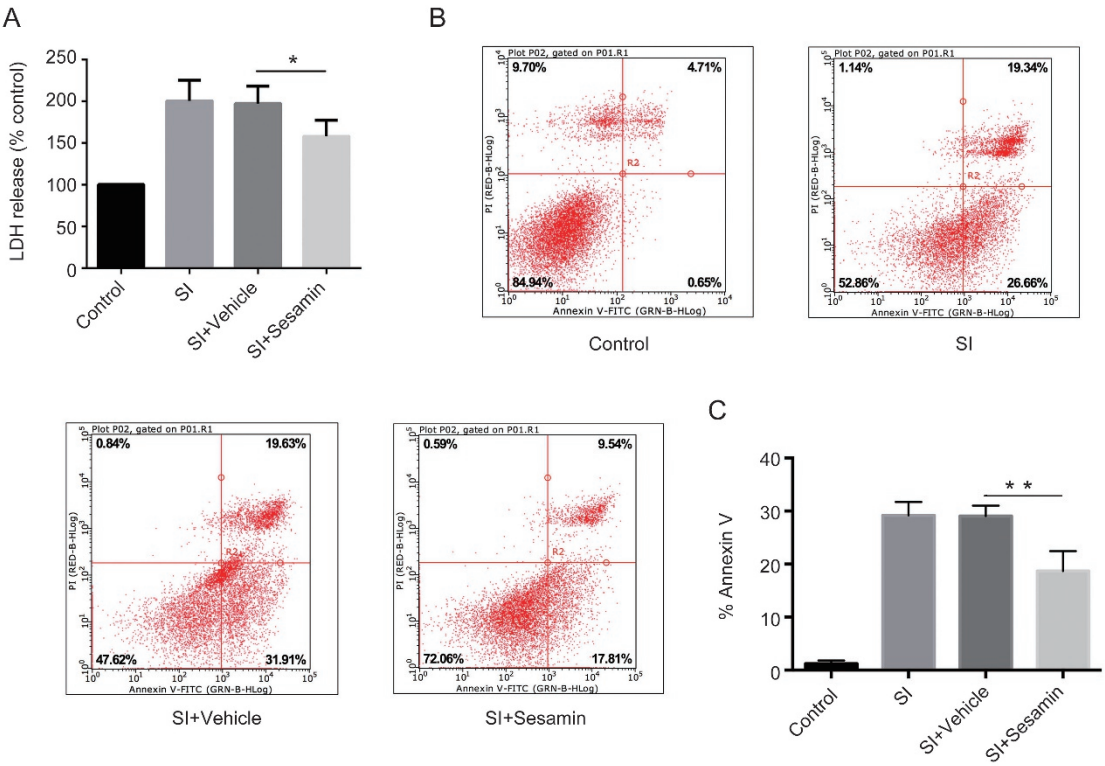

Figure 6. Sesamin (50 μmol/L) inhibits apoptosis in bEnd.3 cells after stretch injury (SI). (A) Bar graphs showing the quantification of LDH released 24 h after SI. (B) Flow cytometry-based assay was used to determine cell apoptosis by assessing the level of annexin V-positive cells. (C) Bar graphs showing a significantly lower percentage of positive cells in the sesamin-treated group compared with the vehicle group. Data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group.

Sesamin modulated stretch injury-induced expression of MAPKs and caspase-3 activation

In this study, we demonstrated that 6 h after stretch injury, the expression of p-ERK and p-p38 proteins was enhanced, while the enhancement was reversed in sesamin-treated cells (Figure 7A, 7B). We also examined the expression of pro-apoptotic and anti-apoptotic members of the Bcl-2 family, which are interrelated with the mitochondrial intrinsic apoptotic pathway. Our results showed that the expression of the pro-apoptotic BAX proteins was significantly increased, whereas that of the anti-apoptotic Bcl-2 protein was markedly reduced after stretch injury (Figure 8A, 8B). Nevertheless, in vehicle-treated cells, the variation inthe expression of pro-apoptotic signaling components was significantly reduced (Figure 8A, 8B). Additionally, the level of the cleaved (activated) forms of caspase-3 increased 2 h after stretch injury, but the level was decreased in the sesamin-treated group compared to the vehicle-treated group (Figure 8A, 8B). These results indicate that sesamin inhibited oxidative stress-induced apoptotic cell death in ECs following stretch injury.

Discussion

Sesamin was recently shown to have neuroprotective functions in many animal and cell models of neurological disease[11, 12, 20, 21]. This study focused on the role of sesamin function in the disruption of the BBB, which is an important component of the neurovascular system. We demonstrated for the first time that sesamin attenuated the BBB damage induced by CCI injury through its anti-oxidant and anti-apoptosis properties in mice with a CCI injury and in an EC stretch injury model. The *in vivo* tests conducted in this study showed that sesamin treatment

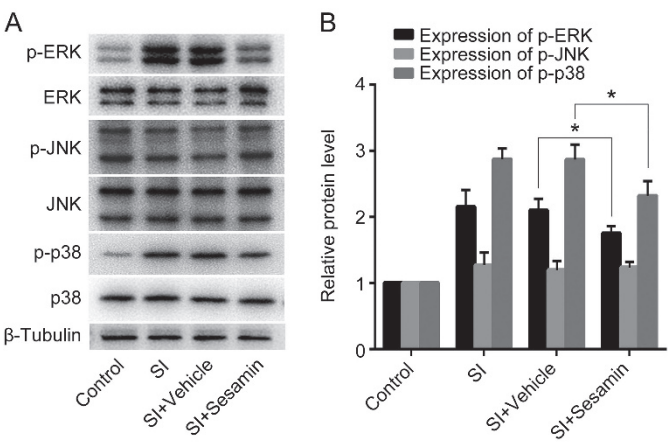

Figure 7. Sesamin (50 μmol/L) modulates the activation of MAPKs in bEnd.3 cells after stretch injury. (A) Protein expression of p-ERK/ERK, p-JNK/JNK, and p-p38/p38 in Western blots. (B) Bar graphs showing quantification of p-ERK, p-JNK, and p-p38 expression. Data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group.

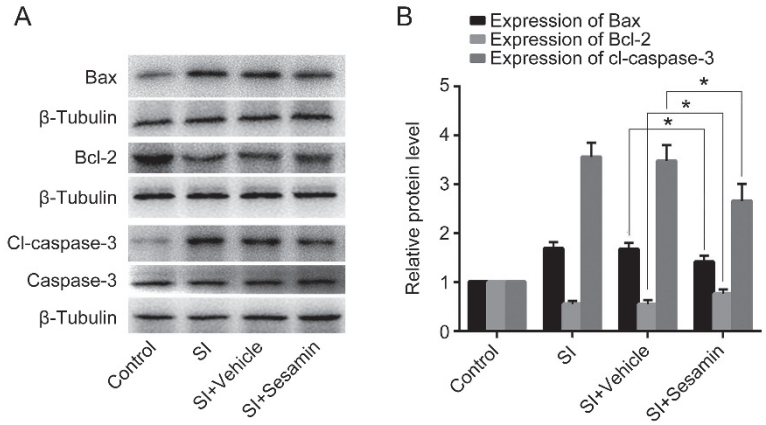

Figure 8. Effects of sesamin (50 μmol/L) on anti-apoptotic and pro-apoptotic proteins in bEnd.3 cells after stretch injury (SI). (A) Protein expression of BAX, Bcl-2, and cl-caspase-3/caspase-3 in Western blots. (B) Bar graphs showing quantification of BAX, Bcl-2, and cl-caspase-3. Data are presented as the mean±SD; $^{*}P<0.05$, sesamin versus vehicle group.

can alleviate brain edema and reduce neural tissue hemorrhaging, thereby protecting the BBB, and we showed that these functions were at least partially based on its protective effect in ECs via changes in MAPKs and the regulation of the expression of pro-apoptotic and anti-apoptotic proteins. Furthermore, the loss of ZO-1 and occludin proteins in ECs induced by the *in vivo* CCI procedure or *in vitro* stretch injury were shown to be significantly attenuated by sesamin treatment. Oxidative stress and apoptosis in bEnd.3 cells were also markedly inhibited by sesamin as demonstrated by the DHE staining test and flow cytometry.

Brain edema, one of the major pathological changes after TBI, comprises two descriptions: cytotoxic and vasogenic. Much convincing evidence has been presented to indicate that AQP4 plays a key role in cytotoxic brain edema, in which water flows in the brain from the blood through capillary endothelium and AQP4[22]. Most investigators demonstrated that the upregulation of AQP4 increased the amount of brain edema[23-25], whereas Hirt reported that early induction of AQP4 expression may limit edema formation but does not prevent early BBB disruption[26]. Our data showed that the expression of AQP4 is correlated with the amount of brain edema in mice at the 1st and 3rd day after the CCI procedure, which indicated that AQP4 expression may contribute to TBI-induced brain edema.

Oxidative stress is a well-known factor that exacerbates brain damage and neurological dysfunction due to its significant role in the etiology of progressive neuropathology in TBI[27-32]. The high metabolic demands on a reduced mitochondrial population as part of the secondary effects of TBI results in abundant ROS, which are the main source of oxidative stress in brain injuries[31]. As a strong neurotoxic factor, ROS can attack DNA, proteins, membranelipids, and transcription factors, causing cell damage and, eventually, cell death[33]. ROS include O_2^-, the hydroxylradical (OH), hydrogen peroxide (H_2O_2), and hypochlorousacid (HOCl). Of these, O_2^- is the most common free radical in TBI[31, 34]. In this study, we performed DHE staining and flow cytometry of bEnd.3 cells 6 h after stretch injury. High levels of O_2^- were detected compared to the control, and these levels were reduced by sesamin treatment. These results indicate that the anti-oxidant properties of sesamin may act as a protective mechanism for ECs in injury-induced oxidative stress.

Apoptotic cell death has been widely reported in the secondary injured cortex of adult mice following TBI[4, 32, 35, 36], and apoptosis can be induced by oxidative stress due to mitochondrial damage[1, 32, 37-40]. Stress-induced apoptosis is regulated by the downregulation of the anti-apoptotic Bcl-2 protein and/or the upregulation of the pro-apoptotic BAX protein. In this study, apoptosis in cells exposed to stretch injury was verified via flow cytometry. Western blotting showed that the expression of the BAX protein was elevated but that the level of Bcl-2 protein was reduced, indicating that stretch injury-induced apoptosis may be associated with oxidative stress. Treatment with sesamin reversed the levels of BAX and Bcl-2 in ECs, suggesting that it may protect against stretch injury-induced apoptosis via its anti-oxidant effects.

ROS-mediated oxidative stress can activate a number of downstream signaling molecules, including MAP kinases[41-43], which are serine/threonine kinases that mainly include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs[44-46]. The phosphorylation states of MAPKs (MKPs) relay signals generated by exogenous and endogenous stimuli to the intracellular space via phosphorylation of a variety of intracellular targets, including transcription factors, nuclear pore proteins, membrane transporters, cytoskeletal elements, and other protein kinases[47]. Our results showed that the phosphorylation of ERK and p38, but not JNK, is markedly increased in bEnd.3 cells after mechanical injury, indicating that the ERK and p38 pathways are activated. This result is consistent with results in PC12 and bv2 cells[38, 48]. These two pathways were suggested to be involved in the regulation of apoptotic cell death in mechanically injured cells[32, 49, 50], which is consistent with our results from LDH assays and flow cytometry.

More importantly, our data demonstrate that sesamin treatment reduced both ERK and p38 MKPs, which suggests that sesamin may play a role as an anti-oxidant and anti-apoptotic agent.

Cellular caspases, which belong to a highly conserved family of cysteine proteases, are the main downstream proteins of the apoptotic pathway, and the cleaved forms of caspases are the key players in the execution phase of apoptosis[51-53]. Several studies have reported that oxidative stress can activate the caspase pathway through a variety of pathways, including the MAPK pathway[1, 4, 32, 37, 39]. Under pro-apoptotic stimuli, the convergence of intrinsic and extrinsic pathways activates caspase-3 as cleaved-caspase-3[1, 53], which executes apoptosis by the cleavage of protein substrates[54]. Studies have demonstrated that the increased expression of cleaved-caspase-3 is closely related to apoptosis in a variety of organisms[55]. In this study, stretch injury elevated caspase-3 activity in bEnd.3 cells, but sesamin pretreatment markedly decreased injury-induced caspase-3 activity in these ECs. These results indicate that sesamin may have a protective function in ECs against apoptosis induced by CCI injury.

Conclusion

This study demonstrated that sesamin attenuated the CCI injury-induced disruption of the BBB. Additionally, our findings suggest that sesamin maintained the integrity of the BBB at least partly via the protection of ECs against stretch injury-induced oxidative stress and apoptosis. Taken together, these findings indicate that sesamin may be a promising potential therapeutic intervention for the prevention of the disruption of the BBB after TBI.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (No 81471245, 81671207, and 81501657), and Shanghai Jiao Tong University Medicine-Engineering Research Fund (YG2016QN20).

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